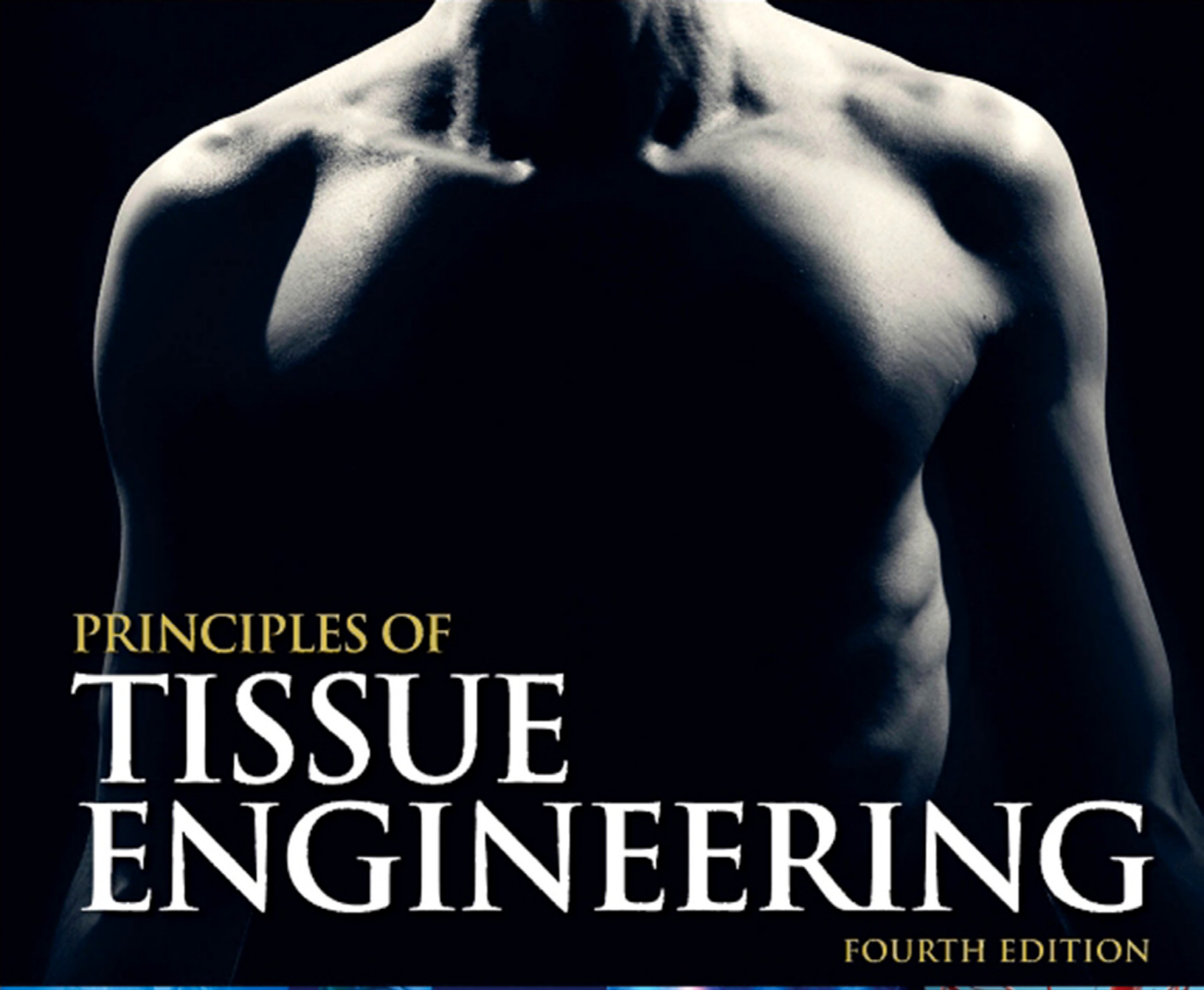
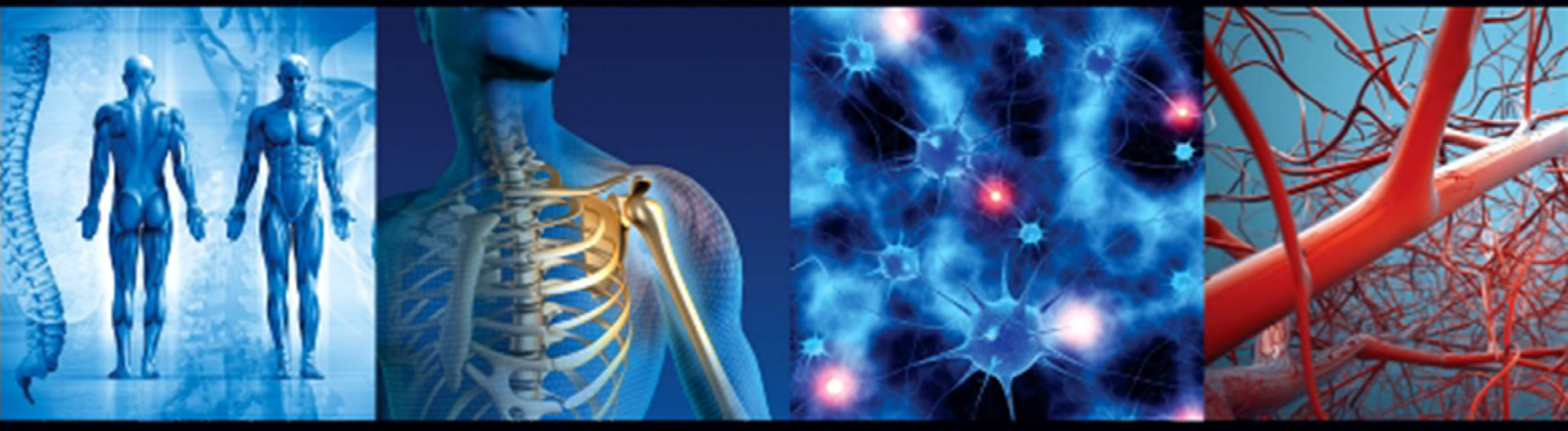


PRINCIPLES OF TISSUE ENGINEERING

FOURTH EDITION



ROBERT LANZA | ROBERT LANGER | JOSEPH VACANTI

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Principles of Tissue Engineering

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Principles of Tissue Engineering

Fourth Edition

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FOREWORD

Robert Langer

Since the mid-1980s, tissue engineering has moved from a concept to a very significant field. Already we are at the point where numerous tissues, such as skin, cartilage, bone, liver, blood vessels and others, are in the clinic or even approved by regulatory authorities. Many other tissues are being studied. In addition, the advent of human embryonic stem cells has brought forth new sources of cells that are beginning to prove to be useful in a variety of areas of tissue engineering.

This fourth edition of *Principles of Tissue Engineering* examines a variety of important areas. In the introductory section, an important overview on the history and challenges of tissue engineering as well as a chapter on stem cells and the movement of engineered tissues into the clinic is examined. This is followed by an analysis of important areas in cell growth and differentiation including aspects of molecular biology, extracellular matrix interactions, cell morphogenesis, and gene expression and differentiation. Next, *in vitro* and *in vivo* control of tissue and organ development is examined. The involvement of biomaterials in tissue engineering is also addressed. Important aspects of polymers, extracellular matrix, materials processing, novel polymers such as biodegradable polymers as well as micro and nano fabricated scaffolds and three-dimensional scaffolds are discussed. Tissue and cell transplantation including methods of immunoisolation, immunomodulation, and even transplantation in the fetus are analyzed.

As mentioned earlier, stem cells have become an important part of tissue engineering. As such, important coverage of embryonic stem cells, adult stem cells, and postnatal stem cells are examined. Gene therapy is another important area, and both general aspects of gene therapy as well as intracellular delivery of genes and drugs to cells and tissues are discussed. Various important engineered tissues including breast tissue engineering; tissues of cardio vascular systems such as myocardium, blood vessels and heart valves; endocrine organs such as the pancreas and the thymus are discussed, as are tissues of the gastrointestinal system, such as liver and the alimentary tract. Important aspects of the hematopoietic system are analyzed as is the engineering of the kidney and genitourinary system.

Much attention is devoted to the muscular skeletal system including, bone and cartilage regeneration, and tendon and ligament placement. The nervous system is also discussed, including brain implants, and the spinal cord. This is followed by a discussion of the eye where corneal replacement and vision enhancement systems are examined. Oral and dental applications are also discussed as are the respiratory system and skin. The concluding sections of the book cover clinical experience in such areas as cartilage, bone, skin and cardiovascular systems as well as the bladder. Even tissue engineered food is evaluated. Finally, regulatory and ethical considerations are examined.

In sum, the 90 chapters in this third edition of *Principles of Tissue Engineering* examine the important advances in this burgeoning field of tissue engineering. This text will be very useful for scientists, engineers, and clinicians engaging in this important new area of science and medicine.

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PREFACE

A lot has happened since the first edition of 'Principles of Tissue Engineering' was published sixteen years ago. Human embryonic stem cells had not yet been discovered. In fact, progress in the field has been so rapid that since the publication of the last edition of this book, the first clinical trials have been carried out using these cells. Indeed, scientists have not only figured out how to reprogram differentiated human cells into induced pluripotent stem (iPS) cells, but a Nobel Prize has already been awarded for the achievement.

The fourth edition of 'Principles' covers all of this recent progress as well as the latest advances in the biology and design of tissues and organs, from mathematical models to clinical reality. It has also been expanded to include the rapidly emerging field of brain-machine interfaces (BMI) for controlling bionic and prosthetic body parts, as well as a new section on the application of tissue engineering techniques for food production, another application of tissue engineering just in its infancy. As in previous editions, the book attempts to simultaneously connect the basic sciences with the potential application of tissue engineering to diseases affecting specific organ systems. While the fourth edition furnishes a much needed update of the rapid progress that has been achieved in the field in the last half decade, we have retained those facts and sections which, while not new, will assist students and general readers in understanding this exciting area of biology and medicine.

The fourth edition of *Principles* is divided into twenty-two parts, an introductory section, and an Epilogue. The organization remains largely unchanged, combining the prerequisites for a general understanding of cellular differentiation and tissue growth and development, the tools and theoretical information needed to design tissues and organs, as well as a presentation by the world's experts of what is currently known about each specific organ system. As in previous editions, we have striven to create a comprehensive book that, on one hand, strikes a balance among the diversity of subjects that are related to tissue engineering, including biology, chemistry, material science, and engineering, among others, while emphasizing those research areas that are likely to be of clinical value in the future.

While we cannot describe all of the new and updated material of the fourth edition, we have expanded and given added emphasis to stem cells, including adult, embryonic, and induced pluripotent stem cells and progenitor populations that may soon lead to new tissue engineering therapies for heart disease, diabetes, and a wide variety of other diseases that afflict humanity. This up-to-date coverage of stem cell biology and other emerging technologies is complemented by two new chapters on the challenges of tissue engineering for food and *in vitro* meat production, which someday may end up a routine part of our food system, potentially reducing environmental pollution and land use. The result is a comprehensive textbook that we believe will be useful to students and experts alike.

Robert Lanza

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Joseph P. Vacanti

PREFACE TO THE THIRD EDITION

The third edition of *Principles of Tissue Engineering* attempts to incorporate the latest advances in the biology and design of tissues and organs and simultaneously to connect the basic sciences — including new discoveries in the field of stem cells — with the potential application of tissue engineering to diseases affecting specific organ systems. While the third edition furnishes a much-needed update of the rapid progress that has been achieved in the field since the turn of the century, we have retained those facts and sections that, while not new, will assist students and general readers in understanding this exciting area of biology.

The third edition of 'Principles' is divided into 22 parts plus an introductory section and an epilogue. The organization remains largely unchanged from previous editions, combining the prerequisites for a general understanding of tissue growth and development, the tools and theoretical information needed to design tissues and organs, and a presentation by the world's experts on what is currently known about each specific organ system. As in previous editions, we have striven to create a comprehensive book that, on one hand, strikes a balance among the diversity of subjects that are related to tissue engineering, including biology, chemistry, materials science, and engineering, while emphasizing those research areas likely to be of clinical value in the future.

No topic in the field of tissue engineering is left uncovered, including basic biology/mechanisms, biomaterials, gene therapy, regulation and ethics, and the application of tissue engineering to the cardiovascular, hematopoietic, musculoskeletal, nervous, and other organ systems. While we cannot describe all of the new and updated material of the third edition, we can say that we have expanded and given added emphasis to stem cells, including adult and embryonic stem cells, and progenitor populations that may soon lead to new tissue engineering therapies for heart disease, diabetes, and a wide variety of other diseases that afflict humanity. This up-to-date coverage of stem cell biology and other emerging technologies is complemented by a series of new chapters on recent clinical experience in applying tissue engineering. The result is a comprehensive book that we believe will be useful to students and experts alike.

Robert Lanza

Robert Langer

Joseph Vacanti

PREFACE TO THE SECOND EDITION

The first edition of this textbook, published in 1997, was rapidly recognized as the comprehensive textbook of tissue engineering. This edition is intended to serve as a comprehensive text for the student at the graduate level or the research scientist/physician with a special interest in tissue engineering. It should also function as a reference text for researchers in many disciplines. It is intended to cover the history of tissue engineering and the basic principles involved, as well as to provide a comprehensive summary of the advances in tissue engineering in recent years and the state of the art as it exists today.

Although many reviews had been written on the subject and a few textbooks had been published, none had been as comprehensive in its defining of the field, description of the scientific principles and interrelated disciplines involved, and discussion of its applications and potential influence on industry and the field of medicine in the future as the first edition.

When one learns that a more recent edition of a textbook has been published, one has to wonder if the base of knowledge in that particular discipline has grown sufficiently to justify writing a revised textbook. In the case of tissue engineering, it is particularly conspicuous that developments in the field since the printing of the first edition have been tremendous. Even experts in the field would not have been able to predict the explosion in knowledge associated with this development. The variety of new polymers and materials now employed in the generation of engineered tissue has grown exponentially, as evidenced by data associated with specialized applications. More is learned about cell/biomaterials interactions on an almost daily basis. Since the printing of the last edition, recent work has demonstrated a tremendous potential for the use of stem cells in tissue engineering. While some groups are working with fetal stem cells, others believe that each specialized tissue contains progenitor cells or stem cells that are already somewhat committed to develop into various specialized cells of fully differentiated tissue.

Parallel to these developments, there has been a tremendous 'buy in' concerning the concepts of tissue engineering not only by private industry but also by practicing physicians in many disciplines. This growing interest has resulted in expansion of the scope of tissue engineering well beyond what could have been predicted five years ago and has helped specific applications in tissue engineering to advance to human trials.

The chapters presented in this text represent the results of the coordinated research efforts of several hundred scientific investigators internationally. The development of this text in a sense parallels the development of the field as a whole and is a true reflection of the scientific cooperation expressed as this field evolves.

Robert Lanza

Robert Langer

Joseph Vacanti

PREFACE TO THE FIRST EDITION

Although individual papers on various aspects of tissue engineering abound, no previous work has satisfactorily integrated this new interdisciplinary subject area. *Principles of Tissue Engineering* combines in one volume the prerequisites for a general understanding of tissue growth and development, the tools and theoretical information needed to design tissues and organs, as well as a presentation of applications of tissue engineering to diseases affecting specific organ system. We have striven to create a comprehensive book that, on the one hand, strikes a balance among the diversity of subjects that are related to tissue engineering, including biology, chemistry, materials science, engineering, immunology, and transplantation among others, while, on the other hand, emphasizing those research areas that are likely to be of most value to medicine in the future.

The depth and breadth of opportunity that tissue engineering provides for medicine is extraordinary. In the United States alone, it has been estimated that nearly half-a-trillion dollars are spent each year to care for patients who suffer either tissue loss or end-stage organ failure. Over four million patients suffer from burns, pressure sores, and skin ulcers, over twelve million patients suffer from diabetes, and over two million patients suffer from defective or missing supportive structures such as long bones, cartilage, connective tissue, and intervertebral discs. Other potential applications of tissue engineering include the replacement of worn and poorly functioning tissues; as exemplified by aged muscle or cornea; replacement of small caliber arteries, veins, coronary, and peripheral stents; replacement of the bladder, ureter, and fallopian tube; and restoration of cells to produce necessary enzymes, hormones, and other bioactive secretory products.

Principles of Tissue Engineering is intended not only as a text for biomedical engineering students and students in cell biology, biotechnology, and medical courses at advanced undergraduate and graduate levels, but also as a reference tool for research and clinical laboratories. The expertise required to generate this text far exceeded that of its editors. It represents the combined intellect of more than eighty scholars and clinicians whose pioneering work has been instrumental to ushering in this fascinating and important field. We believe that their knowledge and experience have added indispensable depth and authority to the material presented in this book and that in the presentation, they have succeeded in defining and capturing the sense of excitement, understanding, and anticipation that has followed from the emergence of this new field, tissue engineering.

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William Chick

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Introduction to Tissue Engineering

1. The History and Scope of Tissue Engineering
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CHAPTER 1

The History and Scope of Tissue Engineering

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INTRODUCTION

From the beginning, humans have yearned for a better existence for themselves, those they love, and for the human race. We have always sought ways to improve the human condition. Injury, disease and congenital malformation have always been part of the human experience. If only damaged bodies could be restored, life could go on for loved ones, as though tragedy had not intervened. In recorded history, this desire was first manifested through myth and magic as in the Greek legend of Prometheus and eternal liver regeneration. Then legend gave way to miracles with the creation of Eve in 'Genesis', or the miraculous transplantation of a limb by the Saints Cosmos and Damien. With the introduction of the scientific method came new understanding of the natural world. The methodical unraveling of the secrets of biology was coupled with the scientific understanding of disease and trauma. Artificial or prosthetic materials for replacing limbs, teeth and other tissues resulted in the partial restoration of lost function. Also the concept of using one tissue as a replacement for another was developed.

In the sixteenth century, Tagliacozzi of Bologna, Italy reported in his work 'Decusorum Chirurgia per Insitionem' a description of a nose replacement that he constructed from a forearm flap. In the nineteenth century, through the scientific understanding of the germ theory of disease and the introduction of sterile technique, modern surgery had its emergence. The advent of anesthesia by the mid-nineteenth century enabled the rapid evolution of many surgical techniques. With anesthetized patients, innovative and courageous surgeons could save lives by examining and treating internal areas of the body: the thorax, the abdomen, the brain, and the heart. Initially, surgical techniques were primarily extirpative — for example, removal of tumors, bypass of the bowel in the case of intestinal obstruction, and repair of life-threatening injuries. Maintenance of life without regard to the crippling effects of tissue loss or the psychosocial impact of disfigurement however was not an acceptable end goal. Techniques that resulted in the restoration of function through structural replacement became integral to the advancement of human therapy.

Now, whole fields of reconstructive surgery have emerged to improve the quality of life by replacing missing function through rebuilding the body's structures. In our current era, modern techniques of transplanting tissue and organs from one individual into another have been revolutionary and lifesaving. The molecular and cellular events of the immune response have been elucidated sufficiently to suppress the response in the clinical setting of

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transplantation and to produce prolonged graft survival and function in patients. In a sense, transplantation can be viewed as the most extreme form of reconstructive surgery; transferring tissue from one individual into another. As with any successful undertaking, new problems have emerged. Techniques using implantable foreign body materials have produced dislodgment, infection at the foreign body/tissue interface, fracture, and migration over time. Techniques moving tissue from one position to another position have produced biologic changes because of the abnormal interaction of the tissue in its new location. For example, diverting urine into the colon can produce fatal colon cancers 20—30 years later. Making esophageal tubes from the skin can result in skin tumors 30 years later. Using intestine for urinary tract replacement can result in severe scarring and obstruction over time.

Transplantation from one individual into another, although very successful, has severe constraints. The major problem is accessing enough tissue and organs for all of the patients who need them. As of this writing in 2012, 115,940 people are on transplant waiting lists in the United States, and many will die while waiting for available organs. Also, problems with the immune system produce chronic rejection and destruction over time. Creating an imbalance of immune surveillance from immunosuppression can cause new tumor formation. The constraints have produced a need for new solutions to provide needed tissue.

It is within this context that the field of tissue engineering has emerged. In essence, new and functional living tissue is fabricated using living cells, which are usually associated in one way or another with a matrix or scaffolding to guide tissue development. New sources of cells, including many types of stem cells, have been identified in the past few years, igniting new interest in the field. In fact, the emergence of stem cell biology has led to a new term; regenerative medicine.

Progress in this field has been so rapid that the 2012 Nobel Prize in Physiology or Medicine was awarded to Drs. Gurdon and Yamanaka for their insights into re-programming of cells and for the production of induced pluripotent stem cells (iPS cells). The last edition of this book had no mention of this technology — such has been the progress.

Scaffolds can be natural, man-made, or a composite of both. A major advance in scaffolding has occurred with the description of gentle decellularization of vital organs with preservation of the architecture of the vascular supply by Harald Ott and colleagues. By perfusion through the skeleton of blood vessels in a bioreactor, cells can be reintroduced and therefore re-animate the organ before transplantation. Again, this technology was not described in our last edition. Living cells can migrate into the implant after implantation, or can be associated with the matrix in cell culture before implantation. Such cells can be isolated as fully differentiated cells of the tissue they are hoped to recreate, or they can be manipulated to produce the desired function when isolated from other tissues or stem cell sources. Conceptually, the application of this new discipline to human health care can be thought of as a refinement of previously defined principles of medicine. The physician has historically treated certain disease processes by supporting nutrition, minimizing hostile factors, and optimizing the environment so that the body can heal itself.

In the field of tissue engineering, the same thing is accomplished on a cellular level. The harmful tissue is eliminated; the cells necessary for repair are then introduced in a configuration which optimizes the survival of the cells in an environment that will then permit the body to heal itself. Tissue engineering offers an advantage over cell transplantation alone, in that organized three-dimensional functional tissue is designed and developed. This chapter summarizes some of the challenges that must be resolved before tissue engineering can become part of the therapeutic armamentarium of physicians and surgeons. Broadly speaking, the challenges are scientific and social.

SCIENTIFIC CHALLENGES

As a field, tissue engineering has been defined for barely two decades. As in any new undertaking, its roots are firmly implanted in what went before. Any discussion of when

the field began is inherently fuzzy. Much still needs to be learned and developed to provide a firm scientific basis for therapeutic application. To date, much of the progress in this field has been related to the development of model systems, which have suggested a variety of approaches. Also, certain principles of cell biology and tissue development have been delineated. The field can draw heavily on the explosion of new knowledge from several interrelated well-established disciplines, and in turn, may promote the coalescence of relatively new, related fields to achieve their potential. The rate of new understanding of complex living systems has been explosive in the past three decades. Tissue engineering can draw on the knowledge gained in the fields of cell and stem cell biology, biochemistry, and molecular biology and apply it to the engineering of new tissues. Likewise, advances in materials science, chemical engineering, and bioengineering allow the rational application of engineering principles to living systems. Yet another branch of related knowledge is the area of human therapy as applied by surgeons and physicians. In addition, the fields of genetic engineering, cloning, and stem cell biology may ultimately develop hand in hand with the field of tissue engineering in the treatment of human disease, each discipline depending on developments in the others.

We are in the midst of a biologic renaissance. Interactions of the various scientific disciplines can elucidate not only the potential direction of each field of study, but also the right questions to address. The scientific challenge in tissue engineering lies both in understanding cells and their mass transfer requirements and the fabrication of materials to provide scaffolding and templates.

CELLS

If we postulate that living cells are required to fabricate new tissue substitutes, much needs to be learned in regard to their behavior in two normal circumstances — namely, normal development in morphogenesis and normal wound healing. In both of these circumstances, cells create or recreate functional structures using pre-programmed information and signaling. Some approaches to tissue engineering rely on guided regeneration of tissue using materials that serve as templates for ingrowth of host cells and tissue. Other approaches rely on cells that have been implanted as part of an engineered device. As we understand normal developmental and wound healing gene programs and cell behavior, we can use them to our advantage in the rational design of living tissues.

Acquiring cells for creation of body structures is a major challenge, the solution of which continues to evolve. The ultimate goal in this regard — the large-scale fabrication of structures — may be to create large cell banks composed of universal cells that would be immunologically transparent to an individual. These universal cells could be differentiated cell types that could be accepted by an individual or could be stem cell reservoirs, which could respond to signals to differentiate into differing lineages for specific structural applications. Much is already known about stem cells and cell lineages in the bone marrow and blood. Studies suggest that progenitor cells for many differentiated tissues exist within the marrow and blood, and may very well be ubiquitous. Our knowledge of the existence and behavior of such cells in various mesenchymal tissues (muscle, bone and cartilage), endodermally derived tissues (intestine and liver), or ectodermally derived tissues (nerves, pancreas, and skin) expands on a daily basis.

The recent description of iPS cells is so promising that they may be the holy grail of therapeutic stem cells in unlimited numbers and with patient specificity to be immunologically neutral. These cells also avoid the ethical and regulatory pitfalls of embryonic stem cells. Much remains to be learned, but data continues to be positive.

As intermediate steps, tissue can be harvested as allograft, autograft, or xenograft. The tissues can then be dissociated and placed into cell culture, where proliferation of cells can be initiated. After expansion to the appropriate cell number, the cells can then be transferred to

templates, where further remodeling can occur. Which of these strategies are practical and possibly applicable in humans remains to be explored.

Large masses of cells for tissue engineering need to be kept alive, not only *in vitro* but also *in vivo*. The design of systems to accomplish this, including *in vitro* flow bioreactors and *in vivo* strategies for maintenance of cell mass, presents an enormous challenge towards which significant advances have been made. The fundamental biophysical constraint of mass transfer of living tissue needs to be understood and dealt with on an individual basis as we move toward human application.

MATERIALS

There are so many potential applications to tissue engineering that the overall scale of the undertaking is enormous. The field is ripe for expansion, and requires training of a generation of materials scientists and chemical engineers.

The optimal chemical and physical configurations of new biomaterials as they interact with living cells to produce tissue-engineered constructs are under study by many research groups. These biomaterials can be permanent or biodegradable. They can be naturally occurring, synthetic or hybrid materials. They need to be developed to be compatible with living systems or with living cells *in vitro* and *in vivo*. Their interface with the cells and the implant site must be clearly understood so that the interface can be optimized. Their design characteristics are major challenges for the field, and should be considered at a molecular chemical level. Systems can be closed, semi-permeable, or open. Each design should factor in the specific replacement therapy considered. Design of biomaterials can also incorporate the biologic signaling that the materials may offer. Examples include release of growth and differentiation factors, design of specific receptors and anchorage sites, and three-dimensional site specificity using computer-assisted design and manufacture techniques. New nanotechnologies have been incorporated to design systems of extreme precision. Combining computational modeling with nano-fabrication can produce microfluidic circulations to nourish and oxygenate new tissues.

GENERAL SCIENTIFIC ISSUES

As new scientific knowledge is gained, many conceptual issues need to be addressed. Related to mass transfer is the fundamental problem associated with nourishing tissue of large mass as opposed to tissue with a relatively high ratio of surface area to mass. Also, functional tissue equivalents necessitate the creation of composites containing different cell types. For example, all tubes in the body are laminated tubes composed of a vascularized mesodermal element such as smooth muscle, cartilage, or fibrous tissue. The inner lining of the tube, however, is specific to the organ system. Urinary tubes have a stratified transitional epithelium. The trachea has a pseudostratified columnar epithelium. The esophagus has an epithelium that changes along the gradient from mouth to stomach. The intestine has an enormous, convoluted surface area of columnar epithelial cells that migrate from a crypt to the tip of the villus. The colonic epithelium is, again, different for the purposes of water absorption and storage.

Even the well-developed manufacture of tissue-engineered skin used only the cellular elements of the dermis for a long period of time. Attention is now focusing on creating new skin, consisting of both the dermis and its associated fibroblasts, as well as the epithelial layer consisting of keratinocytes. Obviously, this is a significant advance, but for truly 'normal' skin to be engineered, all of the cellular elements should be contained, so that the specialized appendages can be generated as well. These 'simple' composites will indeed prove to be quite complex and require intricate designs. Thicker structures with relatively high ratios of surface area to mass, such as liver, kidney, heart, breast, or central nervous system, will offer engineering challenges.

Currently, studies for developing and designing materials in three-dimensional space are being developed utilizing both naturally occurring and synthetic molecules. The applications of computer-assisted design and manufacture techniques to the design of these matrices are critically important. Transformation of digital information obtained from magnetic resonance scanning or computerized tomography scanning can then be developed to provide appropriate templates. Some tissues can be designed as universal tissues that will be suitable for any individual, or may be custom-developed tissues specific to one patient. An important area for future study is the entire field of neural regeneration, neural ingrowth, and neural function toward end organ tissues such as skeletal or smooth muscle. Putting aside the complex architectural structure of these tissues, the cells contained in them have a very high metabolic requirement. As such, it is exceedingly difficult to isolate a large number of viable cells. An alternate approach may be the use of less mature progenitor cells, or stem cells, which not only would have a higher rate of survival as a result of their lower metabolic demand, but also would be more able to survive the insult and hypoxic environment of transplantation. As stem cells develop and require more oxygen, their differentiation may stimulate the development of a vascular complex to nourish them. The understanding of and solutions to these problems are fundamentally important to the success of any replacement tissue that needs ongoing neural interaction for maintenance and function.

It has been shown that some tissues can be driven to completion *in vitro* in bioreactors. However, optimal incubation times will vary from tissue to tissue. Even so, the new tissue will require an intact blood supply at the time of implantation for successful engraftment and function.

Finally, all of these characteristics need to be understood in the fourth dimension of time. If tissues are implanted in a growing individual, will the tissues grow at the same rate? Will cells taken from an older individual perform as young cells in their new 'optimal' environment? How will the biochemical characteristics change over time after implantation? Can the strength of structural support tissues such as bone, cartilage, and ligaments be improved in a bioreactor in which force vectors can be applied? When is the optimal timing of this transformation? When does tissue strength take over the biochemical characteristics as the material degrades?

SOCIAL CHALLENGES

If tissue engineering is to play an important role in human therapy, in addition to scientific issues, fundamental issues that are economic, social, and ethical in nature will arise. Something as simple as a new vocabulary will need to be developed and uniformly applied. A universal problem is funding. Can philanthropic dollars be accessed for the purpose of potential new human therapies? Will industry recognize the potential for commercialization and invest heavily? If this occurs, will the focus be changed from that of a purely academic endeavor? What role will governmental agencies play as the field develops? How will the field be regulated to ensure its safety and efficacy prior to human application? Is the new tissue to be considered transplanted tissue and, therefore, not be subject to regulation, or is it a pharmaceutical that must be subjected to the closest scrutiny by regulatory agencies? If lifesaving, should the track be accelerated toward human trials?

There are legal ramifications of this emerging technology as new knowledge is gained. What becomes proprietary through patents? Who owns the cells that will be sourced to provide the living part of the tissue fabrication?

In summary, one can see from this brief overview that the challenges in the field of tissue engineering remain significant. All can be encouraged by the progress that has been made in the past few years, but much discovery lies ahead. Ultimate success will rely on the dedication, creativity, and enthusiasm of those who have chosen to work in this exciting but still

unproved field. Quoting from the epilogue of the previous edition; 'At any given instant in time, humanity has never known so much about the physical world and will never again know so little.'

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CHAPTER 2

The Challenge of Imitating Nature

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INTRODUCTION

Since the first author on this chapter wrote in the first edition of this book about 'the challenge of imitating nature', the field of tissue engineering has evolved considerably, into what many now call regenerative medicine [1]. This original chapter was written in the late 1990s, when stem cell research was just beginning to accelerate. Since then our knowledge of stem cells and also progenitor cells has greatly expanded, and we now are in a much better position to develop clinical therapies. As was stated then, through the imitation of nature this field has the potential to confront the transplantation crisis, i.e., the crisis caused by the shortage of donor tissues and organs. Data from the Organ Procurement and Transplantation Network indicates that there are more than 110,000 patients in the US waiting for organs, and in contrast in 2011 there were only 28,000 transplantation procedures performed. Compared to 20 years earlier, this represents a four-fold increase in the number of patients on the waiting list for an organ transplant and not even a two-fold increase in transplantation procedures. Perhaps even more important than addressing the transplantation crisis is that, through the imitation of nature, there is the potential for tissue engineering and regenerative medicine to address important, but currently untreatable patient needs. This may be the 'holy grail' for this field; however, if we are to be successful in this, there are a variety of issues that need to be faced.

An underlying premise of this field of tissue engineering and regenerative medicine is that the utilization of the natural biology of the system will allow for greater success in developing therapeutic strategies aimed at the replacement, maintenance, and/or repair of tissue and organ function. Another way of saying this is that just maybe the great creator, in whatever form one believes he or she exists, knows something that we mere mortals do not, and if we can only 'tap' into a small part of this knowledge base, if we can only imitate nature in some small way, then we will be able to achieve greater success in our efforts to address patient needs. It is this challenge of imitating nature that has been accepted by those who are providing leadership to this new area of technology originally called tissue engineering [2,3]. To imitate nature requires that we first understand the basic biology of the tissues and organs of interest, including developmental biology; with this we then can develop methods for the control of these biological processes; and based on the ability to control, we finally can develop strategies either for the engineering of living tissue substitutes or for the fostering of tissue repair or regeneration.

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It should be noted that the concept of a more biologic approach dates back more than 70 years [4]. Since then there has been a large expansion in research efforts in this field, and a considerable recognition of the enormous potential that exists. With this hope, there also has been a lot of hype; however, the future long term remains bright [5,6]. As the technology has become further developed, an industry has begun to emerge. This industry has been very much a fledgling one, with only a few companies possessing product income streams, largely in the skin and orthopedic area, an industry that the first author of this chapter has referred to in the past as being only 'skin deep'; however, from the 'go-go' years of the 1990s, to the more 'sobering years' at the beginning of this century, and then rebounding to today, there now is extensive industrial activity in progress [7]. More recently it has been estimated that there now are more than 175 products in the regenerative medicine pipeline [8]. These products are in various stages of development; however, at least 125 of these are cell-based. Furthermore, an update on 2008 Lysaght et al. study reports a three-fold growth in commercial sales, with 106 companies employing 14,000 employees [9].

Although research in what we now call tissue engineering began to accelerate more than a quarter of a century ago and there were references to tissue engineering in the early 1980s, the term tissue engineering only took on a 'life of its own' when it was 'coined' in 1987 by Professor Y. C. Fung from the University of California, San Diego — who suggested this name at a National Science Foundation meeting. This led to the first meeting called 'tissue engineering' that was held in early 1988 at Lake Tahoe, California [10]. More recently the term regenerative medicine has come into use. For some this is a 'code word' for stem cell technology, while for others regenerative medicine is the broader term, with tissue engineering representing only replacement, not repair and/or regeneration. Still others use the terms tissue engineering and regenerative medicine interchangeably. Whatever name you choose to use, what is important is that it is a more biologic approach that has the potential to lead to new patient therapies and treatments, in some cases where none are currently available.

Tissue engineering also can be viewed as being at the interface of the traditional medical implant industry and the biological revolution. By harnessing the advances of this revolution there will be an entirely new generation of tissue and organ implants as well as strategies for repair and regeneration. Already we are beginning to see increased investments in this field by the large medical device companies, and there has been in more recent years a recognition that there is a convergence of biologics and devices [11]. There also is an increased interest on the part of the pharmaceutical and biotech industries. Long term there is the potential for a literal revolution in medicine; however, this revolution will only occur if we successfully meet the challenge of imitating nature.

As has already been stated, there are a number of issues that need to be addressed. The fourth edition of this book examines these for a variety of enabling technologies and tissue/organ applications. The purpose of this chapter is thus to 'set the stage'; i.e., to provide a foundation for what is to come later in this book. This starts with the next section of this chapter focusing on the basic paradigm that underlies the different approaches that are being taken.

THE BASIC PARADIGM

Tissue engineering in its evolution to regenerative medicine now comprises replacement, repair and regeneration. It is the concept of replacement that dates back the furthest. In this one fabricates a replacement tissue or organ outside of the body which then is implanted within the body. Some of the initial successes were in this category of replacement. This includes such skin substitutes as Integra, a product of Integra Life Sciences and approved by Food and Drug Administration (FDA) in 1996; Apligraf, a product of Organogenesis approved by FDA in 1997; and Dermagraft, a product of Advanced Tissue Sciences approved by FDA in 1999. Dermagraft is now sold by Advanced Biohealing which has been acquired by Shire Medical. Using

Integra as an example, the research on this approach was published in a journal in 1982 [12]; however, as already noted, FDA approval did not come until 1996, a gap of 14 years. This indicates that this field is not only about patients, but that it also requires patience. Furthermore, although all three of these skin substitutes were developed as a 'replacement', these in fact were wound healing implants and were regulated by the US FDA through the Center for Devices and Radiological Health in the category of wound healing. Thus, they were really in the repair category and not replacement. Today these might well be regulated as biologics, and one can only speculate about the length of time today that would be required for FDA approval.

Of course there will be applications where the best approach will be one of replacement. An example of this is the need for a bioartificial pancreas for patients with Type 1 diabetes. Such an implant is under development, and it would involve cells that are glucose responsive and insulin secreting, encapsulated so as to provide immunoprotection since these in most cases would not be cells from the patient. The cells to be used might in fact be derived from embryonic stem cells. Although such a bioartificial pancreas would not have anywhere near all of the functional characteristics of the normal pancreas, it would be a major step forward. Another example would be the tissue engineering of a heart valve replacement. Here it is the pediatric population for whom the need is critical. If an infant has a heart valve defect and thus needs a replacement, as that infant grows into a child and ultimately an adult, there will need to be a series of surgeries so as to implant a prosthetic valve with an ever-increasing diameter. If one could tissue engineer a heart valve that would grow with the child, this would have a major impact on pediatric patients with heart valve defects.

There of course are other examples where replacement will be the preferred approach; however, for many applications the tissue or organ of interest is just so biologically complex that an approach of repair and/or regeneration might be a better strategy than one of replacement. This leads one to what might be considered a basic paradigm for tissue engineering and regenerative medicine. This is illustrated in Fig. 2.1 where the three 'pillars' are cells, scaffolds, and signals. The cells could be stem cells or progenitor cells or even fully differentiated cells. It might be a combination of cell types, even what might be called a primary cell with a partner cell. The scaffolds could either be made of a synthetic biomaterial or be a natural extracellular matrix. Whatever the scaffold, it might be needed in the fabrication of a replacement tissue or used as a delivery vehicle for the cells being used in a cell therapy. The signals could be a variety of types, including growth factors and chemotactic factors. These signals might be ones secreted by the cells employed; in fact, if one actually knew the signals that are necessary, perhaps one would not need cells as one could simply deliver the right signals, however, such a molecular therapy is long ways from being realized.

It should be noted that one of the most successful tissue-engineered products is the small intestine submucosa (SIS). This extracellular matrix product is porcine derived and acellular in nature. It has been used in a variety of applications including both rotator cuff and hernia repair [13,14]. Here SIS is providing the necessary signals to facilitate repair. To date more than two million patients have been treated.

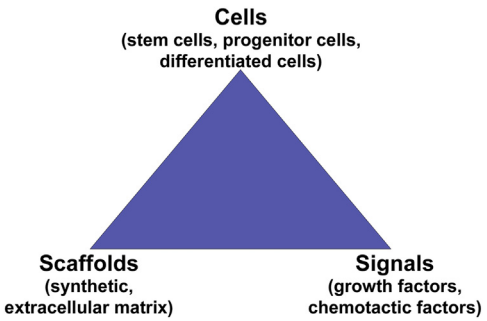

FIGURE 2.1
Illustration of the basic paradigm for tissue engineering and regenerative medicine showing the three 'pillars', i.e., cells, scaffolds, and signals.

If it is the signals that are important, then we need to think about these in a broader way, as the functional characteristics of cells are determined by the cues, i.e., the signals, which make up their microenvironment. This is true *in vivo*, where what might be called 'nature's orchestra' is made up of a 'symphony of signals'. What makes up this symphony? It includes soluble molecules, the extracellular matrix/substrate to which the cells are attached, cell-cell contact, and the mechanical/physical force environment in which the cell resides. Even though everyone recognizes that the *in vivo* microenvironment of a cell is extremely complex, most of the scientific literature does not investigate this 'symphony of signals' but rather is focused on 'soloists', i.e., a single growth factor or a single chemotactic factor. This might be viewed as the reductionist approach, but it does not help us understand the synergy between different signals. It is because of this synergy that we need to go beyond simply understanding 'soloists'. It may be unrealistic to investigate the entire 'symphony' to which cells are exposed, but we at least need to begin to understand how combinations of signals influence cell function. Initially it may not be a symphony that we investigate, but perhaps a 'duet' of signals, maybe even a 'quartet'.

This leads to one final consideration. In learning about 'nature's orchestra' perhaps we should start with what is known about developmental biology. Granted that what we want to do in tissue engineering and regenerative medicine in adults needs to take place on a geometric scale much larger than that in the embryo and with a timescale that will be much shorter. Still, we know that there are species that can regrow their tissues and organs. This has fascinated biologists for at least 200 years; however, for this to happen in a human has always appeared to be in the realm of science fiction. But is it? Perhaps we can learn the nature of the signaling molecules that result in the regeneration of the zebra fish's heart as opposed to the scarring that takes place in the adult human after a major wound. If we can, then we might better understand what the important 'players' are in what we want to do, what we need to do.

The models used in the study of developmental biology are simply one avenue that needs to be focused on in the future. There are other models as well, and these all can contribute to the understanding needed to more fully imitate nature in our therapeutic approaches. These various models are the subject of the next section of this chapter.

MODELING NATURE'S ORCHESTRA

Beyond learning from developmental biology, there are other avenues that need to be pursued by the tissue engineering and regenerative medicine community as it tries to better understand the 'symphony of signals' that are part of nature's orchestra and to address the challenge of imitating nature. This includes learning from those human tissues that do regenerate, the use of animal models, the employment of *in vitro* systems, and also computational approaches. These will be addressed here one by one.

Although most adult human tissues do not repair and/or regenerate, there are examples that in fact do. These include skin and liver, and also in the fetus [15]. For skin, the regenerative process [16] in the adult leads to a recovery of physiological anatomy that is imperfect. In contrast, healing in the early stages of fetal development appears to lead to complete restoration [17]. An entirely unique human tissue that regenerates once a month is the endometrium. It is an amazing tissue in many ways, especially when discussing liver regeneration.

The regenerative capacity of the liver has been known since the time of the ancient Greeks, and the fascination with this remarkable regenerative organ has resulted in a long history of research into the orchestration of liver regeneration. For reviews on liver regeneration see Micholopoulos and DeFrances [18] and Gilgenkrantz et al. [19]. Liver regeneration is possible when even up to 70 percent of the organ is damaged or removed; however, the regenerated tissue is not a recreation of the lost lobes but instead is a compensatory growth of the remaining tissue. Studies utilizing partial hepatectomy suggest that mature liver cells are responsible for growth of the tissue; however, more recent studies of chronic disease suggest

that progenitor cells are key players. The search for the symphony of signals that starts the regenerative process continues, and many of the important growth factors and cytokines in the orchestra have been identified, including hepatocyte growth factor, epidermal growth factor, interleukin-6, tumor necrosis factor-α and transforming growth factor-α.

The liver has a remarkable capacity for regeneration, but it is not unlimited. Liver failure during transplantation will occur when the graft weight to body weight ratio is less than 0.8% for the recipient, or the remaining donor tissue consists of less than 25% of the functional liver. In a chronically diseased liver, regeneration is often impaired in part due to mito-inhibitory factors. Much of what we have learned about the symphony of signals in liver regeneration and failure comes from animal models. Animal studies began in the late 1800s with canine models and has progressed using canine, rabbit, primate, feline, porcine and rodent animal models, with the latter two models being the most utilized today. An in depth review of the various animal models used to study liver regeneration is detailed by Mortenson and Revhaug [20].

As noted earlier, the human endometrium is an amazing tissue, one where rapid scarless regeneration occurs monthly [21]. The endometrium is the innermost layer of the uterus, and it consists of a basalis layer and a functionalis layer onto which an embryo implants. The functionalis layer makes up two thirds of the endometrium and is shed in the absence of pregnancy. In contrast to many other regenerative tissues such as the fetus, this amazing symphony of events takes place in a highly inflammatory environment. The 'conductors' of a woman's monthly endometrial cycle are the ovarian hormones, estrogen and progesterone. The rapid decrease of progesterone triggers a carefully conducted symphony of signals that results in menstruation. Regeneration occurs simultaneously with the tissue destruction making it difficult to study and detect [22]. Endometrial stromal cell proliferation, angiogenesis and the recreation of the microvasculature, and re-epithelialization of the ducts and the endometrial surface occur during the menstrual and proliferative phases of the cycle. Re-epithelialization of the surface by basal gland cells is completed by day two of the cycle, although it is unclear if the epithelial repopulation is due to migration and proliferation of mature epithelial cells or stem cells resident in the glands. While progesterone is a known conductor for the initiation of menstruation, it is unclear what role estrogen plays in regeneration. Estrogen levels increase in the proliferative phase, but there is evidence that estrogen is not essential for complete regeneration [23].

One reason for our limited knowledge on the mechanisms of endometrial regeneration is the lack of animal models. These are limited as menstruation is a rather unique process occurring only in women, a few old world primates, the elephant shrew, and fruit bats. The menstruation models that do exist are reviewed in Salamonsen and Guidice [24]. The macaque is the most utilized primate model due to the similarities in anatomy and menstrual cycle, while the baboon has been widely used in endometriosis studies. Rodent models are now available as menstruation has been induced in mice; alternatively, xenotransplants of human endometrium in a mouse model have been utilized under hormone regulated conditions. These models will help further our understanding of this scarless regenerative process, but none completely recreate the inflammatory human menstrual cycle.

Animal models of course are used in a wide variety of other ways. Such models range from small animals such as the mouse or the rat to large animal models such as non-human primates. Although small animal models do not in general predict what will happen in the human, they do lend themselves to carefully designed experiments that can lead to a better understanding of the signals involved in a particular cellular function. This includes the use of transgenic animals.

In vitro models have been and will continue to be a significant asset to our efforts to achieve a better understanding of signaling mechanisms. Such models already have contributed substantially to our knowledge. As an example, *in vitro* studies have made it clear that for most

cell types their characteristics in a three-dimensional structure can be very different from those when cultured in two dimensions. The exception is the vascular endothelial cell, which exists *in vivo* as a monolayer and thus has been studied successfully as a monolayer in culture. On the other hand, the vascular smooth muscle cell, which exists *in vivo* in a three-dimensional structure, has been demonstrated in culture to have very different characteristics in three dimensions as compared to two dimensions. *In vitro* studies do provide the opportunity to systematically investigate cell signaling [25]. Granted a majority of literature results are for studies of 'soloists'; however, the potential for studies of 'duets' and 'quartets' exists and has led to some relatively recent publications in this area.

As already noted, the endometrium regenerates once a month in the human female. This is a complex process, but one from which the tissue engineering and regenerative medicine community can learn. Because of the limitations of animal models and the difficulties in obtaining biopsies of proliferating endometrium, a hormonally responsive tissue-engineered *in vitro* model of the human endometrium is important if we are to understand the symphony of signals required for regeneration. The first report of a tissue-engineered endometrium was that by Bentin-Ley et al. [26]. This initial approach concentrated on replication of the tissue architecture including the establishment of a ciliated epithelial surface. Bentin-Ley et al. used a collagen I, Matrigel scaffold which is a popular choice for endometrial engineering. Fig. 2.2 shows how the collagen/Matrigel hydrogels are created; Fig. 2.3 shows the ciliated epithelial layer in a tissue-engineered endometrium created by Park et al [27] to study endometrial cancer.

Most of the groups involved in endometrial tissue engineering that have looked at tissue function have focused on receptivity and implantation [28]. Only recently has an engineered tissue been shown to respond to menstruation mimetic cues, where collagenase and MMP-2 activity increases in response to steroid withdrawal and where the shedding of cells occurs [29]. This is an important first step, necessary for the study of regeneration in an engineered tissue. Furthermore, the study of the menstrual and proliferative endometrial phases might help to define the important players in scarless regeneration.

One recent development is that of fabricating human organ-on-a-chip models. Such *in vitro* model systems are being developed for use in drug discovery, toxicity testing, and bioinfectious agents [30]. Just recently Defense Advanced Research Projects Agency (DARPA) made three five-year awards to develop up to 10 organ-on-a-chip models using human cells. A leader in this is the Wyss Institute at Harvard University. Already reported in the literature in 2010 is the development of a human 'lung-on-a-chip' model [31] and more recently a human 'gut-on-a-chip' model has been published [32]. With models such as these it is hoped that the use of animals in the development of a drug or agent might be considerably reduced. Not only are

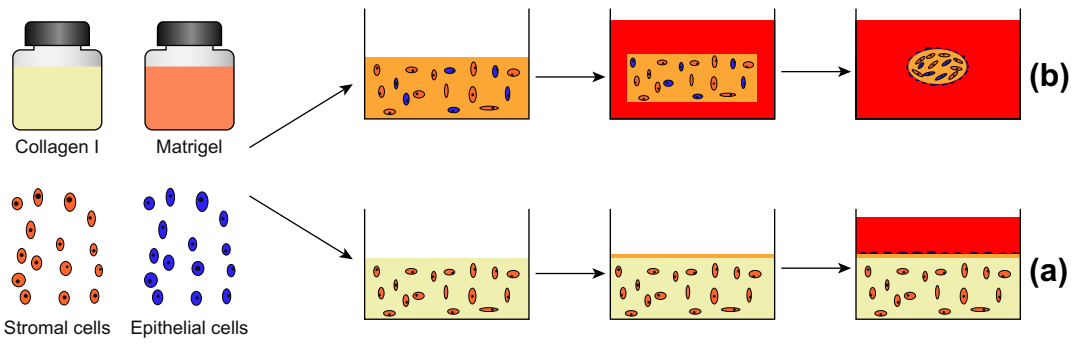

FIGURE 2.2

The most common current approach to creating a tissue-engineered endometrium is the creation of a collagen I and Matrigel cell-seeded hydrogel. A layered approach is shown (a) where collagen I and endometrial stromal cells are combined. After the solution gels a thin layer of Matrigel is added and the endometrial epithelial cells are added above. A combined approach (b) can be used where collagen I, Matrigel, and the endometrial stromal and epithelial cells are mixed together. When liberated from the well the gel will compact and the epithelial cells will eventually coat the outer surface of the tissue.

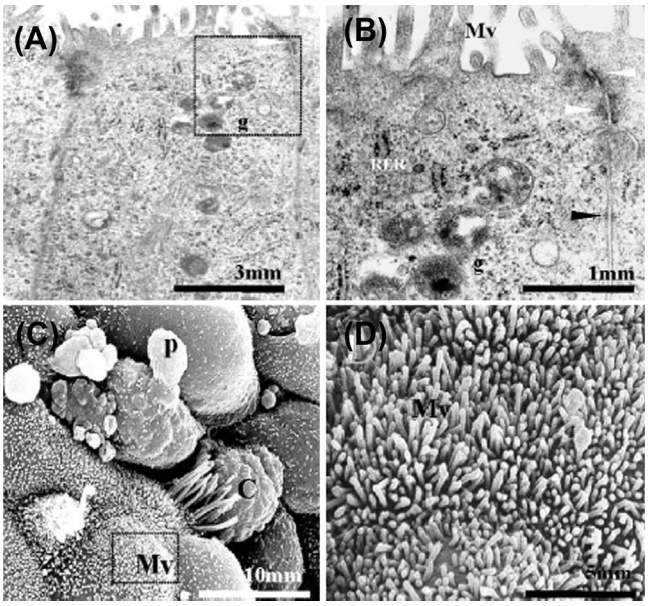

FIGURE 2.3
Electron micrographs of epithelial cell layer on a collagen/Matrigel engineered endometrium. Microvilli and tight junctions (open arrows) are seen in the transmission electron micrographs (A,B). Pinopodes (P), cilia (C) and microvilli (Mv) are seen in the scanning electron micrographs. *Reprinted from Park et al. (2003) with permission from Elsevier.*

animal studies labor intensive, time consuming, and costly, but as already noted, the results obtained also do not necessarily predict what will happen in the human.

Finally, a different kind of modeling that will become increasingly important if we are to understand nature's orchestra is that represented by computational approaches [33–35]. One of the early attempts at this for a eukaryotic cell was the computational model developed by Weisner et al. [36,37] to describe the dynamics in calcium that is observed when a vascular endothelial cell responds to the onset of flow and the associated shear stress. Since then, computational models have become much more sophisticated. Whatever the model being used in a computational approach, it will need to incorporate a hierarchy of time and length scales with many interconnected elements. Furthermore, the intracellular and extracellular dynamics may well be non-linear in nature, and a realistic model will need to include the external environment, transport phenomena, and membrane processes. In each of these not only will the chemistry be important, but also the mechanics. There also are additional complexities including genetic information and the role of miRNAs, as well as other epigenetic features. Clearly this requires a sophisticated model and with this the use of advanced engineering tools and an advanced knowledge of the biology. The development of such a sophisticated computational model initially will be based on experimental results; however, the results obtained from such a computational model can then drive the design of new experiments and may require new, innovative measurement techniques. These new experiments will then lead to an improvement in the computational model. It is this interplay of experiments and computational models that will lead to an improvement in our understanding of the signals that make up the symphony provided by nature's orchestra. And it is this improved understanding that will help us in our efforts to develop therapies that in some way imitate nature.

CELL TECHNOLOGY

The starting point for any attempt to engineer a cell-based therapy is a consideration of the cells to be employed. Not only will one need to decide whether the source to be employed is to be autologous, allogeneic, or xenogeneic, but one will need to ensure that the cells are free of pathogens and contamination of any type whatsoever. As is well known, there are both advantages and disadvantages to autologous vs. allogeneic vs. xenogeneic cells; however, it should be noted that one important consideration for any product or treatment strategy is its off-the-shelf availability. Only with this will the product find application for the wide variety of patients in need throughout the entire healthcare system including community hospitals.

The skin substitutes developed by Organogenesis (Canton, MA) and Advanced Tissue Sciences (La Jolla, CA) represented some of the first tissue-engineered products, and these in fact use allogeneic cells. The Organogenesis product, Apligraf, is a bilayer model of skin including both a dermis and an epidermis involving fibroblasts and keratinocytes that are obtained from donated human foreskin [38]. Apligraf is approved by the FDA. The first tissue-engineered product approved by the FDA was the Advanced Tissue Sciences product, TransCyte. Approved initially for third-degree burns, TransCyte is made by seeding dermal fibroblasts in a polymeric scaffold; however, once cryopreserved it becomes a non-living wound covering. Advanced Tissue Sciences also has a living cell product called Dermagraft. It is a dermis model, also with dermal fibroblasts obtained from donated human foreskin [39]. Even though the cells employed by both Organogenesis and Advanced Tissue Sciences are allogeneic, immune acceptance did not have to be engineered because both the fibroblast and the keratinocyte do not constitutively express major histocompatibility complex (MHC) II antigens.

The next generation of tissue-engineered products will involve other cell types, and the immune acceptance of allogeneic cells will be a critical issue in many cases. As an example, consider a blood vessel substitute that employs both endothelial cells and smooth muscle cells. Although allogeneic smooth muscle cells may be immune acceptable, allogeneic endothelial cells certainly would not be. Thus, for the latter, one either uses autologous cells or else engineers the immune acceptance of allogeneic cells, as will be discussed in a later section. Undoubtedly the first human clinical trials will be done using autologous endothelial cells; however, it would appear that the use of such cells would severely limit the availability of a blood vessel substitute, unless the host's own endothelial cells are recruited. Only by moving to off-the-shelf availability for the clinician does routine use become possible.

There is considerable interest in the use of stem cells, i.e., the 'mother' cells within the body, as a primary source for therapies based on cell and tissue replacement [40—42]. The excitement about stem cells reached a new height with two articles in 1998 [43,44]. These reported the isolation of the first lines of human embryonic stem cells (ESCs). Since then it has been demonstrated that one can reprogram a cell like the fibroblast into a pluripotent cell, what is called an induced pluripotent stem cell (iPS cell), first reported by Takahashi and Yamahaka [45] and further outlined by Takahashi et al. [46]. More recently, Yamanaka has provided an update [47]. Although iPS cells may not be exactly the same as ESCs, they still are of considerable interest, particularly since they can be derived from patients with various diseases and possibly even used as an autologous cell in the therapy for a specific patient. Disease-specific iPS cells also can be used to make novel cell types for drug discovery and screening. Still there are technical challenges to be addressed [48]. This includes new tools for genetic modification and reprogramming. Amniotic stem cell lines also are potentially of interest [49] as well as human umbilical cord blood [50]. The stem cell arsenal thus now includes not only ESCs, but also a variety of other stem cells types including mesenchymal stem cells and iPS cells.

In addition, there are progenitor cells of various types including ones circulating in blood. In some cases, stem cells are being used to derive progenitor cells. One example is the Geron clinical trial for spinal cord injury which, though now stopped, used progenitor cells derived from human ESCs. It may be that it is the use of progenitor cells that offers the best hope for a clinical cell-based therapy.

To take full advantage of stem cell technology, however, it will be necessary to more fully understand how a stem cell differentiates into a tissue-specific cell. This requires knowledge not just about the molecular pathways of differentiation, but even more importantly the identification of the combination of signals leading to a stem cell becoming a specific type of differentiated tissue cell. As an example, it is recognized that there are subtypes of human smooth muscle cells, and recently if was demonstrated how one could generate various human smooth muscle cell subtypes [51]. There also are recognized differences between different subtypes of endothelial cells, e.g., large vessel endothelial cells and valvular endothelial cells.

What are the signals that will drive the differentiation towards one type of endothelial cell versus the other? Only with this type of knowledge will we be able to realize the full potential of stem cells. In addition, however, we will need to develop the technology necessary to expand a cell population to the number necessary for clinical application and to do this in a controlled, reproducible manner. In addition, there are a variety of other issues related to the translation of bench-top science into clinical therapies. These will all be discussed in a later section of this chapter.

ENGINEERING FUNCTIONAL CHARACTERISTICS

Once one has selected the cell type(s) to be employed, then the next issue relates to the manipulation of the functional characteristics of a cell, i.e., the engineering of these characteristics, so as to achieve the behavior desired. This can be done either by manipulating a cell's microenvironment, e.g., its matrix, the mechanical stresses to which it is exposed, or its biochemical environment, or by manipulating a cell's genetic program. In regard to the latter, the manipulation of a cell's genetic program could be used as an ally to tissue engineering and regenerative medicine in a variety of ways. A partial list of possibilities includes the alteration of matrix synthesis; inhibition of the immune response; enhancement of non-thrombogenicity, e.g., through increased synthesis of antithrombotic agents; engineering the secretion of specific biologically active molecules, e.g., a specific insulin secretion rate in response to a specific glucose concentration; or the alteration of cell proliferation.

Much of the above is in the context of creating a cell-seeded construct that can be implanted as a tissue or organ substitute; however, the fostering of the repair or remodeling of tissue also is an essential component of tissue engineering and regenerative medicine. Here a critical issue is how to deliver the necessary biologic cues in a spatially and temporally controlled fashion so as to achieve a 'healing' environment. In the repair and/or regeneration of tissue the use of genetic engineering might take a form that is more what we would call gene therapy. An example of this would be the introduction of growth factors to foster the repair of bone defects. In using a gene therapy approach, it should be recognized that in many cases only a transient expression would be required. Because of this, the use of gene therapy as a strategy in tissue engineering and regenerative medicine may become viable prior to its wide employment in treating genetically related diseases.

With the selection of a source of cells, the next challenge in imitating nature is to develop an organized three-dimensional architecture, one with functional characteristics such that a specific tissue is mimicked. In this it is important to recognize the importance of a cell's microenvironment in determining its function. As already noted, *in vivo* a cell's function is orchestrated by a symphony of signals, a symphony that includes soluble molecules, the mechanical environment to which the cell is exposed, and the extracellular matrix. If we want an organized tissue substitute that replicates the characteristics of native tissue, then attention must be given to each of these components of a cell's microenvironment. If the approach is to seed cells into a scaffold, then a basic question is what type of scaffold? Also, for a given application will it be desirable for the cells to make their own matrix?

The design and engineering of a tissue-like substitute is a challenge in its own right. There are many possible approaches, and one of these, of course, is a cell-seeded polymeric scaffold, an approach pioneered by Langer and his collaborators [2,52]. This is the technology that was used by Advanced Tissue Sciences, and many consider this the classic tissue engineering approach. Another is the use of a cell-seeded collagen gel. This approach was pioneered by Bell in the late 1970s and early 1980s [53,54], and this is being used by Organogenesis in their skin substitute, Apligraf.

A rather intriguing approach is that of Auger and his group in Quebec, Canada [55,56]. Auger refers to this as cell self-assembly, and it involves a layer of cells secreting their own matrix,

which over a period of time becomes a sheet. Originally developed as part of the research on skin substitutes by Auger's group, it has been extended to other applications. For example, the blood vessel substitute developed in Quebec involves rolling up one of these cell self-assembled sheets into a tube. One can in fact make tubes of multiple layers so as to mimic the architecture of a normal blood vessel. A simpler version of this approach is being used to fabricate a tissue-engineered blood vessel at a company called Cytograft Tissue Engineering Inc. Here the initial application is for dialysis, and the blood vessel substitute only involves a single layer, one that is created from a fibroblast sheet. An expanded clinical study carried out by Cytograft was published a few years ago [57,58]. Cytograft is focusing its clinical trials in Europe and Asia, and the Phase III clinical studies for hemodialysis access employ both autologous and allogeneic grafts. Of interest is that the new allogeneic model has the potential of reducing the overall production time to three weeks. Furthermore, it allows thousands of grafts to be fabricated from a single master cell line, thus reducing cost. Cytograft indicates that they plan to have commercial sales and initial revenues in the next year or two (private communication 2012).

A different approach to the creation of a three-dimensional, functional tissue equivalent is the acellular one. Here the use of extracellular matrix as a scaffold represents an attractive approach [59]. An example of this is the use of SIS, which is an extracellular matrix that is porcine derived. As already noted in an earlier section, more than two million patients have been treated for a variety of conditions. SIS is successful because as an extracellular matrix product it provides a rich array of signals that facilitate repair. This has led to a strategy where one employs a decellularized tissue or organ as a scaffold that then can be repopulated with human cells [60,61]. In the last few years, a variety of organs have been 'fabricated' in this manner. Examples include the heart [62], the lung [63], and the liver [64].

In a recent study published by the company Humacyte [65], a tissue-engineered blood vessel (TEBV) showed good patency in both coronary and carotid bypass models. This TEBV was created by culturing human smooth muscle cells on a polyglycolic acid tubular scaffold. The cells secreted an extracellular matrix that, when the polymeric scaffold degraded away and the cells were removed, left a completely formed vascular graft. Perhaps the most important achievement in this study was to produce a small diameter human TEBV with a burst pressure in excess of 3000 mmHg. These vessels were produced in 10 weeks in a pulsating bioreactor and decellularized, and they showed a compliance better than that of a human saphenous vein, but still considerably less than that of human arteries. Humacyte plans to be in their first dialysis patients in Europe next year (private communication, 2011). Another strategy is used whereby the implant is without cells, i.e., acellular, and the cells are then recruited from the recipient or host. A number of laboratories and companies are developing this approach. One result of this approach is to, in effect, bypass the cell sourcing issue, and replace this with the issue of cell recruitment, i.e., the recruiting of cells from the host in order to populate the construct. Because these are the patient's own cells, there is no need for any engineering of immune acceptance.

Whatever the approach, the engineering of architecture and of functional characteristics that allows one to mimic a specific tissue is critical to achieving any success and to meeting the challenge of imitating nature. In fact, because of the interrelationship of structure and function in cells and tissues, it would be unlikely to have the appropriate functional characteristics without the appropriate three-dimensional architecture and the appropriate microenvironment for the cells. Thus, many of the chapters in this book will describe in some detail the approach being taken in the design and engineering of constructs for specific tissues and organs, and any further discussion of this will be left to those chapters.

An important issue, however, is how much of the 'maturation' of a substitute is done *in vitro* in a bioreactor as compared to what is done *in vivo* through the remodeling that takes place within the body itself, i.e., in the body's own bioreactor environment. An interesting approach

has been that of the Campbells and their co-workers, who use the peritoneal and pleural cavities as bioreactors to grow autologous tissues [66]. A bioreactor simply represents a controlled environment – both chemically and mechanically – in which a tissue-like construct can be grown. Although it is generally recognized that a construct, once implanted in the living system, will undergo remodeling, it is equally true that the environment of a bioreactor can be tailored to induce the *in vitro* remodeling of a construct so as to enhance characteristics critical for success to be achieved following implantation [67–70]. Thus, the manufacturing process can be used to influence directly the final product and is part of the overall process leading to the imitation of nature. Again, an important issue is how much of the 'maturation' of a substitute should be done *in vitro* in a bioreactor as compared to what will be done *in vivo* through the remodeling that takes place within the body itself.

TRANSLATION INTO THE LIVING SYSTEM

The final challenge for tissue engineering and regenerative medicine is presented by moving a bench-top concept into the living system. This of course starts with the selection of the cell type(s) to be employed and the processing strategy to be used to scale up to the number of cells required and with the correct phenotype. For a cellular therapy, is it undifferentiated stem cells, progenitor cells, or fully differentiated cells that will be used? Will this approach be an autologous one or an allogeneic one? Also, if the source is to be iPS cells, what will the reprogramming approach be? Can the cell processing strategy be automated?

Beyond the variety of issues related to the cells to be employed, next are the pre-clinical, animal experiments. Unfortunately, there is a lack of good animal models for use in the evaluation of tissue engineering and regenerative medicine therapies. This is despite the fact that a variety of animal models have been developed for the study of different diseases. However, these models are still somewhat unproved, at least in many cases, when it comes to their use in evaluating the success of a cell-based therapy.

In addition, there is a significant need for the development of methods to evaluate quantitatively the effectiveness of a therapy. This is not only the case for animal studies, but is equally true for human clinical trials. In regard to the latter, it may not be enough to show efficacy and long-term patency; it may also be necessary to demonstrate the mechanism(s) that lead to the therapy's success. Furthermore, it is not just in clinical trials that there is a need for more quantitative tools for assessment; it also would be desirable to have available technologies to assess periodically the continued viability and functionality of the treatment that the patient has received.

Important to the success of any tissue engineering or regenerative medicine approach is that it be immune acceptable. This comes naturally with the use of autologous cells; however, if one moves to non-autologous cell systems (as this author believes we must if we are to make these therapies widely available for routine use), then the challenge of engineering immune acceptance is critical to our achieving success in the imitation of nature. It should be recognized that the issues surrounding the immune acceptance of an allogeneic cell-seeded implant are no different than those associated with a transplanted human tissue or organ. Both represent allogeneic cell transplantation, and this means that much of what has been learned in the field of transplant immunology [71,72] can help us understand the immunology and the engineering of immune acceptance for tissue-engineered allogeneic implants and cell therapies. For example, it is now known that to have immune rejection there must not only be host cell recognition of a foreign body, there also must be present what is called the co-stimulatory signal, or sometimes simply signal 2. It has been demonstrated that, with a donated allogeneic tissue, if one can block the costimulatory signal, one can extend survival of the transplant considerably [73]. Thus strategies to do this are under development and may provide greater opportunities in the future for the use of allogeneic cells.

Furthermore, if the approach is one where there is to be an implant, then it must be biocompatible. Even if the implant is immune acceptable, there can still be an inflammatory response. This response can be considered separate from the immune response, although obviously there can be interactions between the two. In fact, it may be that the regeneration that takes place in a species like the newt as opposed to the scarring observed in the human is due to differences in the interaction of the inflammatory process with the immune system. In addition to any inflammatory response, for some types of implants thrombosis will be an issue. This is certainly an important part of the biocompatibility of a blood vessel substitute.

Finally, the challenge of imitating nature does not stop with the design and engineering of a specific treatment or therapy. This is because the patient's need cannot be met by making one construct at a time on a bench top in some research laboratory. Accepting the challenge of imitating nature must include the development of cost-effective manufacturing processes [74]. These must allow for a scale up from making one at a time to a production quantity of 100 or 1000 per week. Anything significantly less would not be cost-effective, and if a product cannot be manufactured in large quantities and cost-effectively, then it will not be widely available for routine use.

Once manufactured, a critical issue will be how the product is delivered and made available to the clinician. The Organogenesis product, Apligraf, is delivered fresh and has a 5-day shelf life at room temperature [38]. On the other hand, Dermagraft, the skin substitute developed by Advanced Tissue Sciences, is cryopreserved and shipped and stored at −70°C [39]. This provides for a much more extended shelf life, but introduces other issues that one must address. Ultimately, the clinician will want off-the-shelf availability, and one way or another this will need to be provided if a product is to have wide use. Although cryobiology is a relatively old field and most cell types can be cryopreserved, there is much that still needs to be learned if we are successfully to cryopreserve three-dimensional cell-based products.

Manufacturing also is an issue for therapies that involve cells without any construct. Whatever the way that cells are to be used, as one moves from bench-top research to a clinical study in a human, ultimately to clinical trials and approval by FDA, there are a variety of issues that will need to be addressed. Some of the needs are as follows:

- A processing system for massive cell expansion
- Systems for the homogenous differentiation of cells
- Methods for the assessment of genetic and epigenetic stability
- Real time in-process assays
- *In vitro* surrogate potency assays

Critical is the need to scale up the number of cells to the level needed for a human, the ability to provide a defined population of cells, i.e., with the appropriate phenotype, and having in place the quantitative assessments needed for quality control [75–78]. In this it would be desirable to automate such a cell manufacturing system.

Finally, the translation of bench-top science to a clinical therapy and ultimately commercialization will only take place if the business model is viable. Commercialization is important because only then can the widest population of patients in need be reached. Commercial successes to date have either been with acellular approaches, or with ones that use allogeneic cells. As attractive as an autologous cell approach is scientifically, in general it will be prohibitive in terms of cost as a clinical therapy, as every patient is an N of one. Even so, Genzyme's Carticel product was autologous, based on research in Sweden [79]; however, what Genzyme provided was a service, not a real product. In the earlier discussion of iPS cells it was commented that they could possibly be used to provide autologous cells for a therapy for a specific patient; however, from the viewpoint of a realistic business model, only an allogeneic iPS cell approach would appear to be viable for a cellular therapy. It is also for this reason that a majority of approaches being commercialized are of an allogeneic nature.

CONCLUDING DISCUSSION

As already noted, if we are to meet the challenge of imitating nature, there are a variety of issues. In this chapter, the authors have attempted to identify if not all, at least many of these. The purpose has been to provide a foundation for what will be discussed in the remainder of this fourth edition. As part of this, it is our hope that we have broadened the perspective of the reader.

Much of the discussion here may have appeared to be focused on the challenge of engineering tissue-like constructs for implantation. As noted earlier, however, equally important to tissue engineering and regenerative medicine are strategies for the fostering of remodeling and ultimately the repair and enhancement of function. As one example, consider a damaged, failing heart. Should the approach be to tissue engineer an entire heart for replacement, or should the strategy be to foster the repair of the myocardium? In this latter case, it may be possible to return the heart to relatively normal function through the implantation of a myocardial patch [80] or even through the introduction of growth factors, angiogenic factors, or other biologically active molecules. Because of the complexity of the heart, it appears to the authors of this chapter that the best strategy is one of repair and/or regeneration; however, to be successful in such an approach there is still much that needs to be done.

Since the first edition of this book, considerable progress has been made; however, at times the 'hype' appears to outpace the progress. The 'hype' is not only about the promise of stem cells. It at times has permeated much of the field. This is unfortunate for researchers speaking about tissue engineering and regenerative medicine need to balance the hope with realism, by making comments that indicate what it will take for the promise offered, i.e., the potential, to be realized.

Beyond the hype and if we are to realize the hope, then the approach of the tissue engineering and regenerative medicine community must be one that is very interdisciplinary. As advocated in the report from the National Research Council entitled 'A New Biology for the 21st Century' [81], this starts with breaking down the silos within the field of biology itself and then integrating into the field computational researchers, engineers, mathematicians, and physicists. As illustrated in Fig. 2.4, such an integration will lead to a deeper scientific understanding, and with the addition of clinicians one can then move to translating the science into therapies. Only with this final integration will our understanding of how to imitate nature lead to the therapies needed for the future.

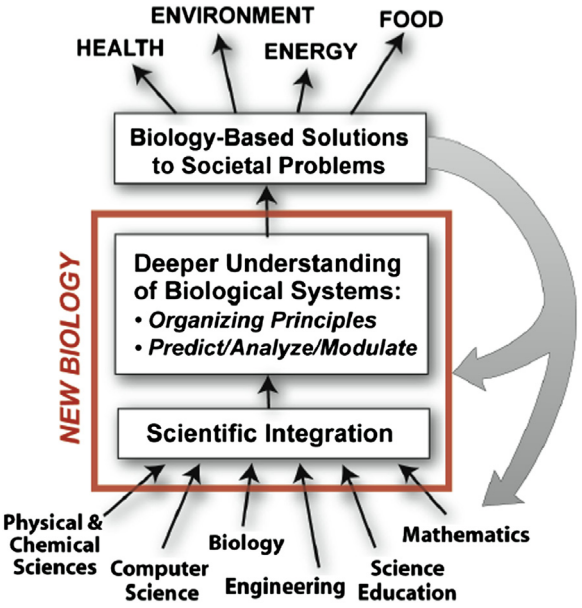

FIGURE 2.4
Illustration of the integration of computational researchers, engineers, mathematicians, and physicists will lead to a deeper biological understanding and provide for addressing the major needs of society. *Reprinted from the National Research Council Report entitled 'A New Biology for the 21st Century' (2009) with permission from the National Academies Press.*

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CHAPTER 3

From Mathematical Models to Clinical Reality

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INTRODUCTION

Tissue engineering aims to understand the principles of *in vivo* tissue growth and development and apply this understanding to the engineering of functional replacement tissue *ex vivo* for clinical use subsequent to disease or injury. However, tissue formation is an inherently complex process — involving multiple biological, chemical, and physical processes that interact on a variety of length- and time-scales — and deciphering general principles of tissue development is a challenging task [1]. Nevertheless, a deep understanding of these principles is necessary to efficiently engineer functional tissue *in vitro*. For this reason, tissue engineering approaches commonly coordinate techniques from a variety of different disciplines, including molecular, cell and developmental biology, chemical engineering and biophysics to dissect this complexity. Recent years have seen an increasing interest in applying tools from the mathematical and computational sciences to the design, development, and implementation of tissue engineering protocols [2,3].

Mathematical and computational models are routinely used to help understand complex systems and optimize industrial processes in the engineering and physical sciences. There are many remarkable examples of the longstanding use of quantitative methods in the cell and molecular biosciences [4]. Recent rapid advances both in available computational power and high-throughput experimental procedures have stimulated an extraordinary cross-fertilization of ideas from the mathematical, statistical, physical and life sciences, particularly within the areas of systems biology and bioinformatics [5]. Concurrently, much progress has also been made in recent years toward understanding the general principles of tissue growth and development. In this chapter we will explore how mathematical models, in collaboration with experimental studies, can help further understanding of these principles. Given the breadth and rapid growth of this field we will necessarily only focus on a few key areas, particularly: understanding molecular basis of stem and progenitor cell fate commitment; spatiotemporal regulation of cellular differentiation; and general principles of tissue morphogenesis.

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MODELING STEM CELL DYNAMICS

Stem cells are present during all stages of development and are uniquely characterized by their capacity to maintain their numbers by self-renewing, as well as differentiate along distinct lineages. Embryonic stem (ES) cells have the capacity to produce all somatic tissues, a property known as pluripotency, and there is much interest in elucidating the molecular mechanisms of pluripotency [6]. Following the seminal work of the Yamanaka group in demonstrating that adult fibroblasts could be reprogrammed to ES cell-like state by the forced expression of just four pluripotency-associated transcription factors (Klf4, c-Myc, Oct-3/4 and Sox2), there is hope of using adult somatic cell-derived induced pluripotent stem (iPS) cells as patient-specific, and less controversial, alternatives to human ES cells [7–9].

In contrast to ES and iPS cells, adult stem cells are typically lineage restricted and thereby constrained in their therapeutic potential (they are generally only multi- or uni-potent) [10]. Nevertheless, due to their regenerative capacities and the relative ease with which they may be obtained, there is also considerable interest in understanding the processes by which adult stem cells orchestrate tissue growth and development, and in applying this understanding to engineering functional replacement tissue in the laboratory [1]. A standard tissue engineering approach to this problem is to harvest appropriate stem cell populations and, using defined culture conditions (which include regulating the chemical, physical/mechanical and geometric properties of the growth environment), recapitulate *in vivo* regenerative processes to produce functional tissue *in vitro*. The ultimate success of this strategy relies on a clear understanding of the molecular mechanisms of stem cell self-renewal and differentiation and the spatiotemporal control of these processes.

Stem cell fate is critically dependent upon interactions with neighboring cells – including differentiated progeny [11] and other stem/progenitor cells [12] – and extracellular components that constitute the stem cell niche [13]. Stem cell interactions with the niche are mediated through intracellular molecular regulatory networks that include transcriptional, signaling, and epigenetic regulatory components [14]. These networks are highly complex, containing multiple feedback and feed-forward loops that allow the cell to respond dynamically to changes in its environment. These types of feedback-based mechanisms are reminiscent of control systems in engineering, and recent years have seen considerable interest in viewing cell fate decisions from a dynamical systems perspective [14]. In the field of synthetic biology, for instance, switches and oscillators have been implemented experimentally in model systems using rational designs based upon mathematical reasoning [15–17]. A general principle that emerges from this viewpoint is that the molecular switches that underpin stem cell fate decisions are ultimately dependent upon positive feedback loops in the cells' underlying intracellular molecular regulatory circuitry [18].

Positive feedback based molecular switches

Positive feedback based switches play a central role during development by initiating all-or-none commitment events in response to external stimuli, and commonly underpin stem and progenitor cell fate decisions, including during hematopoiesis [19]; neural development [20]; and human embryonic and mesenchymal and stem cell fate commitment [21,22]. In order to illustrate the general principle of a feedback-based switch, consider as an example the following dimensionless equations, which describe a simple 'toggle switch' consisting of two cross-inhibitory transcription factors, X and Y[15]:

$$\frac{dx}{dt} = \frac{\alpha_1}{1 + y^{\beta_1}} - x, \tag{1}$$

$$\frac{dy}{dt} = \frac{\alpha_2}{1 + x^{\beta_2}} - y. \tag{2}$$

Here x and y denote the intracellular concentration of X and Y. Equations (1)–(2) describe the rate of change in expression of X and Y over time and state that X and Y are synthesized with effective rates α_1 and α_2 respectively (first terms on the RHS) and decay with constant half-lives (second terms on RHS). The parameters β_1 and β_2 capture cooperativity in repression, allowing for repression by multi-protein complexes and/or binding of multiple regulatory sites on the respective promoters, for instance. Note that although this system is characterized by repressive interactions, it nevertheless represents a simple positive feedback loop since both factors indirectly activate their own expression via inhibiting expression of the other. This kind of switch has been engineered both in *E. coli* [15] and mammalian cells [23] and behaves as a genetic 'memory unit' [15] (indeed it behaves similarly to a SR flip-flop for memory storage in electronic systems).

The dynamics of this system may be explored by plotting changes in expression of X and Y against each other to form the system's *phase plane*. The phase plane for this system is shown in Fig. 3.1 (top row) for different parameter values. For appropriate regimes, this system supports stable expression of *either X or Y*, but not co-expression of both, as one might expect (Fig. 3.1 a, c). For instance, if the effective rate of synthesis of Y is higher than that of X, then Y is expressed whilst X is repressed (Fig. 3.1a); by contrast, if the effective rate of synthesis of X is higher than that of Y, then X is expressed whilst Y is repressed (Fig. 3.1c). In these cases, since only one stable equilibrium point is present the system is said to be *monostable*. In general

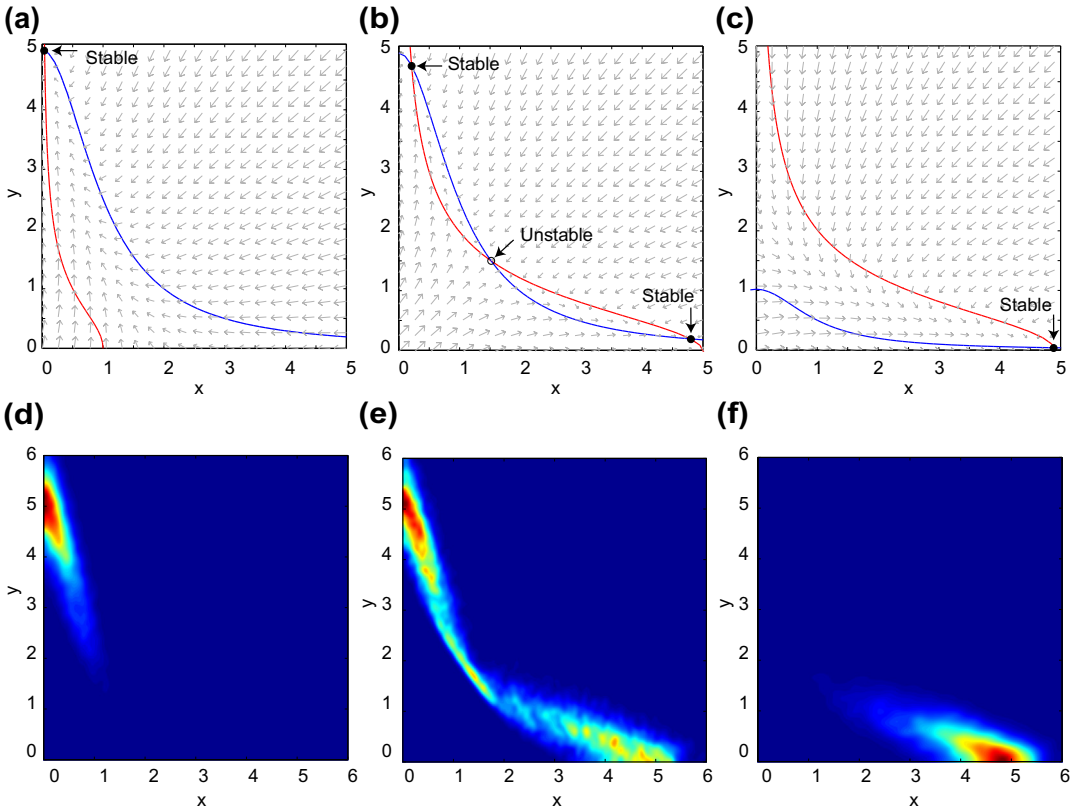

FIGURE 3.1

Bistability and monostability in a genetic toggle switch. (**a**–**c**) Phase planes for Eqs. (1)–(2) for different model parameters showing that this simple molecular switch supports both monostability (**a**, **c**) and bistability (**b**). Arrows show the direction field illustrating the flow towards the stable equilibria from different initial conditions. (**d**–**f**) Corresponding probability density functions, showing that expression variability can arise within a cellular population in the presence of molecular noise. Red shows regions of high probability of finding a cell; blue shows regions of low probability. Here stochasticity in expression of the two genes has been modeled as white noise process with amplitudes $\sigma_1 = 0.55$ (noise in expression of x) and $\sigma_2 = 0.75$ (noise in expression of y). Parameter values: (panels **a**, **d**) $\alpha_1 = 1$, $\alpha_2 = 5$; (panels **b**, **e**) $\alpha_1 = 5$, $\alpha_2 = 5$; (panels **c**, **f**) $\alpha_1 = 5$, $\alpha_2 = 1$. In all cases, $\beta_1 = \beta_2 = 2$.

monostability occurs in the absence of cooperativity ($\beta_1 = \beta_2 = 1$) or if the effective rates of synthesis of the two repressors are unbalanced ($\alpha_1 >> \alpha_2$ or $\alpha_1 << \alpha_2$). However, if the effective rates of synthesis of the two repressors are balanced ($\alpha_1 \approx \alpha_2$) and transcriptional repression is cooperative ($\beta_1, \beta_2 > 1$) then these two alternate states may stably coexist (Fig. 3.1b). In this case the system is said to be *bistable* and it is the capacity of this system to possess different equilibrium patterns of expression depending on parameter values that allows it to act as a switch. Particularly, this system is a *toggle* switch since expression of either of the repressors may be toggled 'on' and 'off' by transiently inducing changes in expression by varying the effective rates of synthesis α_1 and α_2 [15].

Ultimately, the toggle switch described above works since each factor directly represses transcription of the other while indirectly activating its own transcription. However, mutual cross-repression is by no means the only positive feedback motif that can give rise to molecular switches of this kind. For instance, direct auto-regulation, which is also common in eukaryotic genetic regulatory networks [16,24] can give rise to multistability and switching, either on its own [16] or in concert with other mechanisms. For example, if in addition to mutual cross-repression, each of the transcription factors in the toggle switch also directly positively regulates its own expression then a third 'primed' state, in which both factors are promiscuously co-expressed within individual cells, may also be supported resulting in a potentially *tristable* system. This occurs, for instance, during hematopoiesis: the lineage specifying master transcription factors GATA1 and PU.1 each enhance their own expression while repressing that of the other [25]. Since both GATA1 and PU.1 also regulate multiple downstream targets (GATA1 specifies the erythroid/megakaryocyte lineages; while PU.1 specifies the myelomonocytic lineage), the state of this motif affects widespread genetic programs and effectively defines a lineage choice. By differentially regulating multiple downstream targets, positive feedback-based switches such as this can act as input/output (I/O) devices within larger genetic regulatory networks [26]. In the context of development, positive feedback-based I/O devices are important since they provide a mechanism by which the cell populations can convert spatial information (for instance, an extracellular morphogen gradient) into defined commitment responses, resulting in spatial localization of widespread gene or protein expression patterns [16,27]. We will discuss further how spatial localization of intracellular feedback mechanisms can give rise to complex spatiotemporal patterns of expression in the section 'Pattern formation' below.

Variability in stem cell populations

Although useful, the toggle switch model given by Eqs. (1)–(2) is deterministic, and it does not account for environmental fluctuations or variability in gene expression that are inevitably present. Gene and protein expression are intrinsically stochastic processes, [28,29] and cell-cell variability may arise in isogenic cell populations either due to gene expression 'noise', or due to stochastic partitioning errors or asymmetry during cell division [30–32] (Fig. 3.2). For instance, if such expression noise is accounted for in the toggle switch model then cell-cell variability in expression patterns can naturally arise within a population of genetically identical cells (Fig. 3.1, bottom row). This variability is particularly evident in the bistable regime when expression fluctuations can induce transitions between the coexisting stable states (Fig. 3.1e). This kind of cell division-independent, non-genetic population heterogeneity is remarkably robust, and appears to be a relatively widespread feature of both prokaryotic and eukaryotic cell populations [33–35].

It has long been postulated that stochasticity may have an important role in stem cell fate commitment [36–39]. In the 1960s McCulloch, Till and Siminovitch assessed the colony forming ability of adult mouse bone marrow cells in the spleens of irradiated mice [36]. Using a serial transplantation assay they found that the number of colony forming units (CFUs) per colony varied considerably beyond that expected from sampling errors alone, in a manner

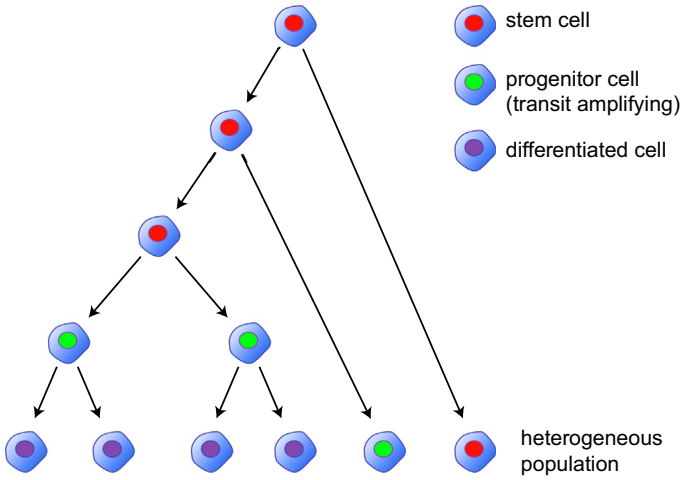

FIGURE 3.2

Heterogeneity in a hierarchically structured population of stem, progenitor and terminally differentiated cells. Stem cells maintain their numbers by symmetric and asymmetric divisions, and give rise to transit amplifying progenitor cells, which in turn produce terminally differentiated progeny. This hierarchy of divisions naturally produces a mixed population containing stem, progenitor and terminally differentiated cell types in proportions that vary with environment, age and disease or insult.

consistent with a 'birth-and-death' process (a simple Markov process, well studied in the statistical physics literature [40]), in which CFU self-renewal and differentiation occur stochastically with defined probabilities [36]. They confirmed this conclusion by numerically simulating colony formation using the birth-and-death model, providing a good early example of the use of computational simulations in stem cell biology. Similarly, Ogawa, Suda and co-workers came to related conclusions concerning the stochastic nature of stem cell fate commitment, in their studies of paired hematopoietic progenitor cells in the 1980s [37–39].

Within the last few years, studies have confirmed substantial variability both in equilibrium expression patterns in a variety of mammalian stem and progenitor cell populations [33,41–45] and the dynamics of cellular differentiation and reprogramming [21,46,47]. In our own recent work we have investigated variability in colony forming ability of primary human bone marrow stromal cell (HBMSC) populations using a two-stage colony forming assay [48]. We found considerable variability both in the sizes of primary (P0) colonies, seeded from single HBMSC cells, and secondary (P1) colonies, formed by detaching cells from P0 colonies and reseeding at clonal density, with apparently little correlation between primary and secondary colony sizes (dependent on the primary colony, transplanted cells produced either small secondary colonies or a wide range of disparate colony sizes). We also observed distinct spatial heterogeneities in expression of mesenchymal and niche-associated markers (including CD146, alpha smooth muscle Actin and Alkaline Phosphatase, see Fig. 3.3, top row) indicating spatial regulation of cell behavior within the colonies. Computational analysis, using a cellular automaton model that accounted for proliferation and migration of individual cells, showed that this behavior is consistent with tissue growth within a structured cellular hierarchy (consisting of stem, transit amplifying, and differentiated cell types, see Fig. 3.2), regulated by cell-cell interactions.

Taken together these results indicate that cell-cell variability in expression patterns and stochasticity in stem cell commitment may be closely related, with stochastic mechanisms allowing dynamic 'priming' of subpopulations of stem cells for different lineages prior to commitment [49]. These observations suggest that cell fate commitment events are regulated at the population, rather than individual cell, level. Thus, intracellular molecular regulatory networks may not control individual stem cell fate commitment *per se*, but rather control the overall structure of the cellular population [36], possibly by modulating molecular noise [50]. In this regard, a more probabilistic view of stem cell fate may be appropriate, in which statistical properties of ensembles of cells, rather than characteristics of individual cells (or averaged properties of populations of cells) are considered. An enhanced quantitative understanding of the intrinsic variability of stem cell populations will require developments

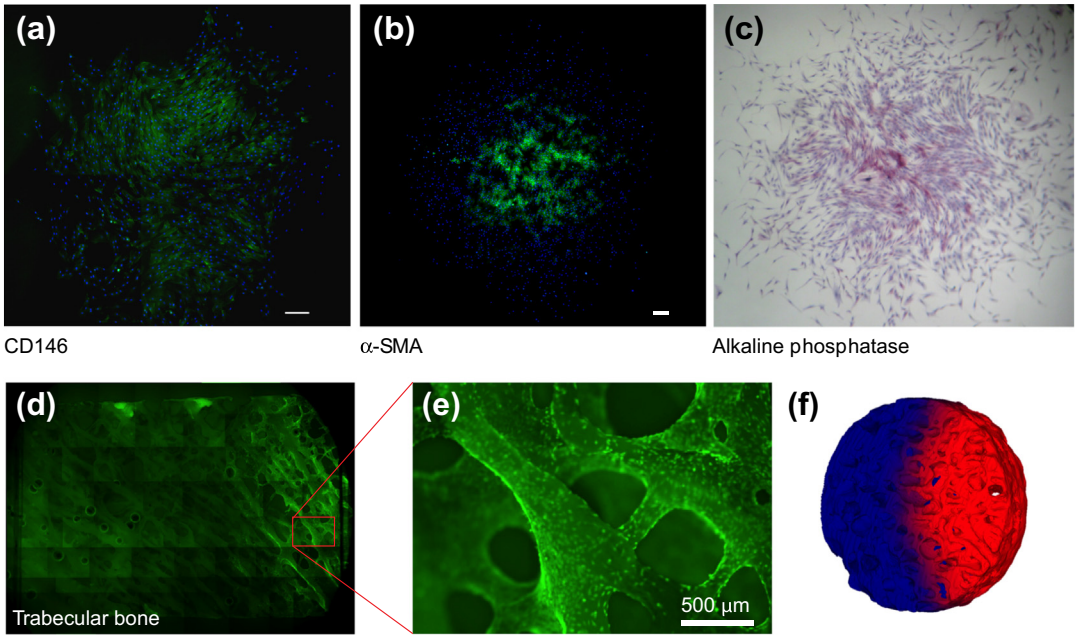

FIGURE 3.3

Tissue growth in monolayer and 3D culture. Panels (**a**—**c**) show intra-colony variability of differentiation and niche markers within primary human bone marrow stromal cell samples seeded at clonal density to form single cell derived colonies. P0 colonies were fixed and assessed for the following mesenchymal markers. (**a**) CD146 (Melanoma Cell Adhesion Molecule) expression measured using mouse monoclonal antibody, in combination with FITC conjugated anti-mouse Immunoglobulin G (IgG) secondary antibody. (**b**) Alpha smooth muscle Actin expression measured using mouse monoclonal antibody in conjunction with FITC conjugated anti-mouse IgG secondary antibody. (**c**) Alkaline Phosphatase activity in red, stained with Naphthol AS-MX Phosphate and Fast Violet B Salts. Staining of all three markers is strongest toward the center of the colony. Panels (**d**—**f**) show cell colonization of complex 3D geometries. (**d**) Slice of human trabecular bone used in a 3D colonization assay. (**e**) Primary human bone marrow stromal cells were seeded onto the trabecular bone sample and cell colonization was visualized over time using Cell Tracker Green labeling (bright green). (**f**) Typical simulation of corresponding mathematical model of cell proliferation and colonization of trabecular bone sample. Domain geometry was obtained from a μCT scan of the trabecular bone sample. Regions of high cell number density are in red; regions of low cell number density are in blue.

both in high-throughput single cell profiling procedures and the mathematical and statistical techniques needed to decipher the complex datasets that result from these experimental advances. Such advances will enable a better understanding of how variability can be controlled to produce more defined stem cell populations, which will ultimately lead to the development of more robust stem cell isolation and expansion protocols for tissue engineering and regenerative medicine applications.

MODELING TISSUE GROWTH AND DEVELOPMENT

Any success in engineering functional tissue *ex vivo* must rely not only on deciphering individual stem cell behavior, but also on developing an enhanced understanding of the mechanisms of morphogenesis *in vivo* including the environmental, chemical, mechanical, genetic and epigenetic processes involved in spatial regulation of cellular differentiation. There have been a number of remarkable recent successes in engineering complex, spatially structured and *functional* three-dimensional (3D) tissues in the lab, including: the optic cup [51]; the anterior pituitary (adenohypophysis) [52]; the trachea [53]; articular cartilage [54,55]; vascularized bone [56]; and, recently, allogeneic veins [57].

The generation of structure either during *in vivo* development or *ex vivo* tissue formation is dependent upon cells translating spatial information concerning their relative location within the developing organism or tissue into different patterns of gene and protein expression based upon their current genetic state and developmental history [58]. The extraordinary

reproducibility of development requires not only that positional information is precisely and robustly defined (that is, insensitive to environmental fluctuations) but also that cellular sensing of such information is correspondingly sensitive and appropriately coordinated. Spatial gradients have long been known to provide positional information for spatial patterning during development (for instance see the work of Wolpert, Tickle and colleagues [59–61]). Since spatial patterns emerge over relatively short length scales, Crick suggested that spatial information may be supplied by diffusion gradients [62], although local mechanisms, such as cell-cell communication, are also important [63]. Consequently, mathematical models of reaction-diffusion processes – which take into account both chemical diffusion, and cellular responses to evolving chemical gradients – have been widely used to study tissue growth and development.

Monolayer tissue growth *in vitro*

One of the simplest reaction-diffusion models is Fisher's equation, which was originally used to study the spread of a favored gene through a population [64], but serves as a good model for expansion of an *in vitro* monolayer cell colony due to proliferation and cellular migration. Denoting the cell number density (the number of cells/m^2 for monolayer culture) at position x and time t by $n(x,t)$, Fisher's equation reads:

$$\frac{\partial n}{\partial t} = rn(N-n) + D\nabla^2 n. \tag{3}$$

This equation describes the rate of change of cell number density in space and time. The first term on the RHS of Eq. (3) describes cellular proliferation. At low cell number densities proliferation is approximately exponential, with linear growth rate rn; however, as the cell number density reaches N, the maximum at confluence, the proliferation rate tends to zero accounting for inhibition of cell division due to cellular crowding. This model is known as logistic growth, and is a good first approximation to two-dimensional expansion of an unstructured population of identical cells. Hierarchically structured populations that contain subpopulations of stem, progenitor or transit amplifying cells may exhibit more complex patterns of proliferation not captured by the logistic model (Fig. 3.2). Mathematical models of growth kinetics in such heterogeneous populations have been considered by a number of authors [48,65–67]. The second term on the RHS of Eq. (3) describes random cell movement with motility coefficient D, similar to a diffusion coefficient. More complex forms of motility, including chemotaxis or haptotaxis, may be included by adapting this migration term [68]. In one spatial dimension, assuming appropriate initial conditions, Fisher's equation predicts that the colony advances as a 'traveling wave' (meaning that the invasion front maintains a constant shape, moving at a constant speed) with wave speed $v = 2\sqrt{rND}$ [64]. In the case that growth is spherical and axisymmetric (for instance, a radially expanding colony), the wave speed approaches this constant asymptotically for large radius [64]. Since the wave speed depends upon both the diffusion coefficient and the linear growth rate, reaction-diffusion fronts may advance much faster than by diffusion alone, and in a precisely quantifiable way. Consequently, Fisher's equation provides an illustrative example of how a simple mathematical model may be used to investigate the relationship between proliferative and migratory parameters and colony growth rate. If the linear growth rate is known (for instance from an independent proliferation assay), then cellular motility coefficients may be estimated using this relationship. For instance, Maini and co-workers have used this model to analyze an invasion front in a wound-healing assay for human peritoneal mesothelial cells [69,70] and we have previously used this relationship to estimate the migration rate of primary HBMSCs [71]. We found that for slowly proliferating migratory cell types, such as HBMSC populations, Fisher's equation provides a good model for colony growth; however for highly proliferative populations, such as the MG63 human osteosarcoma cell line, a sharp-front variation of Fisher's model is more appropriate [71].

Tissue growth on complex surfaces *in vitro*

Fisher's equation as outlined above describes monolayer growth. However, tissue engineering strategies commonly rely on seeding appropriate cell populations onto complex three-dimensional porous biomimetic scaffolds in order to provide geometric support for tissue development [1,72]. In this situation, while good peripheral tissue growth is usually observed, poor growth or necrotic cell death is often seen in the central regions of such constructs (though this is not always acknowledged). Spatial heterogeneity of this kind is usually related to inadequate nutrient supply and/or accumulation of toxic waste products at the scaffold interior, problems that we shall discuss in the following section. However, spatial tissue inhomogeneity may also arise from poor cellular ingrowth due to uneven initial seeding of cells and/or locally adverse scaffold geometries. Numerous groups have investigated the effects of seeding strategies, scaffold geometry, and nutrient supply on subsequent tissue growth, using both experimental and computational methods [73–75]. For instance, we have investigated HBMSC population ingrowth in a complex geometry using an experimental-computational approach which simulated Fisher's equation over a surface obtained from micro computerized tomography (μCT) scanning of trabecular bone [75] and compared simulations with experimentally-derived invasion fronts. We observed that despite the complex geometry, Fisher's equation described tissue ingrowth over the trabecular bone surface surprisingly well, with the average migration front advancing at an approximately constant speed dependent upon geometric properties of the bone surface, such as porosity and tortuosity [75] (see Fig. 3.3, bottom row). Applications of simple models of this kind are useful since they allow quantitative assessment of how scaffold architecture affects key proliferation and migratory parameters that govern the efficacy of tissue ingrowth.

Three-dimensional tissue growth *in vitro*

Nutrient-limited growth has been observed for a wide variety of engineered tissues including articular cartilage, intervertebral fibrocartilage and cardiac tissue [74,76,77]. In the absence of enhanced transport of nutrients and cellular waste products spatial heterogeneities in cell numbers rapidly develop: peripheral cells have access to ample nutrient that they consume, leaving those on the interior nutrient starved. Consequently diffusion-limited viable scaffold-based tissue growth is typically restricted to a few hundred micrometers (approximately the intercapillary distance *in vivo*). Spatial variability in cell numbers can also lead to variability in deposited extracellular matrix (ECM) components that can severely inhibit the formation of mechanically competent tissue (see Fig. 3.4). The problem of nutrient-limited growth and resultant tissue integrity has been examined from a computational perspective by numerous groups both in the context of tissue engineering [74,78–80] and avascular solid tumor growth [81].

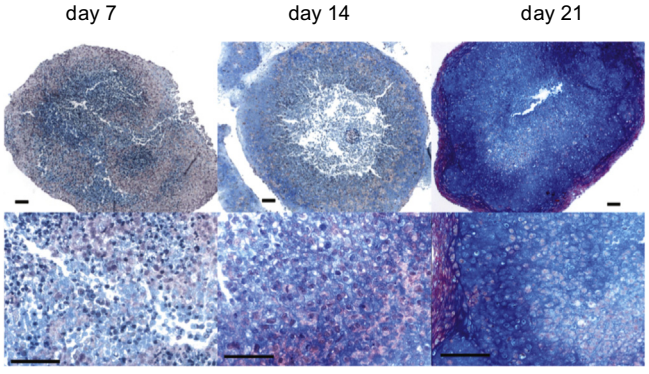

FIGURE 3.4

21-day pellet culture time-course of murine chondrocytic ATDC5 cells. Pellets were stained with Alcian blue and Sirius red to demonstrate presence of proteoglycans and collagenous ECM respectively. Spatial variability in cell numbers is apparent after 7 days and extends throughout the culture period. Cell number variability also leads to variability in deposited ECM components, which affects the structural properties of the engineered tissue. Scale bar: 100 μm.

For example, we used the following mathematical model to investigate the relationship between evolving oxygen concentration profiles and nutrient-limited tissue formation in the early stages of growth in developing engineered cartilage tissue [74]:

$$\frac{\partial c}{\partial t} = D\nabla^2 c - \alpha\beta cn, \tag{4}$$

$$\frac{\partial n}{\partial t} = \beta cn. \tag{5}$$

Here $c(x,t)$ and $n(x,t)$ represent oxygen concentration (mol/m^3) and chondrocyte cell number density (number of cells/m^3 for three-dimensional culture) at position x and time t respectively. These equations describe the rate of change of oxygen concentration and cell numbers both in space and time. The first describes the evolution of the oxygen concentration in the developing tissue: the first term on the RHS of this equation accounts for transport of oxygen which is assumed to occur by diffusion with constant diffusion coefficient D; the second term accounts for oxygen depletion and states that oxygen is consumed at a rate proportional to the local cell number density. The second equation describes changes in cell number density: the term on the RHS accounts for cellular proliferation at a rate proportional to the oxygen concentration (cell death and migration are assumed to be negligible during the initial phase of tissue growth which this model considers). The parameters α and β control the relative rates of cellular proliferation and oxygen consumption. Note that the cell number density and the oxygen concentration are intrinsically coupled to each other: changes in oxygen concentration affect cellular proliferation, which in turn effect changes in oxygen levels. To close the system we assume that oxygen levels are initially constant and the scaffold is uniformly seeded with chondrocytes; and take a no-flux boundary condition at the scaffold center and a continuity condition on the scaffold surface for the oxygen concentration. Although this model is somewhat simplified, it was nevertheless found to provide a good fit to experimentally-derived evolving oxygen and cell number density profiles, indicating that oxygen availability is a rate-limiting process during the early stages of *in vitro* cartilaginous tissue formation. Furthermore, this analysis indicated that in the absence of enhanced transport processes or regulation of cellular proliferation, spatial heterogeneities are inevitable: nutrient supply by diffusion alone will always result in proliferation-dominated peripheral regions that severely restrict viable tissue growth, and therefore the potential for clinical scale-up. This problem has been tackled both by enhancing nutrient transport, for instance using bioreactors in which transport is enhanced by advection or perfusion [82,83], or using printed scaffolds which possess an artificial 'vasculature' which can channel nutrients to the tissue center [84]; and by co-culture with endothelial cells in order to encourage the concomitant formation of *de novo* vasculature within the developing tissue [85].

Pattern formation

During development extraordinarily complex spatiotemporal structures emerge spontaneously, and it is these intricate 'patterns' that ultimately provide tissues and organs with the microscopic and macroscopic structure necessary to their function. Reproducing such pattern-forming processes *in vitro* is a major goal of tissue engineering, and a quantitative understanding of these self-organizing processes will be needed if we are to reproducibly and reliably engineer macroscopically structured functional tissues *ex vivo*. Remarkable progress has been made toward understanding the biochemical and biophysical basis of morphogenesis from both experimental and theoretical perspectives (see [86] and references therein for some examples).

In the 1950s, Alan Turing presented a simple chemical mechanism by which spatial patterns may arise spontaneously in biological systems [87] which has become acknowledged as a milestone in our understanding of development [88]. Turing's mechanism relies upon diffusion of a chemical 'morphogen' destabilizing a spatially homogeneous state, and driving

evolution toward a stable spatially heterogeneous, or patterned, state. This mechanism is both simple and remarkable, since diffusion usually has a homogenizing, or stabilizing, role. However, it can give rise to an extraordinary range of complex patterns. Consequently, it has received much attention in the mathematical biology literature [89]. The biochemical and biophysical basis of morphogenesis has developed considerably since Turing's initial work, and his concepts have been used to help understand a range of mammalian developmental systems including vertebrate limb bud development, angiogenesis and wound healing [64,89,90]. Recent developments have also begun to take into account both chemical mechanisms and biomechanical forces (which are known to play a critical part in the development of tissues such as bone [63]) in morphogenesis [91,92].

An archetypical model of pattern formation is the *activator-inhibitor* model of Gierer and Meinhardt [93]. The generalized Gierer-Meinhardt (GM) model consists of the following coupled equations, which describe reactions between an *activator* with concentration $u(x,t)(\text{mol/m}^3)$ and an antagonist, or *inhibitor*, with concentration $v(x,t)$ (mol/m^3) at position x and time t:

$$\frac{\partial v}{\partial t} = D_u \nabla^2 u + \rho_u + \frac{\rho_u}{(1+\kappa u^2)v} - \mu_u u, \tag{6}$$

$$\frac{\partial v}{\partial t} = D_v \nabla^2 v + \rho_v + \rho_{u^2} - \mu_v v. \tag{7}$$

These equations describe the rate of change of activator and inhibitor concentrations in space and time. Here $D_u, D_v, \rho_u, \rho_v, \rho, \mu_u$ and μ_v are positive constants which characterize the rates of diffusion, production and decay of the two species. The first terms on the RHS of Eqs. (6)—(7) account for diffusion; the second and third terms account for production: the activator enhances its own production (forming an autocatalytic positive feedback loop) and that of the inhibitor, while the inhibitor represses production of the activator; the fourth terms account for decay of both species with constant half-lives. If the inhibitor diffuses more quickly than the activator then this model can give rise to a variety of different spatially inhomogeneous patterns (including various types of spots and stripes, see Fig. 3.5 for some examples). The essential mechanism by which these patterns arise depends on spatial control of positive feedback: small fluctuations away from the spatially homogeneous state are initially strongly locally amplified by the positive feedback present in the system due to activator autocatalysis; however this amplification does not continue uninhibited but rather is spatially confined due to fast diffusion of the inhibitor away from regions of local activation. This type of

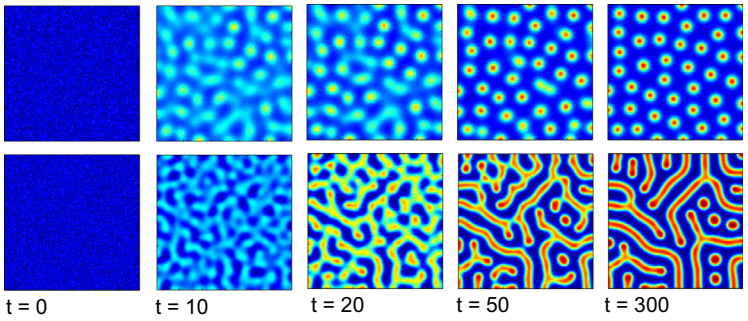

FIGURE 3.5

Spontaneous pattern formation in a simple reaction diffusion model. Simulations of the generalized Gierer-Meinhardt model [93], a classic activator-inhibitor reaction-diffusion process given by Eqs. (6)—(7), show how different kinds of spatial patterns can emerge spontaneously from the same model equations with different parameters. This model has been used to explain experimentally-observed patterns formed by adult vascular mesenchymal cells in culture [94] (**Top row**) Emergence of regular spots. (**Bottom row**) Emergence of stripes.

short-range activation and *long-range* inhibition underpins many examples of biological pattern formation. For instance, using a joint experimental-computational approach, Garfinkel and co-workers have recently shown that this mechanism can account for complex patterns formed by adult vascular mesenchymal cells (VMCs) in monolayer culture *in vitro* [94]. VMCs differentiate and self-organize into a variety of complex spatial patterns, similar to those seen in the GM model, during development, disease progression as well as *in vitro* culture. Garfinkel et al. reasoned that bone morphogenetic protein 2 (BMP-2), which is expressed by VMCs and positively regulates its own transcription, may act as a local activator; while matrix carboxyglutamic acid protein (MGP), a relatively small molecule which inhibits BMP-2 induced differentiation, may play the part of a fast diffusing long-range inhibitor (see [94] and references therein). They found that experimental culture of VMCs demonstrated good qualitative agreement with the patterns formed in a generalized GM model based on spatiotemporal interactions between BMP-2 and MGP. Additionally, their analysis predicted that different patterns would emerge from the same underlying mechanism if the kinetics of BMP-2 activation and MGP inhibition were perturbed. To test this they showed experimentally that addition of MGP to the media preparation shifted the observed pattern from labyrinthine stripes to spots; while addition of warfarin (which blocks MGP inhibition of BMP-2, see [94] and references therein) resulted in 'stripe-doubling', two changes which were predicted by their mathematical model.

Despite the relative success of activator-inhibitor models such as this, Turing-type patterns are difficult to engineer synthetically since they are sensitive to parameter changes and rely on particular disparities in kinetic and transport parameters. Consequently, the importance of these mechanisms in development has been long debated [95]. However, Turing did not intend his model to be an accurate description of any particular morphogenetic process, nor did he intend it to make hard biological predictions. Rather the power of his work was that it initiated a new way of thinking about how complex self-organizing patterns may arise when feedback is spatially controlled. Perhaps the greatest advantage to the tissue engineer of mathematical models of this kind is that they provide examples of how simple chemical, biological and physical processes can interact to spontaneously produce complex spatial patterns in ways that are hard to explore using experiment and intuition alone. In the long term, better understanding of the complex self-organizing mechanisms of tissue and organ morphogenesis from such a mathematical perspective may allow more efficient and reproducible engineering of macroscopically structured tissue.

FROM MATHEMATICAL MODELS TO CLINICAL REALITY

Mathematical methods are routinely used in engineering to optimize complex processes for industrial implementation. Similarly, we anticipate that as tissue engineering protocols make their way from development in the lab to routine use in the clinic, mathematical methods will play an increasingly central role in their optimization and scale-up. Mathematical optimization approaches are already being used to design custom scaffolds for tissue regeneration [72,96–98]. For example, Hollister and colleagues have outlined a strategy for the rational design of porous scaffolds with defined architectural properties that balance conflicting mechanical and transport requirements (reviewed in [72]). This strategy uses a set of mathematical tools from homogenization theory – which allow macroscopic material and transport properties to be inferred from scaffold microstructure – to aid scaffold design *in silico* for practical implementation using novel solid free-form fabrication techniques. Such custom approaches have been successfully used to design optimized scaffolds for bone tissue engineering in craniofacial reconstruction, for instance [97]. As another example, Unadkat and co-workers have recently outlined an algorithm-based strategy to design biomaterial surface micro-topographies that optimally regulate cell function [99]. It is likely that advanced multidisciplinary protocols such as these, which coordinate mathematical, computational,

chemical and materials engineering techniques, will lead to further advances in scaffold design in the near future. Indeed, design optimization is perhaps the area in which there is the most potential for genuinely productive application of mathematical and computational principles to tissue engineering and regenerative medicine. Such sophisticated design strategies are likely to become essential to the future of tissue engineering, and ultimately will help to take tissue engineering from experimental concept to clinical reality.

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CHAPTER 4

Stem Cells as Building Blocks

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LIST OF ABBREVIATIONS

ESC – embryonic stem cell
HSC – hematopoietic stem cell
iPS – induce pluripotent stem cell
MSC – mesenchymal stem cell
ICM – inner cell mass

INTRODUCTION

A human being comes into existence by formation of the zygote, which gives rise to all human stem and non-stem cells. Stem cells are identifiable not only in the developing embryo and fetus, but also in postnatal tissues, thus ensuring the lifetime maintenance of their integrity and homeostasis by replacing the cells lost due to normal turnover, injury or disease [1]. As stem cells play a major role in the process of tissue repair, the concept of their manipulation for applications in tissue engineering and regenerative medicine has attracted considerable attention. The diversity of stem cells imposes difficulties in offering a single comprehensive definition that can be applied to all of these cells. As a general guide, stem cells are classically defined as cells with self-renewal and clonogenic properties capable of multi-lineage differentiation. Self-renewal is the capacity for extensive proliferation and generation of stem cells with the same properties as the parent cell; clonogenicity is the property of a single stem cell to produce a colony of cells through self-renewal [2]. On the other hand, the multi-lineage differentiation capability is the property of a single stem cell to generate different types of mature progenies. It should be noted that in some instances, such as spermatogonial stem cells [3], the cells only differentiate into one mature progeny, the male gamete in this case, and have limited self-renewal properties. These cells are considered as unipotent stem cells. Alternatively, these unipotent cells and other cells, such as neuronal restricted progenitor cells (NRP), with limited properties of self-renewal and multilineage differentiation are called progenitor cells [2].

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Asymmetric cell division has been described in stem cells for a long time. Although this phenomenon has been mainly studied in invertebrates, there is evidence of asymmetric division in some mammalian stem cells, such as hematopoietic stem cells [4]. Asymmetric division gives rise to two daughter cells with different fates: one of them will remain a stem cell and the other differentiates. There are several distinct intracellular and extracellular mechanisms that contribute to asymmetric division. However, the exact nature and regulation of these mechanisms remain to be elucidated, especially in mammalian stem cells. According to recent evidence, mammalian stem cells undergo both asymmetric and symmetric divisions based on factors such as the developmental stage or the health and disease state of the tissue they are residing in [5].

DIFFERENTIATION POTENTIAL OF STEM CELLS

The differentiation potential of stem cells is described with different functional terminologies:

1) **Totipotent** stem cells, i.e., the zygote and its descendants up to the eight-cell stage in mammals, which can form the embryo and the trophoblast of the placenta [6];
2) **Pluripotent** stem cells, such as the inner cell mass of the blastocyst, embryonic stem cells and reprogrammed cells, such as induced pluripotent stem (iPS) cells that can differentiate into all the cells of the three embryonic germ layers;
3) **Multipotent stem** cells, such as mesenchymal stem cells and several other adult stem cells, which can differentiate into multiple cell lineages like osteoblast, chondroblast and adipocyte, but not all the lineages derived from the three germ layers;
4) **Bipotent** stem cells such as mammary gland epithelial stem cells, which can differentiate into two cell lineages such as myoepithelial and luminal cells [7];
5) **Unipotent** stem cells such as spermatogonial stem cells that can differentiate into only one mature cell lineage like male gamete [3].

STEM CELL NICHE

The fate of a stem cell depends not only on the genetics of the particular cell itself, but also on epigenetic cues; i.e., the signals it receives through the microenvironment. The interactions of these exogenous signals with intracellular signaling mechanisms determine a given stem cell's decision to remain quiescent, self-renew, differentiate, migrate, or even undergo apoptosis. The local microenvironment that provides the physical and chemical support for survival and the spatiotemporal cues for the regulation of stem cell function is called stem cell niche [8]. The structure and components of the niche vary in different tissues and are dependent on the developmental stage. For example, during migration of hematopoietic stem cells from fetal liver to fetal bone marrow, the expression pattern of a single hematopoietic stem cell changes according to the function of a given microenvironment and developmental stage [9]. Depending on the tissue and its developmental stage, a niche could be composed of any combinations of chemical, physical and cellular elements. Some of these elements directly interact with the stem cells, others affect the stem cells by modulating the activities of distinct local factors. For example, osteogenic differentiation of mesenchymal stem cells is enhanced by parathyroid hormone through fine-tuning of bone morphogenetic protein signaling [10]. Extracellular fluid and blood in the local vasculature play a significant role in transport of some of these elements, such as hormones and cytokines, to and from the niche.

In a typical stem cell niche (Fig. 4.1), chemical components such as cytokines and growth factors that affect stem cell function, can be secreted from neighboring 'bystander' cells, the stem cell itself or somatic cells located at a distance in the same tissue or other tissues. For example, interleukin-6 and insulin-like growth factor-I are paracrine cytokines and growth

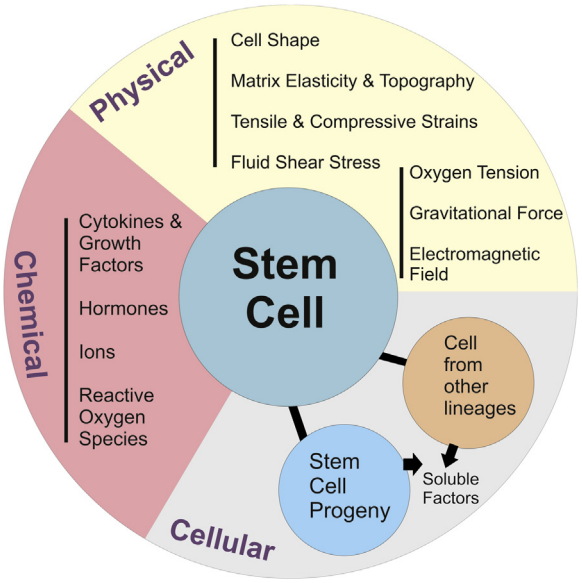

FIGURE 4.1
Composition of stem cell niche including physical, chemical and cellular components. All the components interact dynamically.

factors that promote self-renewal [11] and chondrogenic differentiation [12] of mesenchymal stem cells (MSCs), respectively. Examples of other chemical factors are:

1) Hormones, e.g., parathyroid hormone that increases osteogenic differentiation of mesenchymal stem cells [10];
2) Ions, such as calcium that increase homing of hematopoietic stem cells to bone marrow [13]; and
3) Reactive oxygen species (ROS) that, at low levels can promote self-renewal of neural progenitors [14].

Physical forces, which contribute to the formation of the stem cell niche, originate from different sources of which the extracellular matrix (ECM) plays a significant role as one of the major determinants of cell shape and phenotype. The viscoelasticity and surface topography of the ECM surrounding a stem cell can affect its fate decision *in situ* [15]. For example, differentiating MSCs choose different fates according to:

1) Cell shape, e.g., round morphology favors adipogenesis and flattened morphology osteogenesis [16];
2) Matrix elasticity, e.g., brain-tissue-like elasticity (in the range of several hundred Pascals) favors neurogenesis and bone-like elasticity (in the range of 10–100 kiloPascals) favors osteogenesis [17]; and
3) The matrix's surface topography, e.g., a particular nano-topographic surface pattern can induce the differentiation of MSCs into an osteogenic lineage [18].

The ECM's contribution to the stem cell niche includes its physical properties, structural support and its role as a reservoir of many biochemical cues and as a source for several ligands, which interact with stem cell surface receptors. For example, binding of laminin-5 found in ECM to laminin-specific integrin receptors on the surface of MSCs favors osteogenic differentiation of these cells [19]. Amongst the physical elements of a given niche that contribute to distinct stem cells differentiation are the following:

1) Tensile and compressive strains, e.g., differentiation of MSCs into a vascular smooth muscle phenotype is enhanced by a specific pattern of cyclic tensile strain [20], while their differentiation into a chondrogenic phenotype is enhanced by a certain pattern of dynamic compressive strain [21],
2) Fluid shear stress, which may enhance osteogenic differentiation of MSCs [22],

3) Gravitational force, e.g., simulated microgravity markedly increases the self-renewal capacity of MSCs [23],
4) Electromagnetic field, e.g., pulsed electromagnetic stimulation, which enhances osteogenic differentiation of MSCs [24] or cardiac differentiation of human embryonic stem (hES) cells upon electrical stimulation, [25] and
5) Oxygen tension, e.g., low oxygen tension favors self-renewal of hematopoietic stem cells [26] and endodermal differentiation of mES cells [27].

The cellular components of a particular stem cell niche comprise the stem cells themselves, their progenies and cells from other lineages [28]. For example, the hair follicle stem cell niche in the bulge contains both melanocyte stem cells (McSCs) and hair follicle stem cells (HFSCs). Progenies of the latter are incorporated into the structure of the hair follicle stem cell niche and contribute to the HFSCs' quiescence by secretion of factors such as Fgf18 and Bmp6 [29], while on the other hand, secreting soluble factors, such as TGF-β, that promote McSC self-renewal [30]. Some stem cells establish close interactions with their neighboring cells. For example, hematopoietic stem cells (HSCs) adhere to osteoblasts by activation of Tie2/angiopoietin-1 signaling, which maintains the HSCs in a quiescent state [31]. The establishment of gap junctional contacts between some stem cells and their neighboring cells has also been described. Gap junctions allow diffusion and exchange of small ions, amino acids, metabolites, and second messengers [32]. For example, human MSCs form gap junctions with endothelial cells, a feature which is important for the osteogenic differentiation of these MSCs [33]. The stem cells' response to the niche is highly influenced by the temporal and spatial pattern of expression of specific receptors. For example, in neural stem cells the expression of nuclear hormone receptors changes depending on presence of differentiating cues and the stage of differentiation, leading to a variable response to a particular stimulus at different stages of development [34]. Another important factor is the metabolic state of the stem cells. For instance, while hematopoietic stem cells reside in the bone marrow niche with low oxygen tension, they generate energy mainly via anaerobic metabolism and have low level of ROS, which promotes their self-renewal. Once recruited to the peripheral blood, however, the change of their metabolic state leads to the production of higher levels of ROS. The level of the latter can induce the cells to differentiate, undergo senescence, or lead to apoptosis [35]. Incorporation of some or all of these salient factors into an engineered stem cell niche will be of importance for maintaining the stemness of a particular (tissue-specific) stem cell population or for efficiently manipulating their directed differentiation into a desired (somatic) cell population.

DEVELOPMENTAL ORIGIN OF STEM CELLS

The process of stem cell behavior and differentiation can be understood in the context of the normal processes of development, especially during the embryonic period. Hence, regenerative tissue engineering requires good understanding and application of basic principles of developmental biology. In tissue engineering, stem cells are not just the sources for generation of the right type of somatic cells. Stem cells can also serve as key factors/building blocks for the development of tissues by organizing the stem cells into spatially appropriate models and subjecting them to specific physico-chemical cues. It is necessary to have a comprehensive understanding of the dynamics of early development together with the source and origin of stem cells in order to select the appropriate source of stem cells and design a specific suitable protocol for the use of these cells in specific applications of tissue engineering and regeneration [36]. A detailed review of the initial stages of development in humans is beyond the scope of this chapter and has recently been provided by Sadler, 2012 [37].

In humans, the first eight weeks of development is called the embryonic period. The subsequent intrauterine life after the end of embryonic period is called the fetal period. Most of the named structures of the body appear during the embryonic period. The fetal period is highlighted by maturation of tissues and organs and growth of the body. However, some

TABLE 4.1 Summary of main derivatives of embryonic germ layers

Endoderm	Mesoderm	Ectoderm
Gastrointestinal tract from pharynx to upper rectum	Muscles	Neural crest:
Liver	Bone and connective tissue (except in head)	Melanocytes
Pancreas	Urinary system	Ganglia of cranial nerves and spinal dorsal root
Respiratory epithelium	Reproductive system	Major part of autonomic nervous system
Middle ear epithelium	Circulatory system	Schwann and glial cells
Urinary bladder epithelium	Dermis (except in head and neck)	Adrenal medulla
Thyroid		Bone and connective tissue of head
Parathyroid		Dermis in head and neck
		Nervous system (see neural crest above)
		Sensory epithelium of eye, ear and nose
		Oral epithelium
		Epidermis
		Mammary gland

developmental processes, such as lung development, continue postnatally. An abridged summary of structures derived from each germ layer and neural crest is presented in Table 4.1; a more comprehensive list of the origins of human cell types is provided by Vickaryous and Hall, 2006 [38]. The fate of the differentiating cells is controlled by a set of interacting signaling mechanisms during embryonic development. The overall process of cell fate decision is determined by activation of a few well-known signaling pathways including notch, fibroblast growth factor (FGF), epidermal growth factor (EGF), Wnt/Wingless (Wg), Hedgehog (Hh), transforming growth factor β (TGFβ)/BMPs, non-receptor tyrosine kinase-signal transducers and activators of transcription (JAK-STAT), Hippo, JUN kinase (JNK), nuclear factor kappa B (NF-κB), and retinoic acid receptor (RAR). A particular cell fate decision is determined by the spatial and temporal activation and interaction of these pathways. See Perrimon et al., 2012 for a detailed review [39]. For details of the molecular mechanisms that control specification of endoderm see Grapin-Botton and Constam, 2007 [40]; for mesoderm see Lim and Thiery, 2012 [41]; and for ectoderm see Jiménez-Rojo et al., 2012 [42], for neural crest derivatives see Nelms and Labosky, 2010 [43].

Embryonic stem cells

In both mice and humans, embryonic stem (ES) cells are classically derived from the explanted ICM of the pre-implantation blastocyst. In mice, cultured ICM cells differentiate to early epiblast before giving rise to ES cells; i.e., in mice the origin of mouse ES cells is the early epiblast. Primordial germ cells (PGCs) have also been speculated as the secondary origin of mouse ES cells. It is believed that a longer term culture of ICM cells can lead to appearance of PGCs in the epiblast, which can subsequently give rise to pluripotent cells with the same phenotypic characterization as ES cells derived from early epiblast [44]. In humans, the phenotype of ES cells closely resembles the phenotype of stem cells derived from the post-implantation mouse epiblast (EpiSCs), and hence, it is believed that human ES cells originate from epiblast at a later stage of development i.e., the origin of human ES cells is the late epiblast [44]. More recent data show the possibility of capturing human cells also at the early epiblastic stage. For example, derivation of human ES cells under 5% oxygen tension generated human ES cells with two active X chromosomes, which is one of the characteristics of early epiblastic cells [45].

Non-embryonic stem cells

In terms of the origin of stem cells derived from the fetus proper, supportive fetal tissues, and postnatal tissues, three main theories are currently considered [46].

1) During development, stem cells undergo hierarchical differentiation by decreasing their differentiation potential so that pluripotent stem cells differentiate into multipotent and unipotent stem cells, which in turn, differentiate into the mature cells. Some of these multi- or unipotent cells, which have been developed after somatic lineage specification, escape further differentiation and remain in their respective tissues as stem cells. This is the most widely accepted theory for the origin of postnatal stem cells. For example pancreatic beta cells develop from progenitors, which were committed to be definitive endoderm, foregut endoderm, and endocrine precursor cells [47].
2) Some stem cells escape differentiation before or at early lineage specification, migrate to and colonize the differentiating tissues and acquire the specific tissue stem cell phenotype based on the signals in their niche. HFSCs could be the example of this hypothesis [48]. Another example would be tissue-specific differentiation of neural crest derived progenitor cells of the sympathoadrenal cells into chromaffin cells of the adrenal medulla [49].
3) In adults, there are rare populations of pluripotent stem cells, which can circulate in the body and acquire a particular tissue-specific stem cell phenotype upon integration into different tissues [50].

FETAL STEM CELLS

Detailed studies of stem cells in fetal tissues and their supporting structures, such as placenta, amniotic fluid and umbilical cord, suggest that these cells originate from specific embryonic germ layers. For example, mesenchymal stem cells isolated from chorionic villi [51] and amniotic membrane [52], which are of fetal origin, can be tracked to the chorionic and amniotic extraembryonic mesoderm, respectively. Similarly, stem cells isolated from the maternal part of the placenta, i.e., from decidual tissue, [53] are of maternal origin and from the embryonic mesoderm layer. Epithelial stem cells isolated from the amniotic membrane [54] originate from the epiblast. Amniotic fluid is a known source of stem cells. Amniotic fluid sampling is usually performed after 15th week of gestation for diagnosis of fetal diseases. The cellular composition of this sample depends on the presence or absence of congenital abnormalities, but in general most of these cells are derived from the fetal skin, pulmonary, digestive and urinary tracts and from the amniotic epithelium. Amniotic fluid may also contain cells originating from the mother and from trophoblasts. Amniotic fluid stem cells express both embryonic [55] and mesenchymal stem cell markers [56]. These markers are often expressed by the same cells [57]. Clonal derivatives of these cells can differentiate into cells of all three embryonic germ layers [58]. However, further studies are needed regarding the origin, characterization, maintenance, and differentiation potential of amniotic fluid stem cells before translation into any clinical applications can be considered [59].

Umbilical cord stem cells

The umbilical cord is of fetal origin and contains several types of stem cells. Stem cells have been identified in the umbilical epithelium, which is believed to be derived from the amniotic epithelium, and hence from the epiblast [60]. Stem cells with predominant mesenchymal properties have been isolated from subamnion [61], intervascular matrix known as Wharton's jelly [62], perivascular stroma [63] and subendothelium [64] of the umbilical cord. All these stem cells are believed to originate from extraembryonic mesoderm. The origins of hematopoietic and mesenchymal stem cells in cord blood are the same as of fetal blood, since the umbilical circulation is part of the fetal circulation. Cord blood hematopoietic stem cells are a mixed population of cells at different commitment stages, as characterized by the differential combination of CD34, CD45 and CD133 markers [65]. Other cells isolated from the umbilical cord blood, which reportedly have stem cell properties, include unrestricted somatic stem cells (USSCs) and very small embryonic-like stem cells (VSELs). USSCs are a CD45-negative population [66] with the ability to differentiate towards derivatives of all three germ layers. USSCs exhibit a high *in vitro* proliferation capacity, no spontaneous differentiation, and

immunophenotypic features that overlap with fetal MSCs [67]. There are speculations that these cells may represent the precursor cells for MSCs [66], but their precise developmental origin and molecular mechanisms of stemness remain to be elucidated. Initial studies suggest that their extensive differentiation capacity may be due to incomplete DNA methylation and reversible histone modification of key pluripotency genes including Oct4, Sox2, and Nanog, which epigenetically keep these cells in an uncommitted state [68]. Very small embryonic-like stem cells (VSELs) are cells of very small diameter (3–6 μm) with high nucleo-cytoplasmic ratio, which express the main pluripotency markers including Oct-4, Nanog, and SSEA-4 [69]. They are found in low abundance in human cord blood and are mobilized to peripheral blood as the result of specific conditions, such as skin burn injury [70]. Murine VSELs have the capability to differentiate into cells of all three germ layers and are believed to originate from early migrating primordial germ cell-like cells derived from epiblast [71]. Human VSELs may not show the same differentiation potential as their murine counterparts [72]. However, more studies are needed to define the molecular and cellular characteristics of these intriguing cells in humans.

Fetal hematopoietic stem cells

Fetal hematopoietic stem cells are generated during the process of prenatal hematopoiesis, which takes place in several anatomical locations. In human, extraembryonic mesoderm surrounding the yolk sac is the first site of hematopoiesis. In this site, blood islands appear at about the day 17 of embryonic development [73]. After establishment of vascular connections between the yolk sac and the embryo, these yolk sac-derived hematopoietic cells enter the embryo and colonize the liver at day 23 (first hepatic colonization). Starting from day 27 of development, a few groups of hematopoietic stem cells are detected as the cells adhering to the aortic endothelium in the pre-umbilical area. These cells originate from intraembryonic splanchnic mesoderm [74] and lead to definitive hematopoiesis, which is characterized by differentiation into erythroid and myelolymphoid lineages. The liver is colonized by these cells at day 30 (second hepatic colonization) and bone marrow in the week 11. For detailed reviews, see Baron et al., 2012 [75].

MESENCHYMAL STEM CELLS

MSCs have been widely believed to be derived from embryonic mesoderm. However, a report published in 2007 suggested that these cells may originate from more than one embryonic layer [76]. So far, only a few follow-up experiments have been performed to elucidate the developmental origin of MSCs. These experiments suggested that the earliest MSC cells in the embryonic trunk are derived from the neural crest rather than the mesoderm [76] and identified neural crest derived stem cells in the embryonic circulating blood [77]. During later stages of development, these cells of neural crest origin are replaced with mesenchymal stem cells from other sources, but some of the neural crest derivatives persist in adult tissues. This hypothesis could explain the finding that, for example, bone marrow mesenchymal stem cells are not a homogenous population [78] and that some of these 'MSCs' may differentiate to cells outside the mesodermal lineage. The detailed description of different types of mesenchymal stem cells, their functions and applications for tissue engineering will be presented in Chapter 36.

It is important to apply the knowledge of the origin and development of stem cells in the design of protocols for engineering tissue constructs *in vitro* and also for the use in regenerative tissue repair, especially following a tissue loss. The latter process mimics some of the very early developmental events and involves a progressive remodeling of the existing tissue and progressive phenotypic characterization of stem cells. Consideration of this principle could help in designing and implementing a correct protocol, which stimulates stem cells to follow their normal developmental pathway and hence, enhance tissue repair through a remodeling process [79].

STEM CELL DIFFERENTIATION METHODS

When in their *in situ* microenvironment, stem cells are subjected to the physico-chemical cues and cellular elements of the niche. According to the specific physiological or pathological condition, a niche can have a spectrum of properties, which maintain stem cells in their undifferentiated state or coax them to differentiate into particular phenotypes. Niche elements and their unique properties have been extensively used for the design of protocols for the maintenance and differentiation of stem cells *in vitro*.

Soluble factors such as cytokines, growth factors, and hormones are the most widely used niche elements for *in vitro* differentiation of stem cells due to their relative ease of manipulation. In comparison to other methods that put stem cells in contact with other cells or their products or use genetic manipulation, it seems that the probability of inducing any aberrant phenotype in the differentiated cells is lower when using a soluble factor as the main method of differentiation. There are many examples for the use of this niche element. For instance, differentiation of embryonic stem cells to a distal pulmonary phenotype can be effectively directed by removal of inhibitory soluble factors in the serum [80], followed by a stepwise differentiation into definitive endoderm and distal lung epithelial cells using first activin A and wnt3a and then a cocktail of soluble factors including Fgf2 designed to mimic the natural microenvironment of mature distal lung cells [81]. A most recent variation to this protocol has shown that superior results can be obtained by a more refined stepwise direction of embryonic stem cells to distal lung epithelium by stepwise provision of niche-specific soluble factors and direction of ES cells into five distinct phases of induction of definitive endoderm, anteriorization of endoderm, induction of Nkx2-1+ foregut endoderm, proliferation and specification of lung progenitors, and their maturation [81].

Use of defined extracellular matrix (ECM) components or their ligand sequences is another strategy, which has been adopted to control the fate of differentiation of stem cells. For example, collagen type I promotes osteogenic differentiation of bone marrow mesenchymal stem cells by activations of $\alpha 1\beta 1$ and/or $\alpha 2\beta 1$ integrins [82]. Therefore, use of collagen type I for coating of 2D dishes or fabrication of 3D scaffolds is one of the effective methods for differentiation of MSCs into the osteoblast lineage. Furthermore, the DGEA (Asp-Gly-Glu-Ala) ligand sequence of collagen type I can be used instead of the whole type I collagen and can effectively induce osteogenic differentiation in mesenchymal stem cells [83]. However, Pimton et al. (2011) [84] recently showed that murine embryonic stem cells lack collagen integrin receptors and instead express $\alpha 5\beta 1$, the fibronectin receptors, which are subsequently further upregulated during the initial stages of meso-endodermal differentiation. Hence the choice of permissive ECM components to induce directed differentiation will depend on a clear understanding of the integrin profiling of naïve and differentiating stem cells, which may be stage- and species-specific. In addition to extracellular matrix proteins, synthetic materials that exhibit bioactive properties can also induce stem cell differentiation. For example, osteogenic differentiation of mesenchymal stem cells can be induced by many permissive scaffolds, such as a mixture of bioactive glass-polyvinyl alcohol [85] or hydroxyapatite-containing electrospun chitosan fibers [86].

As described above under the 'stem cell niche', differentiation of stem cells is also controlled by several physical factors. However, as in most cases, integration of these factors into the differentiation protocols needs special equipment and expertise. Physical stimuli including mechanical strain, fluid shear stress, hydrostatic pressure or electrical stimulation are not always in routine use in many stem cell laboratories. Specifically designed dynamic bioreactors are usually needed for subjecting the stem cells to physical factors. In addition to control of physical factors, some of these bioreactors will also enable control of the chemical micro-environment of the cells. For example, when murine embryonic stem cells are cultured in a low shear, low gravity, 3D rotating wall, bioreactor system, the efficiency of their differentiation into pulmonary epithelial cells is significantly enhanced in comparison to a static culture system [87]. This issue will be further discussed in Chapter 15.

To take advantage of the cellular component of the stem cell niche, *in vitro* co-culture of stem cells and other cells has been practiced for many years. The most widely used example of this method is the co-culture of embryonic stem cells (ESCs) and embryonic fibroblasts, which maintains the 'stemness' of ESCs and inhibits their spontaneous differentiation. Enhancement of the differentiation of ESCs into the distal pulmonary epithelial cell phenotype by co-culture of ESCs and embryonic mesenchyme is another example of this method [88]. Although the effectiveness of the co-culture method has been demonstrated in several studies, the difficulty of its administration has prompted the use of cell-conditioned media as an alternative. For example, a medium conditioned by A549 cells, an established cell line from a human lung adenocarcinoma with properties of type II alveolar epithelial cells, directs the differentiation of embryonic stem cells into type II pneumocytes [89]. A medium conditioned by particular 'mature' cell types contains the entire repertoire of soluble factors secreted by these cells ('secretome'), which can affect the process of differentiation of the target stem cells. In order to take full advantage of the competence of a mature cell to direct the fate of differentiation of stem cells, Quin et al. (2009) [90] used a method developed by Hakelien et al. (2002) [91] to permeabilize embryonic stem cells and expose them to the extracted cytoplasm of the mature A549 cells, and hence, effectively direct them to differentiate into type II pneumocytes [90]. This method exposes the stem cells to the whole gamut of intracellular signaling molecules of the mature cells and directs/induces the stem cells to differentiate along the mature cell's phenotype.

Genetic manipulation of stem cells is another means for induction and control of differentiation with high efficacy. This is usually achieved by transfecting the cells with a DNA construct, which encodes the expression of a certain protein. For example, over-expression of bone morphogenetic protein-2 in MSCs has been known to increase their osteogenic differentiation [92]. On the other hand, genetic manipulation can be used for purification of differentiated derivatives of stem cells. For example, Wang et al., (2007) [93] generated a pure population of lung alveolar epithelial type II cells derived from human ESCs using a unique human SPC promoter-neomycin transgene. Although many more successful examples of this approach have been reported so far, the potential risks of application of recombinant DNA technology in the clinical setting should always be considered. The concerns are the safety of the viral-based vectors, the risk of malignant transformation of the transfected cells and the possibility that over-expression of a particular protein may lead to unpredictable physiologic effects.

TRANSDIFFERENTIATION AND REPROGRAMMING

It has been known for many years that differentiation is not a one-way, terminal process. Under certain conditions, an adult differentiated cell can switch its phenotype to that of another mature cell type. This phenomenon is called transdifferentiation. For example, after near total destruction of pancreatic β cells in mice, pancreatic α cells spontaneously transdifferentiated into β cells [94]. Additionally, pancreatic exocrine cells can be transdifferentiated into insulin-producing β cells by over-expression of three genes involved in development of pancreatic β cells, including *Ngn3*, *Pdx1* and *Mafa* [95]. Transdifferentiation is highly facilitated when the cells are from closely related lineages or are derived from the same embryonic layer. For example, both the pancreas and liver are endoderm-derived organs. Hence, using the appropriate sets of lineage-specific reprogramming transcription factors, hepatocytes can be turned into pancreatic β cells and vice versa [96].

Transdifferentiation of one somatic cell into another is not the only strategy for turning an adult cell into one with another phenotype. Mature cells can be turned into pluripotent, ESC-like cells through a process called reprogramming. In addition to the scientific novelty and exciting possibility that they offer for answering specific mechanistic questions related to the process itself, reprogramming could yield patient-specific immunocompatible pluripotent cells on

demand. This opens up many potential clinical applications for these cells, while circumventing the ethical issues related to the use of ESCs. The first evidence of successful reprogramming was provided some 50 years ago by transfer of the nucleus of an intestinal cell of a frog to the enucleated fertilized egg of another frog, which led to the birth of live frogs [97]. This method, which is also known as cloning, is difficult to perform, has a very low efficiency, and needs availability of fertilized eggs, which in case of the human, imposes further practical and ethical restrictions. Also, cloned animals show phenotypic abnormalities which make cloning a challenging technique. In 2006, Yamanaka's group reported that retroviral insertion of a combination of genes for four key embryonic 'stemness' transcription factors Oct3/4, Sox2, c-Myc, and Klf4 could reprogram somatic cells into pluripotent cells, which are known as 'induced pluripotent stem (iPS) cells' [98]. However, the random integration of these transgenes and the risk of insertional mutagenesis raised concerns about the clinical safety of these cells. Hence, this technique is continually being improved upon to avoid random integration and overcome the low efficiency of the process. Although iPS cells are considered to be the surrogates of ES cells, emerging evidence shows a number of genetic and epigenetic differences between them. For example, mis-imprinting of the genes at the Dlk1-Dio3 locus on chromosome 12 has been reported in iPS cells [99]. These and other differences should be addressed by better definition of the epigenetics of pluripotent stem cells and by improving the methods of reprogramming. It should also be noted that iPS cells will be carriers of the same disease/genetic disorder as their somatic parental cells, which may limit their use, especially for treating conditions with significant genetic components. Reprogramming will be covered in detail in Chapter 35.

Finally, while the Yamanaka approach, recently recognized with the 2012 Nobel Prize for Medicine, is exciting in terms of generating quasi-embryonic stem cells, it may not be necessary to take reprogramming all the way back to first having to generate iPS cells, which then can be differentiated into the desired mature somatic cells. Recent studies suggest that essentially all mature somatic cells can potentially be transdifferentiated into any other cells in the body [100]. For example, using diverse combinations of cardiac-enriched defined transcription factors, mouse fibroblasts can be transdifferentiated into functionally mature cardiac myocytes [101]. If harnessed correctly and efficiently, transdifferentiation and/or reprogramming could be used as an additional strategy for the repair of damaged tissue using the patients' own mature cells, hence avoiding the need for the isolation and expansion of rare adult stem cells [102]. However, as the mechanisms and different aspects of this phenomenon are not yet very well understood, many more studies will be needed before this technology can be translated into the clinical arena.

CHALLENGES AND LOOKING FORWARD

Challenges

Although stem cell research offers many hopes for the better management of numerous human diseases, the progress of clinical applications of stem cells in the context of tissue engineering and regenerative medicine has been rather slow so far. This may be in part due to the fact that we still do not have a comprehensive understanding of the mechanisms, which lead to a particular disease and which we then hope to treat with stem cells. A clear definition of what we expect to be modulated and improved by stem cell therapy in different conditions will help us to design more suitable protocols for the clinical translation of stem cell research findings. On the other hand, there are also still many gaps in our knowledge of both the *in vivo* and *in vitro* biology of stem cells, including their microenvironment, signaling, differentiation and proliferation pathways, asymmetric cell division and response to stimuli. The potential safety risk of genetic manipulating stem cells for clinical applications is another issue that needs further in-depth study. More work needs to be done in the field of the *in vivo* biology of human stem cells. Specifically, in the natural *in vivo* setting, each stem cell resides in a niche, which is specific to that stem cell and given tissue. Unfortunately the routine culture of stem

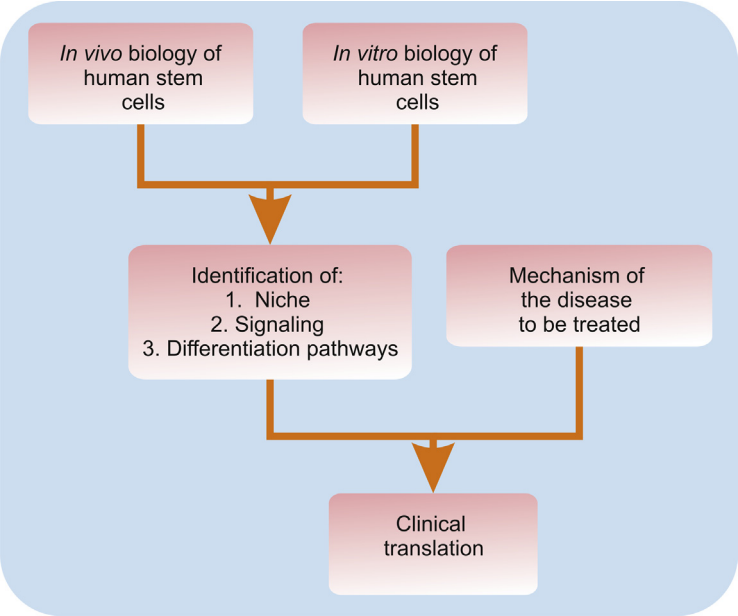

FIGURE 4.2
Major issues to be addressed for successful application of stem cells in tissue engineering and regenerative medicine.

cells in many laboratories is often performed under conditions that are quite distinct from the stem cell niche *in situ*. These differences can profoundly affect the behavior of stem cells not only *in vitro*, but also after implantation *in vivo*. It is therefore highly recommended to identify the natural stem cell niche at a specific developmental stage, from undifferentiated to fully differentiated, and provide the right niche during their *in vitro* manipulation (Fig. 4.2). Understanding the behavior of these cells in normal and pathological conditions *in vivo* will increase our ability to take full advantage of their therapeutic potential in different clinical scenarios.

Looking forward

Overall, there will be an increased demand for faster translational research in the coming years. Due to the technical complexities, interventions that involve a single cell lineage and their secretion products are expected to have a higher rate of progression in the translational phase. A stem cell therapy for restoring the dopaminergic system in Parkinson's disease is an example of such an intervention. We believe that both autologous and allogenic sources will find their ways into the stem-cell-mediated therapeutics. This is because genetic factors contribute to the pathogenesis of some diseases in the first place, and are retained in autologous cells, which continue to carry the same genetic risk factors. We are close to entering the logarithmic phase of growth in the clinical translation of stem cell research, hence we should be prepared for this new and challenging phase by clearly and comprehensively addressing the regulatory issues involved in the clinical application of stem cells as building blocks for regenerative engineering and cell-based therapies.

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CHAPTER 5

Moving into the Clinic

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INTRODUCTION

In the early 1930s Charles Lindbergh, who was better known for his aerial activities, went to Rockefeller University and began to study the culture of organs. After the publication of his book about the culturing of organs *ex vivo* in order to repair or replace damaged or diseased organs, the field lay dormant for many years. Indeed, delivering respite to failing organs with devices or total replacement (transplant) became far more fashionable. Transplantation medicine has been a dramatic success. But in the late 1980s scientists, engineers, and clinicians began to conceptualize how *de novo* tissue generation might be used to address the tragic shortage of donated organs. The approach they proposed was as simple as it was dramatic. Biodegradable materials would be seeded with cells and cultured outside the body for a period of time, before exchanging this artificial bioreactor for a natural bioreactor by implanting the seeded material into a patient. These early pioneers believed that the cells would degrade the material, and after implantation the cell-material construct would become a vascularized native tissue. Tissue engineering, as this approach came to be known, can be accomplished once we understand which materials and cells to use, how to culture these together *ex vivo*, and how to integrate the resulting construct into the body.

Most major medical advances take decades to progress from the laboratory to broad clinical implementation. Tissue engineering was such a compelling concept that the process moved much faster. As we will discuss later, the speed at which tissue engineering solutions can be implemented is inherently faster than traditional drug development strategies. For this reason, coupled with what was probably unexplained exuberance, business investors saw an immediate role for industry in delivering tissue-engineered products to patients.

Traditionally, new fields are seeded with foundational research, development, and engineering prior to implementation, but an apparent alignment of interests caused many to believe that companies could deliver products immediately and that the traditional foundational aspects could wait. The race to clinical implementation of a tissue-engineered medical product began with the incorporation of Advanced Tissue Science (ATS) in 1987. ATS and Organogenesis, an early competitor, began their quest by focusing on seeding biodegradable matrices with human foreskin fibroblasts. In the early days, Organogenesis, Integra, and Ortec focused on bovine-derived scaffolds, while ATS focused on human-derived scaffolds. Other companies focused on developing tissue engineering products using scaffold alone or cells alone. The path to implementation has been very different for each class of company, as will be summarized later. With hindsight, one might say that the choice of living skin equivalents as a first commercial product was probably driven by the willingness of the Food and Drug Administration (FDA) to regulate them as Class III devices rather than biologics. This attractive feature of the products was supplemented by large predicted market sizes and the ease of culturing skin cells.

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As these products moved from the laboratory to the clinic, development issues such as which cells, materials, and bioreactors were supplanted with industrial challenges such as scale-up and immunocompatibility. At the same time that ATS and Organogenesis were rapidly growing, the view emerged that delivery of tissue-engineered products to patients would require an allogeneic off-the-shelf solution with ease of use and long storage life in the United States — and this persists today. This business-driven decision predicated the development of allogeneic, cell-based therapies. Interestingly, in a study sponsored by the National Science Foundation, the World Technology Evaluation Center discovered that non-US investors were focused on autologous cell therapies resulting from a belief that allogeneic therapy would be unsuccessful because of the need to suppress the patient's immune system. This difference in emphasis between US and non-US investors continues today. Both approaches have genuine advantages and disadvantages. However, once it became clear that cell-seeded scaffolds could trigger dramatic changes in natural wound healing, thereby inducing *de novo* tissue formation and function, clinical implementation through industry progressed from skin to a wide array of tissues. In addition, pockets of excellence arose at major medical centers, where new innovations were tested clinically in relatively small numbers of patients.

So one is left with several questions: What have we learned from these early adoptions of tissue engineering? How can these lessons drive sustainable innovation that will both heal and generate a return on investment? Is broad clinical implementation of tissue engineering limited by the nature and structure of regulatory bodies? This chapter seeks to answer these questions by looking historically at selected high-profile clinical tissue engineering programs and looking forward with a suggested generic approach to rapid clinical translation in this new era of advanced medical therapies.

HISTORY OF CLINICAL TISSUE ENGINEERING

What is clinical tissue engineering?

As mentioned earlier, in the early 1990s the term *tissue engineering* was generally used to describe the combination of biomaterials and cells *ex vivo* to provide benefit once implanted *in vivo*. What emerged over the next decade, however, were biomaterials designed to alter the natural wound healing response and cell-only therapies. Lessons learned in the development of each led to the fusion of these tools under the rubric of tissue engineering. Today, there remains confusion about what the term *tissue engineering* truly encompasses. A related term, *regenerative medicine*, has emerged recently. The boundaries of what falls under each of these terms are unclear. We do not seek to provide a definitive answer in this chapter, but herein we discuss the use of biomaterials and cell-seeded biomaterials. We exclude the use of cell-only therapies.

Thus, we define *clinical tissue engineering* as:

> 'the use of a synthetic or natural biodegradable material, which has been seeded with living cells when necessary, to regenerate the form and/or function of a damaged or diseased tissue or organ in a human patient.'

We see clinical tissue engineering as a set of tools that can be used to perform regenerative medicine, but not all regenerative medicine has to be done with that set of tools.

Two-dimensional clinical tissue engineering

The earliest clinical applications of tissue engineering revolved around the use of essentially flat materials designed to stimulate wound care. Tissue-engineered skin substitutes dominated the market for almost a decade. Another small and slim tissue that found a clinical application was cartilage. Later in the 1990s, thin sheets of cells were produced in culture and then applied to patients using a powerful cell-sheet technology. In both the applications,

engineered tissue equivalent is relatively easy to culture *ex vivo* because oxygen and nutrient delivery to thin, essentially two-dimensional, materials is not challenging. In addition, once the construct has been cultured *ex vivo*, integration into the body is not an insurmountable barrier for thin materials.

TISSUE-ENGINEERED SKIN SUBSTITUTES

Since the inception of tissue engineering there has been a focus on the regeneration of skin. A number of drivers led to this early focus, not least of which was the mistaken assumption that skin is simple to reconstitute *in vitro*. Skin cells proliferate readily without signs of senescence. Indeed, fibroblasts and keratinocytes have been cultured *in vitro* for many years with ease. Interestingly, other highly regenerative tissues, such as the liver, are populated with cells that cannot be proliferated *in vitro*. The clinical need for effective skin wound healing was also a major driver. One in seven Medicare dollars is spent on treating diabetes-induced disease in the United States. The largest component of that cost goes toward treating diabetic ulcers. This attractiveness drew many tissue engineering efforts into the wound care market.

REGENERATIVE BIOMATERIALS

For almost two decades scientists have explored the use of processed natural materials as biodegradable scaffolds that induce improved healing from skin wounds. One of the first products to market was the INTEGRA® Dermal Regeneration Template. INTEGRA® is an acellular scaffold designed to provide an environment for healing using the patient's own cells. The INTEGRA® label describes the product as follows:

> INTEGRA® Dermal Regeneration Template is a bilayer membrane system for skin replacement. The dermal replacement layer is made of a porous matrix of fibers of cross-linked bovine tendon collagen and a glycosaminoglycan (chondroitin-6-sulfate) that is manufactured with a controlled porosity and defined degradation rate. The temporary epidermal substitute layer is made of synthetic polysiloxane polymer (silicone) and functions to control moisture loss from the wound. The collagen dermal replacement layer serves as a matrix for the infiltration of fibroblasts, macrophages, lymphocytes, and capillaries derived from the wound bed. As healing progresses, an endogenous collagen matrix is deposited by fibroblasts, simultaneously the dermal layer of INTEGRA® Dermal Regeneration Template is degraded. Upon adequate vascularization of the dermal layer and availability of donor autograft tissue, the temporary silicone layer is removed and a thin, meshed layer of epidermal autograft is placed over the 'neodermis'. Cells from the epidermal autograft grow and form a confluent stratum corneum, thereby closing the wound, reconstituting a functional dermis and epidermis. INTEGRA® is now one of many processed natural materials used to stimulate healing. Since the material is not vascularized at point of use, it is best used in thin (two-dimensional) applications. INTEGRA® is an FDA-approved tissue engineering material widely used in patients today. It does not, however, contain biological factors that are released during the tissue-remodeling process.

Another class of products, the thin extracellular matrix-based materials, does release natural factors as the material degrades, and these factors serve to reset the natural tissue-remodeling process, thereby producing a healing outcome. The most common Extra Cellular Matrix (ECM)-based material is derived from the submucosal layer of pig small intestine. The Cook OASIS® Wound Matrix label describes the product as follows:

> The OASIS® Wound Matrix is a biologically derived extracellular matrix-based wound product that is compatible with human tissue. Unlike other collagen-based wound care materials, OASIS is unique because it is a complex scaffold that provides an optimal environment for a favorable host tissue response, a response characterized by restoration of tissue structure and function. OASIS is comprised of porcine-derived

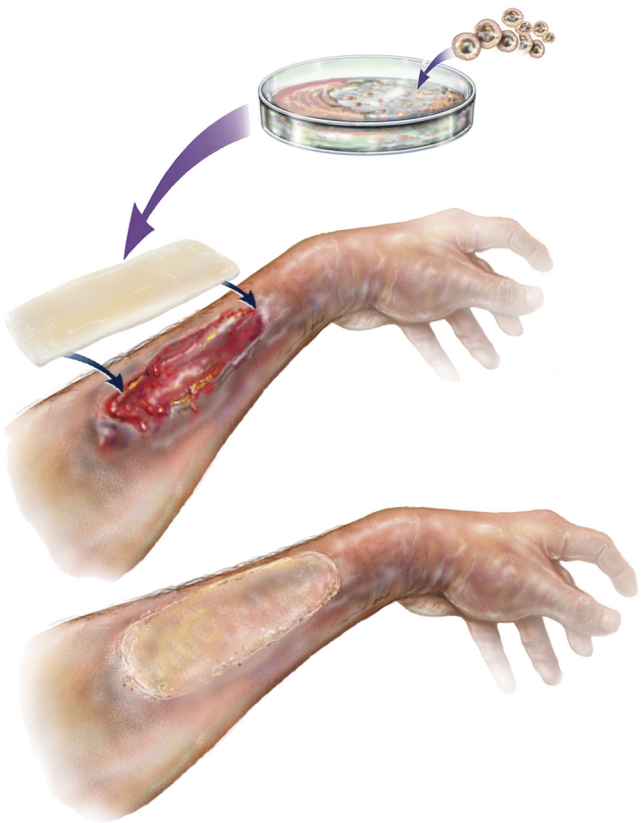

FIGURE 5.1

acellular small intestine submucosa. The OASIS Wound Matrix is indicated for use in all partial- and full-thickness wounds and skin loss injuries as well as superficial and second-degree burns.

Regenerative biomaterials, or materials designed to alter and enhance the natural tissue-remodeling process, are being used in hundreds of thousands of patients worldwide. These materials recruit a patient's own cells into the healing process post-implantation, and their relative simplicity makes them compelling clinical tools for indications where a thin, essentially two-dimensional material will achieve the desired result. In an elegant series of accomplishments, natural matrices have been applied for skin wounds and many other tissue-replacement therapies.

Cultured skin-substitute products (Fig. 5.1), where cells are seeded onto a biodegradable matrix and cultured *ex vivo* prior to shipment and use, have been extraordinarily difficult to market. Given this reality, it is interesting that the purveyors of the two leading skin equivalents are the true early pioneers of tissue engineering. Both Advanced Tissue Sciences and Organogenesis engaged in a valiant effort to use human fibroblasts and biomaterials to regenerate skin. They were challenged by a changing regulatory landscape, an ongoing struggle with reimbursement issues, and the highly complex need to manufacture and ship a living product. A full case study of ATS or Organogenesis would be of tremendous value to the next generation of tissue engineering companies, but is beyond the scope of this chapter. Dermagraft®, the Advanced Tissue Science product now manufactured by Shire, uses skin cells isolated from neonatal foreskins prior to seeding onto a polymeric scaffold. Apligraf®, Organogenesis' product, used similar technology to seed and culture cells on collagen-based scaffolds. Tissue-engineered skin equivalents continue to be developed. As technology improves and multilayer systems progress to broad clinical use, the market should also increase from today's anemic levels of $15 million/year. The FDA and reimbursement issues have greatly impacted clinical use of cultured skin equivalents. The FDA treated these products as devices yet held them to biologic standards, and this led inevitably to their being reimbursed as biologics.

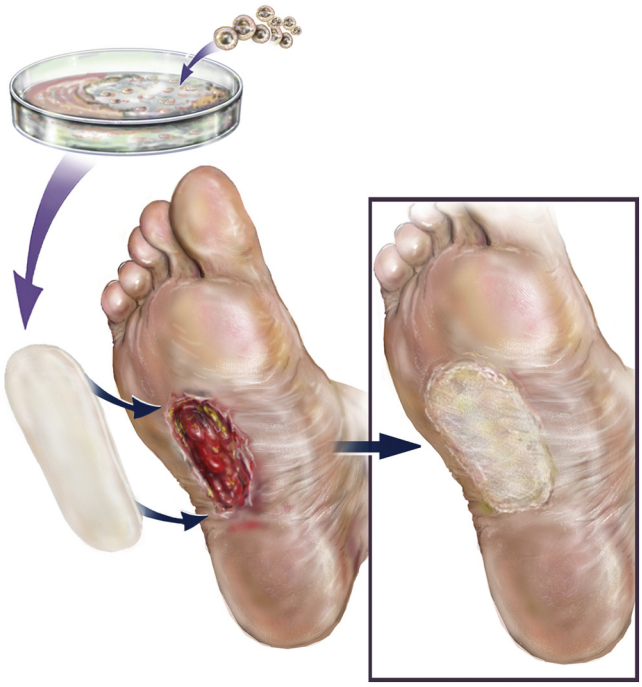

FIGURE 5.2

Another approach to clinical skin remodeling is the use of autologous cell-based therapies (Fig. 5.2). One attractive feature of using a patient's own cells is, of course, the lack of an immune response, but the manufacture of patient-specific yet inexpensive skin replacements is very complex. Epicel® from Genzyme Biosurgery uses irradiated mouse fibroblasts as a feeder layer from which to grow patient-specific keratinocytes. Co-culture with animal-derived cells may raise regulatory and infectious disease questions requiring manufacturing practices that increase the cost of goods. In 1995, Genzyme began expanding patient-specific chondrocytes (Fig. 5.3). Small biopsies were sent to Genzyme, where they were cultured and returned to the surgeon for implantation. The product, Carticel®, was approved as a biologic by the FDA in 1997. At time of treatment, a patient typically receives 10 million to

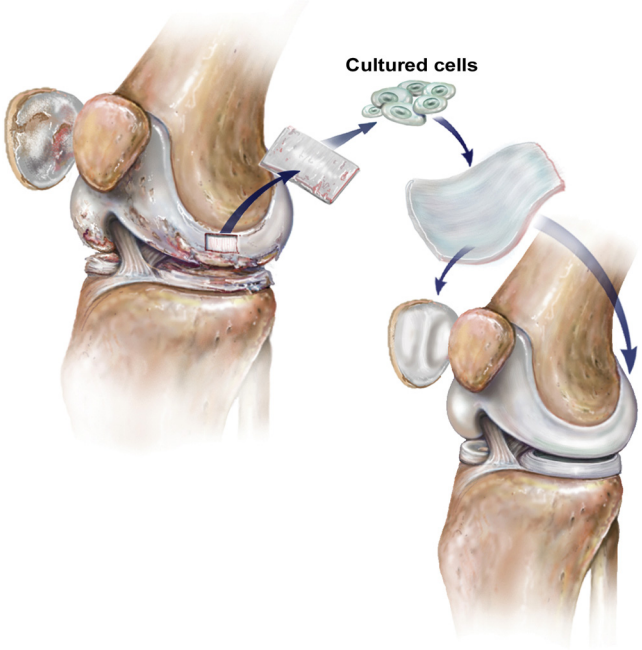

FIGURE 5.3

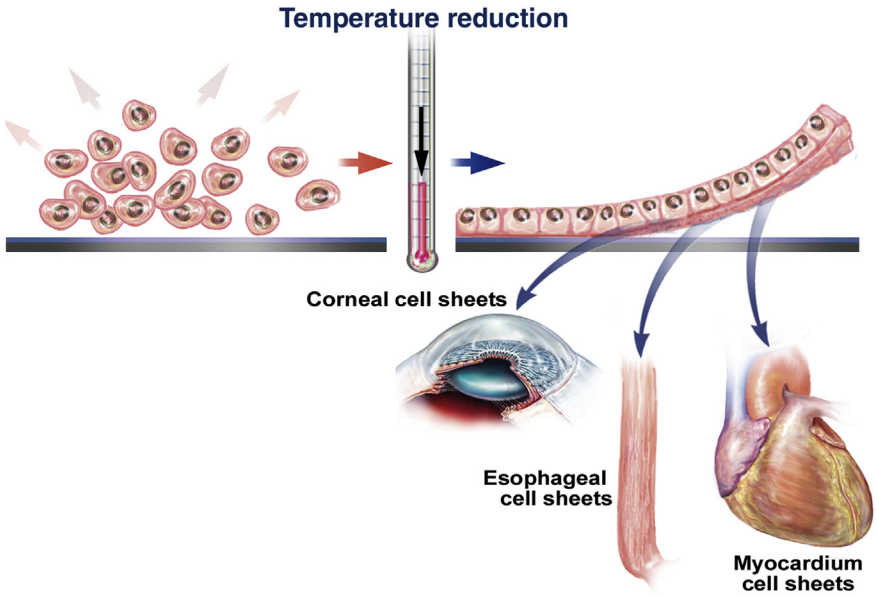

FIGURE 5.4

15 million cells after five weeks of custom *ex vivo* culturing. As with skin remodeling, a number of companies have focused on the use of acellular regenerative materials. Approved products are currently sold in many countries around the world that are based on collagen and/or extracellular matrices (ECMs). Thousands of patients around the world have benefited from orthobiologic approaches to cartilage replacement. Although patient-specific cartilage replacement therapy has also provided benefit, it is a good example of the difficulty of delivering individualized therapies while deriving a profit. The considerable infrastructure required to culture tissue safely in this manner presents unique challenges for the manufacturer to overcome.

Many research groups around the world have sought to improve on the efficacy of Carticel®, focusing on cell-based and regenerative material-based approaches. Although cartilage segments *in vivo* and *in vitro* are generally small and non-vascularized, the biomechanical properties of those tissue-engineered cartilage products have not overall achieved the standards required for clinical application (Fig. 5.4). Okano at Tokyo Women's Hospital has invented a remarkable technology that produces intact cell sheets for clinical applications. In general, when human cells are cultured *in vitro* they adhere to their culture dish substrate. Traditional culturing techniques extract cells by adding enzymes and other materials that digest cell-surface and cell-cell contacts. Cells processed in this manner are delivered as single cells for clinical application. Okano envisioned an alternative for removing cells that has had a dramatic clinical impact. Okano covalently bonds a layer of *N*-isopropylamide to the surface of the culture dish prior to adding cells and has shown that, under normal growth temperatures cells adhere, but when slightly chilled the entire sheet of cells is repelled from the dish without disrupting the cell-cell contacts and can be lifted from the surface rather like a Post-it note®. For a first clinical application, Okano's team cultured corneal epithelial cells and used the resulting sheets to replace the damaged corneal epithelia of dozens of patients. Okano has reported significant success and, although the number of patients in need of corneal epithelial replacement is limited, this cell-sheet technology has real potential for broader clinical tissue engineering application.

ENCAPSULATED PANCREATIC ISLETS

The use of biomaterials to immunoisolate pancreatic islets of Langerhans has been studied since the mid-1990s. If one could build a cage that surrounded the islet and had a mesh size

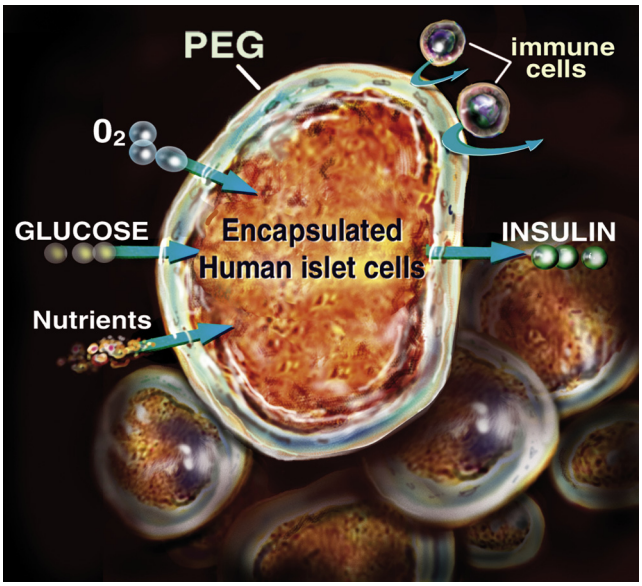

FIGURE 5.5

small enough to prevent the approach of antibodies to the islet but large enough to enable nutrient diffusion, it may be possible to diminish a patient's dependence on insulin, post-transplant. Alginate-encapsulated islets have been studied for many years, and an ongoing clinical trial (Novocell) is using interfacially polymerized Polyethylene Glycol (PEG)-encapsulated islets (Fig. 5.5). The success of these trials is not yet known, but porcine islets have already been shown to be protectable in a short-term discordant xenotransplantation model. Interestingly, our own work has shown that even a molecular-scale PEG cage can immunoisolate islets, and this has now been shown to eliminate insulin dependence in diabetic animals.

Three-dimensional clinical tissue engineering

BONE REGENERATION

Since the turn of the century, Dr. Yilin Cao has led a remarkable clinical tissue engineering approach to craniofacial reconstruction in Shanghai, China. Regeneration of craniofacial bone in patients has now been reported by using demineralized bone and autologous cells. Using tissue engineering to rebuild lost bone is novel, but it is not the only regenerative medicine approach being applied to the challenge.

Peptide-based therapy is an established treatment for stimulating bone formation. Bone morphogenetic protein (BMP) is the most common drug currently employed to induce bone growth. In a novel application of BMP, Medtronic developed a spine fusion device containing a collagen sponge infused with the peptide that is now in clinical use (Fig. 5.6). Deployed in the spine, the device and BMP induce native bone to fill the cavity within the device.

Although not often identified as such, this combination of a biodegradable material and a tissue-formation-inducing biologic molecule is clinical tissue engineering at its best.

BLADDER

Early successes in the tissue engineering field were gained in relatively simple tissue structures, organizations, or functions, such as chondrocytes, or two-dimensional cellular structures with limited organ function required. Tengion advanced a technology pioneered by Anthony Atala to augment or replace failing three-dimensional internal organs and tissues, requiring functionality and a vascularization platform using autologous progenitor cells, isolated and cultured *ex vivo*, and seeded onto a degradable biomaterial optimized for the body tissue it is

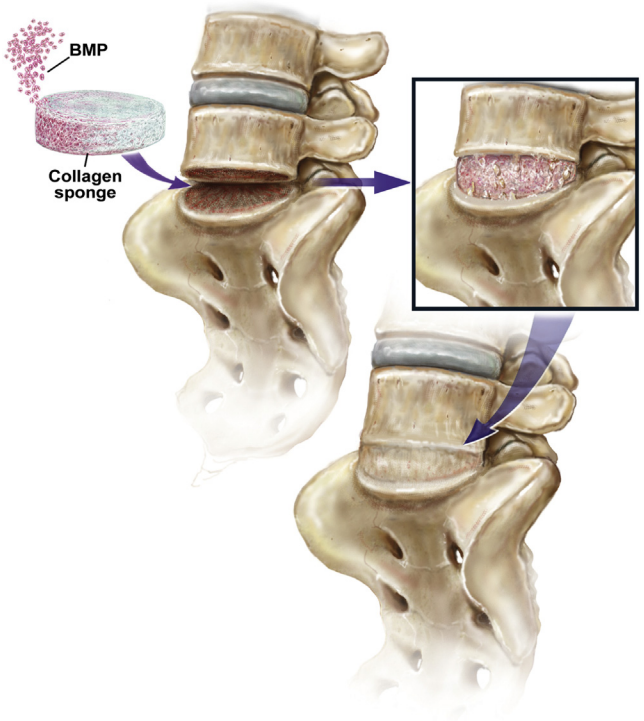

FIGURE 5.6

intended to augment or replace. This cell-seeded neo-organ construct is implanted into the patient for final regeneration of the neo-organ (Fig. 5.7). Using the neo-organ construct as a template, the body regenerates healthy tissue, restoring function to the patient's failing organ. This autologous organ and tissue regeneration avoids many of the negative implications of traditional donor transplantation techniques, such as requisite immunosuppression and limited donor supply. Tengion's initial focus on the genitourinary system was based on a bladder augmentation and ultimately an organ replacement for patients who have undergone radical cystectomy, or removal of the bladder. Tengion developed a robust focus on manufacturing capabilities to support neo-organ construct production in accordance with regulatory standards.

BLOOD VESSEL

At Tokyo Women's Hospital, Dr. Toshi Shin'Oka has used patient-specific tissue engineering to replace malformations of pediatric pulmonary arteries. Working with a biodegradable matrix designed by one of the 'fathers' of biomaterials, Dr. Ikada, Shin'Oka seeded a tubular material with the patient's own bone marrow cells at the time of vessel reconstruction (Fig. 5.8). In a series of clinical experiments, Shin'Oka demonstrated that the biodegradable scaffold's strength during the degradation period was sufficient to allow complete natural vessel replacement without rupture. This first successful clinical replacement of a pediatric blood vessel with a tissue-engineered construct designed to become as natural as the patient's own vasculature was performed in almost 50 patients. As one considers these historical events and the advances in tissue engineering over the past 80 years, we are seeing that tissue engineering is moving toward the regeneration and repair of increasingly complex tissues and even whole-organ replacement. This field holds the realistic promise of regenerating damaged tissues and organs *in vivo* (in the living body) through reparative techniques that stimulate previously irreparable organs into healing themselves.

Regenerative medicine also empowers scientists to grow tissues and organs *in vitro* (in the laboratory) and safely implant them when the body is unable to be prompted into healing itself. We have the technological potential to develop therapies for previously untreatable

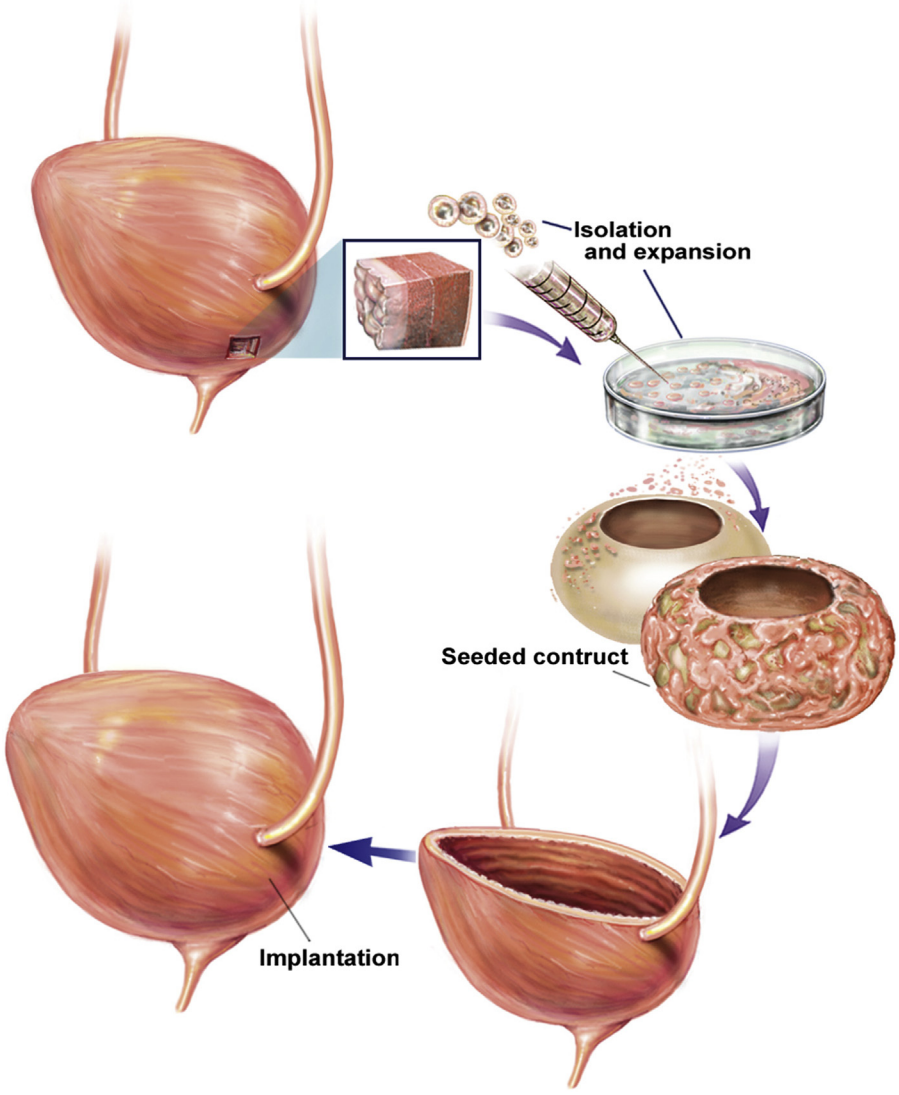

FIGURE 5.7

diseases and conditions. Examples of diseases regenerative medicine could cure include diabetes, heart disease, renal failure, and spinal cord injuries. Virtually any disease that results from malfunctioning, damaged, or failing tissues may be potentially cured through regenerative medicine therapies. Having these tissues available to treat sick patients creates the concept of *Tissues for Life* [1].

STRATEGIES TO ADVANCE TOWARD THE CLINIC

Since the mid-1980s we have had many opportunities to learn how one might quickly convert tissue engineering technology into regenerative medical products from the bench to the bedside. Establishing a plan to move toward clinical testing rests on a strategy of defining the unmet medical need (patient population), determining the intended use of the tissue-engineered/regenerative medical product (TERMP) that addresses the need, and defining the processes necessary to ensure that the product can be reproducibly manufactured to be both safe and effective once it is placed into the patient. A requisite scientific basis for partial or complete structural and/or functional replacement of a diseased organ or tissue requires a definition of what constitutes a successful outcome (i.e., primary clinical endpoint) in the appropriate patient population conducted under adequate and well controlled studies.

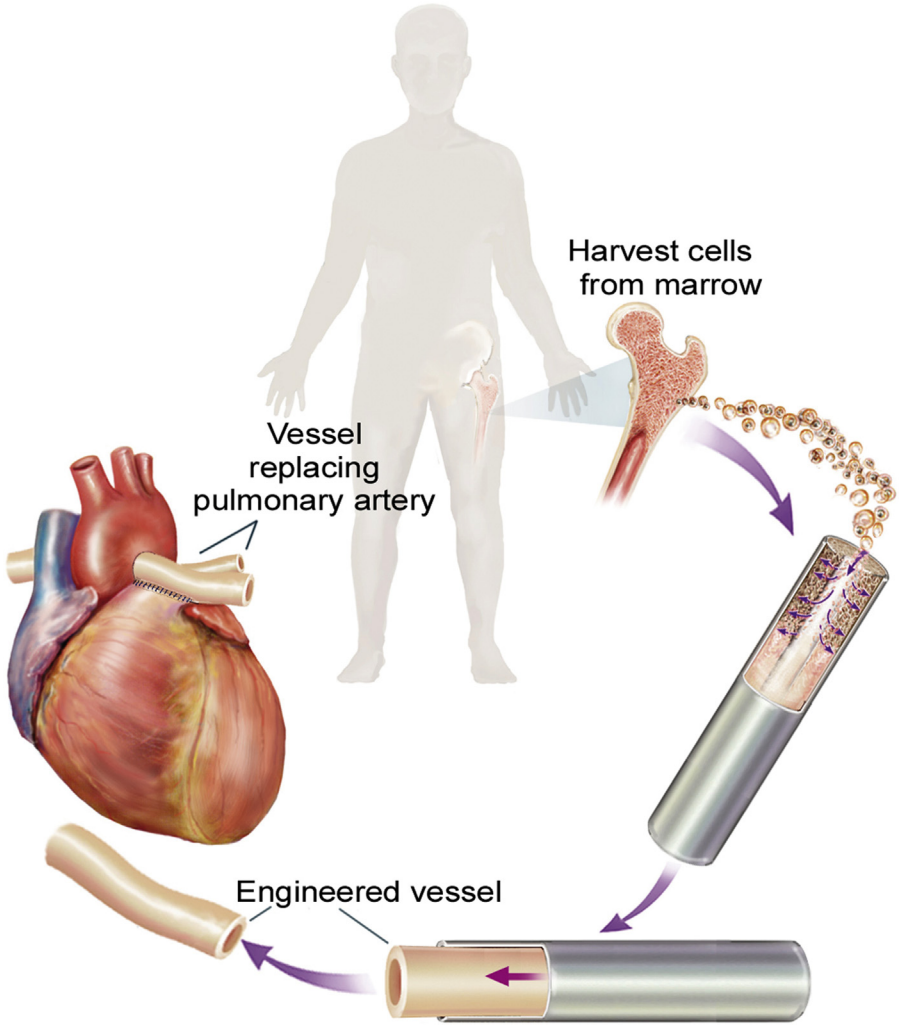

FIGURE 5.8

Ultimately, any clinical testing will require the application of existing regulatory guidelines for testing, clinical trial design and manufacturing a product prior to use in a human subject. With this information in hand, initial steps into clinical testing phases can be contemplated.

As we have already seen, sound scientific strategies are not effective in clinical translation unless there is a balancing sustainable business strategy. Naturally, the scientific data, clinical plan and business strategies must be woven together, in terms of both specific outcomes and timelines. As we will discuss in detail later, the significant differences between traditional drug therapies and tissue engineering therapies actually offer the opportunity to accelerate the bench-to-bedside process. We take the position that instead of a 10- to 15-year development cycle, tissue engineering therapies can be brought to market in 8—10 years, notably, when the product under development is for an orphan population. In considering the exploratory clinical testing phase (Phase I and II) with a scientifically based program, final product characteristics must be defined as well as standardizing the production processes and anticipating what justifications will indicate readiness for entering into the next phase of clinical testing (confirmatory studies). Table 5.1 presents an overview of a prototypical product development process.

Transitioning into an initial exploratory clinical evaluation rests on understanding the objectives for the first regulatory review as they relate to the specific product characteristic, the process to make the product, translational medical study results, and how preclinical

TABLE 5.1 Overview of a potential testing program to support clinical entry of a prototypical Tissue Engineered/Regenerative Medical Product (TERMP)

Cellular/chemistry manufacturing control
Define product production and early manufacturing processes
Establish cell, tissue and biomaterial sourcing for GMPs
Validate product processing and final product testing scheme
Characterize adventitious agents and impurities for each element
Define lot-to-lot consistency criteria
Validate quality control procedures
Translational medicine studies
Complete *in vitro* and *in vivo* testing
Define toxicity testing of raw materials composing the TERMP
Evaluate biomaterial biocompatibility
Establish immunogenic and inflammatory responses to each component
Develop rationale for animal and *in vitro* models to test product effectiveness
Define of endpoints for establishing TERMP durability
Clinical trials
Develop rationale for safety and clinical benefit (Risk/Benefit analysis)
Design exploratory and confirmatory trials
Select patient population and define inclusion/exclusion criteria
Identify investigational comparators and control treatments
Establish primary and secondary study endpoints
Consider options for data analysis and potential labeling claims

information demonstrates the desired clinical outcome [2,3]. In a rapidly changing field where regulatory agencies are still maturing in their decision-making processes, the decisions made during the initial clinical evaluation phase can have far-reaching impact. Table 5.2 provides an overview of data that will be needed prior to entering into an exploratory clinical trial. Regulatory considerations for the types of data required and bioprocess technologies must be in place prior to initiating clinical trials. Indeed, the regulatory environment is much more defined today than in the early days of tissue engineering because of scientific advances and

TABLE 5.2 Information needed prior to evaluating a TERMP in clinical studies*

	In vitro	*In vivo*
Raw materials supply		
Cells*	+	±
Scaffold	+	±
Manufacturing process controls — In-process and potency		
Cellular Processing*	+	−
Biomaterial processing	+	−
Final Combination Product*	+	+
Translational Medicine studies		
Safety and Efficacy	+	+
Endpoint Selection	+	+
Translation into Clinical Design	±	+

**for products comprised solely of acellular scaffold material, evaluation of cellular components is not needed.*

TABLE 5.3 Regulatory considerations for the development of a tissue engineered or regenerative medical product

	Description	Impact
Manipulation of cells	For structural and non-structural tissues, manipulation is minimal if it involves centrifugation, separation, cutting, grinding and shaping, sterilization, lyophilizing or freezing (e.g., cells are removed and reintroduced in a single procedure) Manipulation is not minimal if cells are expanded during culture or growth factors are used to activate cells to divide or differentiate Defined in 21 CFR 1271.3(f) – see also (FDA, 2005) 4	More extensive regulatory requirements applied to TERMP when manipulation is more than minimal
Cell source and application	Homologous use is interpreted as the augmentation tissue using cells of the same cellular origin. Examples include applying bone cells to skeletal defects and using acellular dermis as a urethral sling. Non-homologous use examples include using cartilage to treat bladder incontinence or hematopoietic cells to treat cardiac defects. Defined in 21 CFR 1271.3(c) – see also (FDA, 2001) 7	Non-homologous use triggers additional requirements for entering clinical trials
Scaffold characterization	Final scaffold composition and design determine whether the TERMP is characterized as a device, a biologic or a combination product. Defined in Quality Systems Regulations (QSRs) in 21 CFR 820 (FDA, 2005) 4 – see also (FDA, 1999) 11	Devices are held to the QSRs in 21 CFR 820 (FDA, 2005) 4, biologics are required to comply with Good Manufacturing Processes (GMPs) (FDA, 1991)12 and combination products are often required to comply with both sets of regulations and guidelines

insights gained from various attempts to commercially develop tissue engineering and regenerative medical products. Once again, understanding the regulatory environment is foundational to successfully implementing the development plan and using the scientific objectives laid out in Tables 5.1 and 5.2. Several regulatory considerations have significant impact on the development plan necessary to bring a TERMP to clinical testing: extent of cellular manipulation, cell source and use, and scaffold characteristics (Table 5.3). With a scientific foundation, an established product characterization, and application of appropriate regulatory considerations established, three additional considerations come into play for a particular technology to be transitioned from the bench to the bedside: raw materials testing, manufacturing process controls testing, and translational medicine.

Raw materials testing

CELLS

Cellular components of a TERMP are raw materials encompassing viable cells from the patient (autologous), other donors (allogeneic), or animals (xenogeneic). Standards for cellular quality have been extensively reviewed and considered by regulatory bodies and generally focus on controlling introduction of infectious diseases and cross-contamination from other patients. These standards also consider potential for environmental contamination from the

facility and equipment and the introduction of infectious agents from materials used to process cells (e.g., bovine-derived material that may contain infectious agents). For TERMPs that have cells placed onto a scaffold, scientific and regulatory considerations focus on ensuring that both the raw materials comprising the scaffold and its three-dimensional characteristics are biocompatible [4]. Biocompatibility testing involves evaluation of the scaffold's potential cytotoxicity to cells being seeded, potential toxicity that may be inflicted on the recipient's tissues once implanted, as well as the consequences of immune and inflammatory responses to the TERMP after implantation. Biocompatibility extends through the *in vivo* regeneration process; therefore, biocompatibility should be evaluated in parallel with demonstrating that the scaffold maintains the necessary biomechanical properties to support new tissue or organ growth.

SCAFFOLD

Synthetic, natural, or semisynthetic materials are readily available from various commercial sources, but the quality control of a material varies substantially between medical and research grades. As testing of a potential TERMP moves from research bench to clinical testing, scaffold composition and designs must be controlled for reproducibility of production and product characterization. Final production must consider quality management and organization, device design, production facility environmental controls, equipment, component handling, production and process controls, packaging and labeling control, distribution and shipping, complaint handling, and records management, as outlined in 21 CFR 820 [5]. However, during the exploratory phase and transition from bench to clinical testing, the most relevant of these guidelines are process validation and design controls. Typically, a design input phase is a continuum beginning with feasibility and formal input requirements and continuing through early physical design activities. Engineering input on final prototype specifications follow the initial design input phase and establish the design reviews and qualification. For a combination TERMP, defining quality for the chemical polymer (e.g., PGA) or natural material (e.g., collagen), including any residues introduced during machine processing (e.g., mineral oil), can require QSR integration into a product that would otherwise be regulated as a biologic. Since many TERMPs are combination products, testing of scaffold, cells, and the cell-seeded scaffold (i.e., construct) are required to ensure that, in exploratory clinical trials, the product is sterile, potent, fit for use, and composed of the appropriate raw materials to function properly following *in vivo* placement.

Manufacturing process controls and testing

CELLULAR PROCESSING

In-process controls generally focus on sterility, viability, and functional analysis of cells from isolation, through expansion and before they are placed on the scaffold. Release criteria generally ensure that cells remain viable and functioning properly after being attached to the scaffold. Functional evaluations of cells and potency assessments of their 'fitness for use' are performed after cells are combined with (or seeded onto) a scaffold. Taken together, these tests determine whether the final product can be released from the production facility for surgical implantation in the clinical setting.

BIOMATERIAL PROCESS AND TESTING

The focus of biomaterial process testing is to evaluate the *in vivo* behavior of the scaffold material following implantation. Characterizing the scaffold degradation profile ensures that breakdown time and other degradation attributes will support the regenerating tissue long enough for it to acquire the appropriate functional and structural integrity as the scaffold material degrades. Defining scaffold-breakdown products identifies the biochemical factors that may impact reparative, inflammatory, immunologic, and regenerative processes once the product is placed into the body. Measuring biomechanical properties such as stress-strain

relationships, Young's modulus, and other characteristics ensures that the scaffold portion of the combination product will perform properly during the *in vivo* regenerative phase.

Final combination product testing

Analytical methods for final product testing vary substantially, depending on the composition of the TERMP. In general, any product intended for customization to individual patients (e.g., autologous products) requires confirmation that release and potency standards are met via non-destructive test methods. Such test methods are typically novel and specific to each product type and are frequently based on a battery or 'matrix' of tests that evaluate cellular function and physical parameters of the scaffold. In contrast, lot-testing strategies, statistical sampling, and more routine analytical methods are available for scaffold-only products and cell-based products produced in large lots (e.g., allogeneic and xenogeneic cellular products). In the future we may see allogeneic therapies that are customized for patient-specific needs. Naturally, such innovations will require a combination of analytic approaches.

Translational medicine

Safety and efficacy evaluation of a TERMP is conducted in animals, and the findings are foundational to designing the first clinical trial protocol. These translational studies are the basis for safely transitioning a potential product into clinical testing. Since the regenerative process invoked by components product involve multiple homeostatic (e.g., metabolic), defense (e.g., immune), and healing (e.g., inflammation) pathways, animal studies provide an approach to understanding the inherent function of the TERMP (i.e., if the product contains cells, it can be considered a living 'tissue') and the inherent response of the body to a product composed of biomaterials with or without cells. Animal studies are a regulatory necessity, but we must also remind ourselves that many therapies function effectively in animals and fail in humans. The reverse is not often discussed.

Some therapies may fail preclinical testing and never enter clinical trials, but this is not to say that some of those therapies would not be excellent when applied in humans. An interesting example of this conundrum can be found in artificial-blood therapies. The US Army received approval for clinical use of a natural blood substitute in trauma applications during the recent war in Iraq. In a post-approval attempt to understand why the product worked so effectively in humans it was tested in a porcine model of hemorrhagic shock. The pigs did not do well with the therapy. The investigators proceeded to test the product in rodents and demonstrated that the rodents died when injected with the blood substitute that worked so effectively in the clinic. If preclinical animal testing had been performed first, this excellent product would never have been submitted for clinical evaluation.

Translational medical studies can be conducted in large animals (e.g., dog) or small (e.g., rat). Selection of the correct animal model should be based on the similarities of the pathophysiology, physiology, and structural components intended for treatment in the clinical setting. Exploratory clinical trials for most medical products utilize normal human volunteers as the first line of clinical testing. However, some products can be tested outside of the intended clinical population. As such, the animal model employed in translational medicine should resemble the human condition as closely as possible — immune status, inflamatory response, and healing pathways, as well as the medical approaches used to treat the human condition (e.g., surgical procedure) and monitoring methods to follow a clinical benefit or risk (e.g., imaging). Many pivotal preclinical experiments are performed in academia, and the importance of complying with the good laboratory, manufacturing, and tissue practice regulatory standards is critical at this phase. There is no such thing as Good Laboratory Practices/Good Manufacturing Practices/Good Tissue Practices (GLP/GMP/GTP)-light, and many academic animal facilities are not compliant to the degree needed by the FDA. This issue will increase in

TABLE 5.4 Test categories described in ISO10993-1

In vitro assays	*In vivo* assays
Cytotoxicity	Irritation
Pyrogenicity	Sensitization
Hemocompatability	Acute systemic toxicity
Genotoxicity/Genetic tests	Sub-chronic Toxicity
Local tolerance	

significance as the FDA increases its post-approval auditing of preclinical compliance. Since the regenerative response starts at the moment of TERMP implantation and concludes with the final functioning neo-tissue or neo-organ, animal studies provide an understanding of how to evaluate the early body responses, as well as longer-term outcomes reflecting the desired benefit — an augmented or replaced tissue or organ. Since most products are surgically implanted for the life of the patient, the duration of a translational study would extend to the time when final clinical outcome is achieved. Regulatory agencies have given considerable thought to the duration of translational studies, and many are of long duration — months to years. Nonetheless, since the final outcome is frequently achieved in a shorter period of time, the potential to conduct shorter-duration studies based on final patient outcome may present a rational solution to testing clinical utility in the shortest possible time while ensuring a high benefit:risk outcome. Understanding which endpoints are available and appropriate for clinical testing is achieved through translational studies. Standards are provided for the proper safety evaluation of TERMPs, whether they are regulated as a device [6] or a biological product (e.g., 351 or 361). Although the optimal testing strategy will typically be product specific [7], some basic guidelines for testing device-like products can be found in the ISO10993-1 guidance document. These testing guidelines cover a number of *in vitro* and *in vivo* assays (Table 5.4).

A scaffold-only product that is similar to an already tested material or medical device can be accelerated through the testing process using a 510K approach under an existing PMA [8]. Appropriate translational testing approaches will follow the biocompatibility flowchart for the selection of toxicity tests for 510(k)s [4]. If the device requires an IDE/PMA level of testing, then the translational studies will be more extensive and influenced by the length of time that the TERMP is in contact with the body of the recipient. As already mentioned, many regenerative medical devices or the tissues that replace the initial implant are in bodily contact for longer than 30 days and are therefore considered permanent devices. These products require a full range of *in vitro* and *in vivo* testing approaches prior to clinical testing. If the TERMP is cell-based or the primary mode of action is mediated through the cellular constituents of a scaffold-cell combination product, then the device requires an IND/BLA. The testing approach for these products will usually involve an assortment of studies that evaluate scaffold and cellular components through appropriate endpoint selection and experimental design for both *in vitro* and *in vivo* translational studies. An example of a preclinical development program for a cell-based product is presented in Table 5.5.

Although specific testing approaches are not defined absolutely, the scope and testing approaches for a specific TERMP can frequently be predicted by evaluating the development approach used for related technology platforms. A number of tissue engineering technologies can bridge from bench to clinical application. Testing a TERMP prior to moving into clinical evaluation is based on:

1) Scientific information demonstrating that the potential clinical product can invoke a response in the body of potential therapeutic benefit;
2) Demonstration of a controlled and reproducible manufacturing process; and
3) Demonstration of the safety of each component and the final product.

TABLE 5.5 General translational medicine testing paradigm for a cell-based tissue-engineered regenerative medical product

Cellular component	Scaffold	Combination
Phenotype characterization Genetic stability Biocompatability Biomechanical properties Degradation profile	Early stage (Acute) Late stage (Chronic)	Early stage (Acute toxicity) Late stage (Chronic toxicity)

This stage in the development of a prototypical clinical product is typically the first point of regulatory authority and governance body interactions and an area where procedural approaches for establishing controls are frequently reviewed and clarified.

BRINGING TECHNOLOGY PLATFORMS TO THE CLINICAL SETTING

General

Technology platforms that intend to recapitulate a tissue (e.g., skeletal muscle, bone, cardiac muscle) or an organ may address a range of unmet medical needs, from simple cosmetic defects in the body (tissue-focused technologies) to life-threatening maladies (organ and organ system replacement) (Fig. 5.9). Bringing a TERMP technology to clinical testing may rest on the scope of unmet medical need and the availability of alternative therapies. The array of available alternative therapies influences the early testing strategy of a particular product by

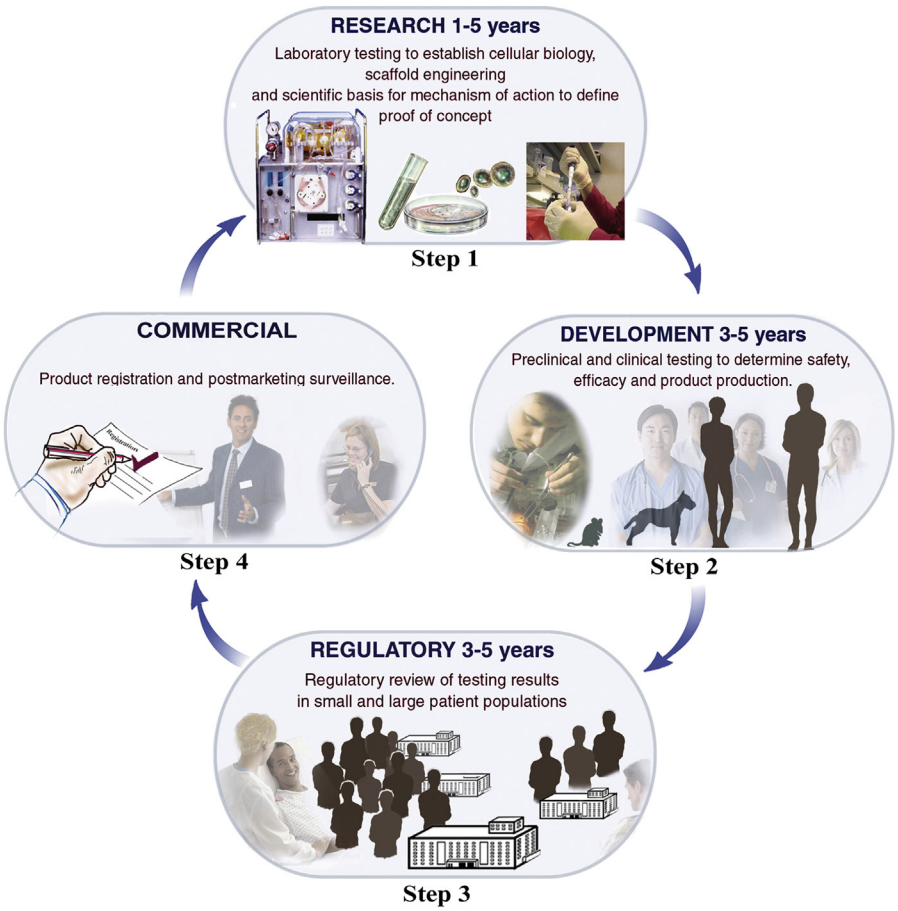

FIGURE 5.9

determining comparable products to be evaluated, selection of animal models, appropriate endpoints and amount of preclinical information needed to enter into clinical testing. Ultimately, the safety and efficacy of the prototype product are balanced by a risk-benefit analysis versus other available products, which directly influences the ability to test it in human trials.

Tissue-focused technologies

Tissue-focused technologies, such as bone and tendon repair, may move into clinical testing through routes that have been established by previous successes (e.g., Depuy's Restore®). If animal models and alternative therapeutic approaches are established, comparing the benefit of a proposed product to an existing therapy may be an appropriate approach to potential clinical testing. Ultimately, comparing the benefit of the TERMP versus the 'gold standard' commercial product or surgical therapy is the foundational rationale to evaluate potential human use. Depending on the raw materials composing the TERMP, the primary mode of action may drive the testing strategy for novel products. The primary mode of action is defined by the scientific studies demonstrating the range of bodily responses invoked by the product and the range of long-term outcomes. Products that elicit an immune response (e.g., allogeneic, xenogeneic, or genetically modified cells) will need to include an evaluation of immunotoxicity, immunomodulation, and/or potential for rendering the recipient sensitive to infectious diseases. Those products whose production employs animal materials will require testing for adventitious infectious agents or the use of materials from certified sources. Testing for potential endogenous infectious agents prior to clinical testing is especially relevant for products that contain, or whose production process includes, xenogeneic cells. Products using scaffold material for which there is little or no previous human testing will require testing that follows established FDA Guidelines (see G95-1) [4]. Biodegradable scaffolds have a testing paradigm similar to that used for a non-biodegradable material, with additional requirements for defining the degradation profile, breakdown products released, route of excretion, and response of the body to the material as it breaks down.

Ultimately, the final safety/efficacy testing strategy may rest with the regulatory pathway selected through a process established by the 'Office of Combination Products'.

Organ-based technologies

Tissue-engineered/regenerative medical technologies offer the promise of alleviating the vast organ shortage that exists worldwide. In spite of this great promise, the pathway to clinical testing with a product that replaces an entire organ is the least clearly defined. Although the clinical benefit of such a TERMP may be definitive, the endpoints readily discernible, and the animal models established, the delivery mechanism, procedures for connecting the neo-organ to other parts of the body, may pose substantial development hurdles and actually preclude clinical testing.

The complexity of whole-organ replacement by these types of products spans defining what is actually being replaced through defining what ancillary products may be needed if all organ functions are not included in the product characteristics. Traditional therapeutic approaches have generally focused on one pathway or target (e.g., pharmaceutical) or possibly two therapeutic benefits, such as structural and functional restoration (e.g., cartilage repair products). However, those products replacing an entire organ (e.g., kidney) or body part (e.g., limbs) will need to consider broad functional testing of both exocrine/excretory and endocrine functions before clinical testing can be considered. The transition to clinical testing of more complex TERMPs will have commensurate preclinical testing requirements to demonstrate not only the functionality of each component being replaced or augmented, but also the biological responsiveness of the integrated organ to native homeostatic mechanisms (e.g., integration with blood pressure or glucose control). Matters such as percutaneous conduits, skin infections, and controlling biofilms may be substantial development hurdles for the use

of products outside the body. For products intended to be used inside the body, solutions for vascular connections, waste product release pathways (e.g., urinary tract and GI), clinical monitoring of neo-organ development, and establishing how long it takes to achieve the desired clinical outcome may all need to be established before clinical testing can be considered. Biosensors and integration of biosensors with TERMPs replacing whole or major portions of an organ's function are becoming a reality. Moving into clinical testing with such products requires definition of *recovery pathways* in the event of product failure; definition of alternative therapies to be used in association with the product if not all organ functions are replaced; understanding TERMP longevity and how to replace the product if the product/neo-organ wears out; and understanding the rate of product failure for proper clinical management. In spite of these hurdles, the lure of replacing an entire organ is considerable. The benefit to society of replacing a kidney or pancreas is unimaginable. As scientific advances in *in vitro* organ growth are made and regenerative templates for entire organs are pioneered (e.g., through such technologies as organ printing), the potential to replace, regenerate, repair, and restore entire organ systems is being considered. Tissue engineering approaches may yield solutions for some of the most devastating human conditions, including congenital agenesis, cancer, degenerative disorders, and infectious diseases. However, entry into clinical testing with such products has not yet been defined.

TRANSITION TO CLINICAL TESTING

Defining and testing a prototype

Prior to beginning a clinical testing program, the TERMP's specific characteristics must be defined to the point that the product can be repeatedly and reproducibly manufactured for *in vitro* and *in vivo* testing as defined earlier. Product characteristics should be sufficiently stable to allow for data-driven demonstration of their clinical utility. Once a prototype is defined, its characteristics are evaluated in a series of tests to define the limits of the initial design criteria that allow for durability testing of the product design by establishing failure points, limits of TERMP application, and the achievement of design criteria. Anticipating the clinical conditions, complications, and untoward events that may arise during clinical testing also establishes a prototype's potential for clinical utility. A TERMP is seldom introduced as a final functioning neo-tissue; therefore, characterizing the pharmacological responsiveness, electrophysiological parameters, and phenotypic and structural features of the neo-tissue or neo-organ that emerges following implantation is key to demonstrating the product's ultimate clinical benefit.

Specific design elements of a final TERMP prototype that will be tested in humans are the culmination of a series of biological, physical, and chemical evaluations obtained during the prototyping phase. This characterization also defines sourcing and control of raw materials, assembly processes (aka: in-process testing) and release criteria.

Additionally, the product's shelf life, shipping conditions (temperature, humidity, nutrients, etc.), stability, sterility, and method of use are established before clinical testing. Any unique surgical procedures, clinical management practices during and after implantation, and recovery times are estimated based on the translational medical results using the final prototype product with the fully embodied characteristics.

Extending existing technology

Using previously tested technology platforms can accelerate the entry of any TERMP into clinical testing. Most products are combination products based on multiple technology platforms. Using one or more already-approved scaffold materials, cell-processing methods, culture media components, or transport containers greatly reduces the number of variables that need to be tested in product prototyping and preclinical testing phases. Additionally,

historical data available for any technology can help develop testing strategies for a final prototype and even establish early clinical phase designs.

Production of TERMPs in GMP facilities

With established product characteristics, standard operating procedures and clinical production processes, a GMP-qualified facility can be deployed to manufacture the first clinical prototype. GMP facilities not only meet GMP guidelines, but they have specialized facility designs and highly trained personnel to produce faithfully the first clinical prototypes in a controlled and reproducible fashion. Considerations for GMP facilities include capacity limitations, availability restrictions, and costs to build, operate, and maintain. Furthermore, utilization of a particular GMP facility may be constrained by the controls needed to generate a particular product. Deploying contract manufacturing is a strategy that can hasten product-prototype production in a manner that complies with regulatory guidelines. TERMP technologies can vary substantially, so it is not uncommon for small GMP facilities to be custom-built to meet the needs of a particular technology platform. Facility design considerations are outside of the scope of this chapter, but a GMP-qualified facility that can provide the required clean-room processing, shipping, and receiving procedures and Heating Ventilation Air conditioning (HVAC) systems for airflow maintenance should be identified before any consideration can be given to initiating clinical testing. This should be done as early as possible, but no later than the final stages of the prototyping phase, to ensure that the necessary facility design capable of producing products in compliance with GTPs and GMPs is available. Contract manufacturing operations (CMOs) have emerged that produce scaffold-only and scaffold-plus-cell products. These operations have staff skilled in various aspects of product manufacturing and generic facilities that can accommodate a variety of cellular methods and biomaterial-handling needs. A technology transfer plan [9] should be established before engaging a CMO, to ensure optimal product generation and the success of the first clinical trial.

Medical and market considerations

Entering clinical testing of TERMPs will not achieve the promise of impacting major unmet medical needs without consideration of market demands. These demands include third-party payers' willingness to support costs, follow-up care, and subsequent patient morbidity. The availability of lower-cost alternatives may be the most significant and practical barrier to clinical testing of a TERMP. Tissue engineering technologies address medical needs unmet by pharmaceutical agents or devices, but these needs may be met by the modification of medical practices, lower-cost alternatives (e.g., cadaveric skin), or currently accepted medical procedures (e.g., tissue transplantation). Exploratory clinical testing strategies can incorporate these alternative approaches to establish the comparative clinical benefit of a prototype product. As the science and technology of tissue engineering becomes more established and regulatory pathways are clarified, products will become more broadly applied. Strengths and limitations of TERMP technologies will determine market size and application to unmet medical needs. At present, products have few competitors in the market place, and the opportunities are driven largely by reducing a particular technology to practice.

Regulatory considerations and governance bodies

Multiple FDA review organizations oversee TERMPs, depending on their characteristics. For device-like products, Center for Device of Radiological Health (CDRH) is the regulatory center, for biological products it is Center for Biological Evaluation Research (CBER), and for combination products, the Office of Combination coordinates a time-bound process that begins with a Request for Designation to assign the combination product to the appropriate center. For example, scaffold and cell TERMPs having a cell-based primary mode of action would most likely be regulated by CBER's Office of Cellular, Tissue and Gene Therapies, with varying involvement from CDRH. These regulatory organizations conduct evaluations

TABLE 5.6 Regulated practices for consideration when taking a TERMP to clinical testing

Good tissue practices – 21 CFR 1271
Good manufacturing practices – 21 CFR 210 and 211
Good laboratory practices – 21 CFR 58
Good clinical practices – 21 CFR 50
Quality systems regulations – 21 CFR 820*

**Replaced cGMPs for TERMPs regulated as devices.*

under different regulatory authorities, depending on the designation of the product and the extent of clinical testing required. Lower-risk products that are minimally manipulated and intended for homologous use are considered under Section 361 of the Public Health Services Act and must comply with current good tissue practices (Table 5.6). Higher-risk products (e.g., cartilage that is implanted to provide bladder support) that are modified through tissue culture or genetic manipulation and not intended for homologous use are regulated under Section 351 of the Public Health Service Act and must comply with both the current good tissue practices and good manufacturing practices (Table 5.6) and go through a premarket approval review through an IND/BLA under 21 CFR 312/601 or IDE/PMA 21 CFR 812/814. In moving toward clinical testing, non-governmental groups guided by governmental regulations provide oversight of studies conducted in animals and humans. Animal care, use, and housing are governed by an institutional animal care and use committee (IACUC) whose operations are defined and established in 9 CFR 1–3. Although not a direct part of regulatory requirements to engage in clinical testing, institutions conducting animal studies in support of human trial testing are regulated by good laboratory practices (GLPs) and comply with United States Department of Agriculture (USDA) guidelines. Human subject testing is also governed by an institutional review board (IRB). IRB conduct and necessity are controlled by 21 CFR 56 whenever an application is submitted for a research and marketing permit. Specific IRB conduct may vary somewhat between institutions, but the IRB is consulted about necessary preclinical data prior to consideration of human subject testing in that institution.

CLINICAL TRIAL PLANNING

Clinical trials are human research studies designed to assess the effect of a new TERMP or treatments in study volunteers. The treatment may be a novel technology, application, or new use for a previously untreated or untreatable ailment. Trial design can vary substantially depending on the clinical hypothesis but can be classified as exploratory or first-in-human trial and confirmatory trial for product registration. Trials generally evaluate safety and efficacy in the defined human population, and frequently serve to compare the new technology with a current standard of care. These trials generally involve volunteer patients with specific health issues which the investigator believes will benefit from this new treatment.

In the US, the National Institutes of Health classifies clinical trials based on their purpose (http://www.ich.org/about/organisation-of-ich/coopgroup/pandrh/topics-under-harmonisation/article/good-clinical-practices.html). For example:

1. Compassionate use trial is a means to provide experimental medicinal products before the FDA has given final approval for use in humans. The FDA must approve compassionate use, usually only after a case-by-case analysis. This use is granted only for individuals who are very ill and have no other treatment options available to them.
2. Diagnostic trials are conducted to define tests or procedures that have better accuracy, sensitivity, or turn-around time for diagnosing a particular disease or condition than is currently available. This type of trial usually includes volunteers who exhibit symptoms of the disease or condition being studied.

3. Expanded access trials allow for the distribution of experimental drugs to participants who are not responding to currently available treatments for their illness and who are also unable to participate in ongoing conventional clinical trials.
4. Prevention trials are designed to discover better ways of disease prevention in people who have never had the disease or to prevent reoccurrence of a particular disease. These types of trials may include the use of medicines, vaccinations, dietary supplements such as vitamins and minerals, or changes in lifestyle.
5. Quality of life trials are sometimes referred to as supportive care trials. During these trials, ways to improve comfort and quality of life for individuals with a chronic illness are investigated.
6. Treatment trials test novel treatments which may include new combinations of drug therapies, new approaches to surgery or radiation therapy, or application of tissue-engineered and regenerative medicine products.
7. Screening trials are designed to address the best and most practical way to detect diseases or health conditions.

Principally, there are two main types of treatment studies; randomized and non-randomized. In a randomized controlled trial, abbreviated RCT, study volunteers are allocated at random to receive one treatment type over another. One of these treatments is the control, which is usually the standard of care. In some trials the control may be no treatment at all. The randomization is designed to prevent any bias that the investigator has from affecting the study results and their subsequent interpretation. Randomization procedures should include equal treatment group sizes to allow for adequate statistical powering, they should have low selection bias to prevent the investigator from inferring the next group which the next study volunteer will go to, and there should be a balance in covariates across the groups.

In a double-blind randomized trial, neither the study volunteers nor the clinical investigators know who belongs to the control group and the test group. Only upon completion of the study is this information revealed. Double-blind randomized studies have proven to be extremely successful towards eliminating subjective bias of both the study subjects and the clinical investigators, since neither knows to which group the study volunteers belong. In contrast, single-blind studies, in which the investigator knows but the study volunteer does not, runs the risk of the volunteer becoming biased as a result of their interaction with the investigator. When both investigators and study volunteers know to which group the volunteers are in, the RCT is called unblinded or open.

To alleviate as much bias as possible from these types of studies, it may be desirable to have the people assessing the data be blinded. Since the clinical outcomes are measured after the study participants receive the full course of the treatments, RCTs are quantitative studies, which make them one of the simplest and more rigorous ways of determining a cause-and-effect relationship between treatment and outcome in clinical research. Despite the widespread acceptance of the scientific merits of randomization, there may be reluctance to advocate such a trial in TERMP due to the lack of blinding for a surgically implanted product. Such experimental requirements are generally tested in the preclinical studies to allow TERMP to be tested in patients who may benefit from the therapeutic mechanism.

A non-randomized clinical trial is one in which the study volunteers are not assigned by chance to different treatment groups; they may choose which group they want to belong, or they may be assigned to the groups by the clinical investigator. The reporting of a non-randomized trial requires special care, especially when claims are made about efficacy. Reporting should address the potential biases that could affect the conclusions (Glossary of Clinical Trial Terms. http://clinicaltrials.gov/ct2/info/glossary). This is especially true for trials which use a comparison with a historical control group. Nonetheless, a non-randomized study is valid for evaluating pilot studies of novel therapies, provided that the potential biases are

recognized. Non-randomized studies are also compelling when the treatment outcome is so remarkable that it cannot be explained by the combination of the potential biases.

Clinical trials are typically divided into four phases, each of which is treated as a separate entity requiring its own clinical investigation. A description of each is provided below:

- Phase I – Initial studies on a small group of healthy volunteers to determine if there are any adverse effects of the treatment. These studies may include patients who do not have the disease for which the new medicinal product is designed to treat. At this phase, the overall safety of the treatment is unknown and the purpose of the study is to define safety.
- Phase II – This is a controlled clinical study to evaluate the effectiveness of the product for a particular illness in volunteers with the illness. Phase II can commence only after the safety of the treatment has been demonstrated during Phase I. The treatment group is generally larger than in a Phase I trial. The purpose is to determine if there are any common safety risks and establish early indications of potential efficacy.
- Phase III – Controlled and uncontrolled trials, often randomized and involving multiple centers, include a greater number of volunteers than in Phase II. The goal is to gather additional information to evaluate the overall effectiveness and benefit-risk relationship of the medicinal product, including any side effects, overall safety, and quality of life.
- Phase IV – After regulatory agency approval, this phase involves post-marketing studies to gather additional information regarding the product's risks, benefits, and optimal use. The purpose is to allow for detection of any rare or long-term adverse effects among a larger patient population which may not have been evident during the previous phase trials.

TERMP represent a relatively new therapeutic modality for clinical trial design and regulatory agency oversight. Although TERMP products are being actively developed and registered for commercialization, the number of examples for TERMP product-prototypes that have advanced to late stage clinical development remains relatively limited so generalizations on clinical development are not readily available. This has posed challenges not only for regulatory authorities in how these products are categorized but also in how manufacturers need to think about testing and characterizing their product. A recent publication by Lee et al. provides an excellent overview of the FDA process and some of the key scientific considerations, which may be applicable to developers of TE/RM products. These authors emphasize several key points to consider when planning to conduct clinical trials with TE/RM products:

1. The appropriate regulatory pathways need to be identified as early as possible during product development
2. Engage regulatory agencies, the example used being the FDA, early on during product development. The FDA encourages such informal exchanges so that they can have a preview of what they will be seeing during the anticipated formal filings. At the same time, the product developer can gain valuable insight into what the agency will be looking to see in the filing
3. Be up-to-date and well versed in all relevant agency guidance documents and regulations
4. Anticipate scientific and clinical questions that will be asked at the clinical phase of product development
5. Plan for changes in product design and manufacturing

A complete list of guidance documents from the three FDA centers in charge of regulating medicinal products can be found on their respective websites (CFR– Code of Federal Regulations, part 312 – investigational new drug application, subpart A– General provisions, Section 312.3 Definitions and interpretations. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=312.3; Top contract research organization http://www.jazdhealthcare.com/healthtech/leaf/Health-Information/Clinical-Research/Contract-Research-Organizations-CROs.htm; Overcoming Cost Escalation with Design and Construction Strategies http://www.tradelineinc.com/reports/CCDDBD31-2B3B-B525-8E9C98EBAD6AE55F).

The actual clinical plan including protocol, clinical opinion leader network, clinical site identification, patient population to be tested and, initiation of the clinical trial can be based on established approaches for clinical trial conduct of approved medical products. However, the specific aspects unique to TERMPs require consideration prior to trial initiation.

Clinical design and protocol: Patient population, number of clinical sites, primary outcome, secondary outcome, I/E criteria, endpoint selection

The clinical trial design for a TERMP is generally composed of four major sections:

1) Study design including duration and randomization;
2) Patient population to be studied including controls;
3) Inclusion/exclusion criteria;
4) Outcome measures including safety and efficacy endpoints.

Logistics for setting up a clinical trial including identifying clinical centers, physician investigators, monitoring, data collection and reporting are beyond the scope of this chapter.

STUDY DESIGN

Study designs for TERMP remain an area of active evaluation and can include the use of single or multi-group studies, with or without randomization. As pointed out above, general study designs used for other medicinal products (e.g., devices) can serve as a basis for investigator selection and regulatory dialog.

PATIENT POPULATION

Establishing the most appropriate patient population for testing a TERMP is critical to advancing toward commercialization. Most TERMP trials will involve patients with the disease target. Such trials will have significant preclinical safety evaluation programs to ensure the patient volunteers are adequately protected and the product being tested can be safely administered. One approach not infrequently used with TERMPs is to identify a specific subpopulation that can be studied using adequate and well controlled trials. These 'orphan' populations are generally defined by US regulatory authorities as being composed of fewer than 200,000 patients/year, or when there are more than 200,000 patients/year, where the R&D costs will not be recovered upon sale of the product (http://www.tradelineinc.com/reports/CCDDBD31-2B3B-B525-8E9C98EBAD6AE55F).

INCLUSION/EXCLUSION CRITERIA

A key component of any clinical trial focuses on the criteria that will be used to screen subjects willing to enter it. These criteria require careful consideration with TERMP studies, because the first-in-human trials frequently are conducted in patients with the disease condition being targeted. Consequently, allowing patient volunteers who are insufficiently affected by the disease condition may prevent detection of either a safety or efficacy signal, especially in small studies with few patients; however, such patients are more capable of being treated in the event of an adverse outcome linked to the TERMP being tested. Conversely, allowing patient volunteers who are severely affected by the disease may provide results that are highly indicative of an effective treatment but equally sensitive to potential adverse effects of the TERMP understudy.

Inadequate definition of these criteria will lead to highly variable results that will not provide a successful outcome due to the inability to detect either a safety or efficacy signal. Balancing these type I and type II errors requires clear definition of the inclusion/exclusion criteria and establishing endpoints that are consistent with product evaluation and the patient population's response to both the disease and TERMP effects.

OUTCOME MEASURES — SAFETY AND EFFICACY ENDPOINTS

Selection of endpoints will vary between exploratory and confirmatory trials. All clinical trials must consider a robust evaluation of the safety for both the TERMP and product delivery approach being used. Safety outcomes can include symptomatology, hematology, and analysis of various body fluids and tissues. For TERMP, the use of imaging to evaluate both safety and efficacy is a common approach — safety might be biocompatibility and efficacy may be the appropriate tissue or organ structure. Additionally, patient quality of life, function of the TERMP once implanted and durability of the TERMP are key outcome measures.

ESTABLISHING A REGULATORY PATHWAY

Substantial clarification about appropriate regulatory pathways for evaluating TERMPs has occurred in recent years and is currently most advanced in the United States. Since several regulatory pathways exist for these products and most of the product characteristics consist of a scaffold and cells isolated from a specified source, the Office of Combination Products (OCP) serves as the most common entry point for establishing regulatory authority (21 CFR 3) [10]. Notably, some regenerative products may fit into existing regulatory pathways for drugs, devices, or biologics. Since regulatory pathways for individual products are well established, consideration here will focus on TERMPs composed of a combination of materials (biologics, drugs, and/or devices). A sponsor seeking to obtain regulatory guidance for a combination product prepares a Request for Designation (RFD) document laying out key information requested by the FDA (Table 5.7). This document presents the sponsor's recommendation and rationale for how the combination product should be regulated. The FDA's decision on how to regulate a combination product is based on the primary mode of action, a judgment that focuses on the scaffold and cellular components of the TERMP. If the product is sufficiently close to a product already regulated by a particular center and pathway, the FDA's decision about requirements for clinical trials may mirror that product's regulatory pathway. The FDA has 60 days from the time of RFD submission to render a decision. Once a pathway has been identified, the sponsor can engage that particular reviewing authority for the optimal study plan to support their first clinical trials.

Specific guidance on engaging the Office of Combination Products and establishing communications with the FDA can be found on the FDA website (FDA, updated regularly). Interacting with this office prior to clinical testing can assist in linking to the proper regulatory authority and necessary regulatory guidelines. For some products the primary mode of action is not readily apparent, and the primary mode of action assignment may be based on the most relevant therapeutic activity, intended therapeutic use, similarity of the product to an existing product, or the most relevant safety and efficacy questions. This designation is then used to establish the most relevant regulatory center and potential regulatory pathway for entry into the clinics and ultimate product registration. A current assignment algorithm and flowchart can be obtained on the FDA website.

TABLE 5.7 Request for designation — information requested by FDA

Name of product
Composition of product
Primary mode of action
Method of manufacture
Related products currently regulated by the FDA
Duration of product use by the patient
Science supporting product development
Primary route of administration

CONCLUSIONS

It is inevitable that regenerative medicine-based products will represent an important class of treatments for future patients. These products have the potential to satisfy significant unmet medical needs with an almost unimaginable benefit — a cure, not just a treatment. Regenerative medicine products can be customized to heal the specific needs of the patient in need. Currently, many regenerative medical products have little downside risk, since they eliminate rejection — autologous products representing the clearest example. These products may offer unmatched benefit-risk profiles with the potential to be rapidly approved for introduction into the appropriate patient populations and bring reductions in health care costs and substantial patient benefits, particularly when there are no medically acceptable alternatives.

The path to clinical entry has already been paved for these breakthroughs, which emerge from applying established processes — in cell biology and scaffold engineering — in a knowledgeable way. It is possible that regenerative medical products can be brought to market more rapidly and efficiently than traditional medical products (e.g., pharmaceuticals).

The logistical advantages include development that can occur quickly with patient studies (rather than time-consuming and costly large-scale preclinical studies to define unknown risks), smaller trial sizes (customized nature of the products), and long-term follow-up that occurs post-registration (these products, once implanted, become part of the patient). One could easily envisage that once there is a dramatic success that combines effective therapy with compelling clinical data, industrial scale efforts will open the floodgates to developing treatments for diseases that today fill patients with fear and little hope. The responsibility of tissue engineers for today will be to deliver on the promise of the hope and bring forward the promise of their scientific endeavors.

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CHAPTER 6

Tissue Engineering: Future Perspectives

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CLINICAL NEED

Tissue engineering combines the principles of materials and cell transplantation to develop substitute tissues and/or promote endogenous regeneration. The approach was initially conceived to address the critical gap between the growing number of patients on the waiting list for organ transplantation due to end-stage failure and the limited number of donated organs available for such procedures [1–3]. This need continues to grow internationally [4]. Increasingly, tissue engineering, as a subfield of regenerative medicine, will also focus on even more prevalent conditions in which the restoration of functional tissue would answer a currently unmet medical need. The development of therapies for patients with severe chronic disease affecting major organs such as the heart, kidney, and liver, but not yet on transplantation waiting lists, would vastly expand the potential impact of tissue-engineering technologies. A notable example is congestive heart failure, with over 5 million patients in the United States alone, across all ethnic groups, who might benefit from successful engineering of cardiac tissue [5,6]. Similarly, diabetes mellitus is now recognized as an exploding epidemic with well over 16 million patients in the United States and more than 217 million worldwide [7]. Patients with type 1 diabetes have lost most or all of their pancreatic β cells due to autoimmune attack, and potentially could be treated by transplantation of surrogate β cells or neo-islets. However, a significant fraction of patients with type 2 disease, by far the more prevalent form, also have insufficient pancreatic β-cell mass, and might benefit from receiving β-like cells [8]. A recent report from the US National Academy of Sciences entitled *Stem Cells and the Future of Regenerative Medicine* highlighted these and other conditions, including osteoporosis (10 million US patients), Alzheimer's and Parkinson's Diseases (5.5 million patients each), severe burns (0.3 million), spinal cord injuries (0.25 million) and birth defects (0.15 million), as targets of regenerative medicine [9].

CURRENT STATE OF THE FIELD

Significant progress has been realized in tissue engineering since its principles were defined [10] and its broad medical and socioeconomic promise was recognized [11–13]. Yet to date only a handful of complex products incorporating cells together with scaffolds have gained regulatory approval; notably bioartificial skin grafts and replacement cartilage, and these have only achieved limited market penetration [14]. Nonetheless, recent clinical reports

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with multiple years of patient follow-up document the maturation of the field and validate the development of neo-tissues and neo-organs. Furthermore, the tissue engineering and stem cell industry is rapidly becoming economically robust; annual commercial revenues and spending approached equivalence at about $3.5 billion in 2011 [15]. The combination of advances in both clinical development and commercialization indicates that these technologies will reach increasing numbers of patients in the years to come.

Studies in research models and humans have supported the principle that cell-seeded scaffolds generally perform much better than synthetic scaffolds alone [16]. In one early clinical study, tissue-engineered vascular grafts (TEVG), utilizing autologous bone marrow cells seeded onto biodegradable synthetic conduits or patches, were implanted into 42 pediatric patients with congenital heart defects [17,18]. Safety data were encouraging; there was no evidence of aneurysms or other adverse events after a mean follow-up of 490 days (maximum 32 months) post-surgery. The grafted engineered vessels remained patent and functional. Most importantly, the vessels increased in diameter as the patients grew. However, detailed analysis demonstrated that donor cells did not contribute directly to the long-term development of regenerated vascular tissue, but appeared to play a trophic role in mobilizing host cells from nearby vessels. The investigators who carried out this pioneering study on TEVG now hypothesize that comparable benefits might be obtained with cell-free vascular grafts that would elute cytokines from sophisticated scaffolds to enhance endogenous regenerative responses [19].

Studies of urinary bladder constructs in a canine model convincingly demonstrated the promise of whole organ tissue-engineering technology [20]. As with TEVG, tissue-engineered bladders grafted into juveniles responded to growth regulation as the host matured [21]. The first human studies of bladder augmentation with tissue-engineered constructs yielded very encouraging clinical data [22]. Seven pediatric patients in need of cystoplasty because of high-pressure or poorly compliant bladders received grafts comprising autologous urothelium and smooth muscle cells expanded *ex vivo* and seeded onto a biodegradable collagen or collagen-PLGA composite scaffold. Serial follow-up data obtained over 22 to 61 months (mean 46 months) post-surgery provided evidence for safety and efficacy of the procedure and highlighted advantages over previous surgical approaches. Some of the patients have now been followed for more than 11 years and continue to do well. More recently, 2 phase II clinical trials of tissue-engineered bladder augmentation were carried out under the sponsorship of Tengion Inc. Sixteen human subjects received neo-bladder constructs with autologous cells seeded on scaffolds (www.clinicaltrials.gov, NCT00512148 and NCT00419120). As of this writing, results from these trials have not yet been reported in the peer-reviewed literature.

The promise of tissue engineering in the field of urology has been amplified by a successful observational clinical trial of autologous, cell-seeded, tubularized urethral constructs in five boys with a mean age of 11 years [23]. Subjects were followed for at least 3 years to more than 6 years after surgery, and maintained wide urethral calibers without strictures.

Tissue-engineered bladders and urethras remain experimental, and considerable work remains to be done before they are likely to achieve regulatory approval and broad medical use. Nevertheless, advances in cell biology, biomaterials and bioreactor technologies, and improved understanding of basic regenerative mechanisms, bode well for future application to substantial numbers of patients [24,25]. The need for improved procedures to enable diversion of urine from the kidneys in patients with advanced bladder carcinoma is particularly acute [26]. A clinical trial is underway to test the safety and efficacy of a simpler construct, a tissue-engineered autologous neo-urinary conduit, in cancer patients after radical cystectomy (Tengion Inc., www.clinicaltrials.gov NCT01087697).

Tissue-engineered trachea and bronchi also have entered the early stages of clinical testing. A case report from 2008 describes the treatment of a subject with end-stage airway disease by

replacing the left main bronchus with a decellularized human trachea reconstituted by autologous epithelial cells and mesenchymal stromal cell (MSC)-derived chondrocytes [27]. Subsequent efforts focused on scaffold improvements and identification of an alternative cell source for seeding, as graft production for the initial subject had required almost 3 months [28,29]. Two further case reports document airway tissue engineering using scaffolds seeded with autologous bone marrow-derived cells. An adult subject with lung cancer received a tracheobronchial construct comprising a synthetic nanomaterial scaffold seeded with autologous marrow mononuclear cells [30]. At five months post-implantation the grafted airway appeared to function well and the subject was tumor-free. A 12-year-old child with a congenital tracheal defect was implanted on an emergency basis, after the failure of conventional stents, with a decellularized human tracheal scaffold seeded with autologous MSCs and epithelial patches [31]. Follow-up of each patient indicated that epithelial cells eventually covered the grafted scaffolds, albeit very slowly. Restoration of the epithelium was not apparent in the pediatric subject until a full year post-implantation, and biomechanical strength of the engineered trachea was poor until about 18 months. Nevertheless, the child's health improved significantly; at a two year follow-up, his airway was functional and he had returned to school.

CURRENT CHALLENGES

Observations from the limited numbers of human cases reported to date highlight the potential of tissue engineering to address unmet medical needs. However, both technical and economic hurdles must be overcome before therapies based on these technologies will reach the millions of patients who might benefit from them.

The bladder, urethra, and airways exemplify hollow or tubular structures with relatively thin walls. The engineering of tissues with more complex three-dimensional architectures remains a long-recognized challenge. Solutions will depend on advances in all of the key technologies of tissue engineering, including the design of scaffolds, the choice of cells and the methods for their culture and seeding onto the scaffolds, and maturation of constructs in bioreactors prior to implantation. A particularly critical issue will be to overcome the mass transport limit and provide sufficient oxygen to the engineered tissue prior to vascularization. One potential answer lies in the development of biomaterials that release oxygen at a controlled rate to sustain implanted cells until new blood vessels have formed [32,33]. In addition it will be important to enhance angiogenesis, to give the regenerating tissue access to sufficient oxygen and nutrients from the circulation as rapidly as possible. The use of angiogenic factors, improved scaffold materials, printing technologies, and accelerated *in vitro* maturation of engineered tissues in bioreactors may help to address this problem. Of particular interest is the invention of novel scaffold materials designed to serve an instructive role in the development of engineered tissues, both for parenchyma and vasculature. Another approach utilizes decellularized whole organs that retain the vascular channels and many extracellular matrix (ECM) components from the native tissues. Methods to prepare improved cell-scaffold constructs by growth in bioreactors before implantation will serve a complementary role in generating more robust clinical products.

A second key challenge centers on a fundamental dichotomy in strategies for sourcing of the live component of engineered tissues — the use of autologous versus allogeneic or even xenogeneic cells. It appears most cost-effective and efficient for manufacturing, regulatory approval, and wide delivery to end users to employ a minimal number of cell donors, unrelated to recipient patients (i.e., allogeneic), to generate an 'off-the-shelf' product. On the other hand grafts generated from autologous cells, obtained from a biopsy of each individual patient, in principle present no risk of immune rejection because of genetic mismatches, thereby avoiding the need for immunosuppressive drug therapy, with its attendant high cost and side effects. Thus, the autologous approach, though perhaps more laborious and costly, appears to have a major advantage. Nonetheless, there are many tissue-engineering

applications for which appropriate autologous donor cells may not be accessible by biopsy. Therefore, new sources of cells for regenerative medicine are being sought, mainly from among progenitor and stem cell populations. However, any use of unrelated donor cells inevitably raises the potential barrier of immune rejection, especially via the direct allorecognition pathway [34].

The path-breaking discovery that pluripotent stem cells can be generated by the reprogramming of normal adult cells, for which Shinya Yamanaka was awarded the 2012 Nobel Prize in Physiology or Medicine, in principle enables the production of autologous cells of virtually any type desired for tissue engineering [35]. Furthermore, evidence that some stem cell populations and tissues derived from them can be grafted successfully despite histocompatibility mismatches offers encouragement for allogeneic tissue-engineered products that could be delivered without immunosuppression.

Our goal here is to review recent advances in tissue engineering in light of the issues raised above. We will gaze into an admittedly imperfect crystal ball and attempt to highlight the technologies that will most profoundly impact medicine.

FUTURE DIRECTIONS

Smarter biomaterials

Scaffolds provide mechanical support and shape for neo-tissue construction *in vitro* and/or through the initial period after implantation as cells expand, differentiate, and organize [36]. Materials that mainly have been used to date to formulate degradable scaffolds include synthetic polymers such as poly(L-lactic acid) (PLLA) and poly(glycolic acid) (PLGA), and polymeric biomaterials such as alginate, chitosan, collagen and fibrin [37]. Composites of these synthetic or natural polymers with bioactive ceramics such as hydroxyapatite or certain glasses can be designed to yield materials with a range of strengths and porosities, particularly for the engineering of hard tissues [38]. Broadly, the conceptual understanding of biomaterials is evolving to include a wider range of substances such as biopolymers, nanoparticles, carbon nanotubes and quantum dots, and to focus increasingly on functional interactions with cells and tissues [39].

EXTRACELLULAR MATRIX

A scaffold used for tissue engineering can be considered an artificial ECM [40]. It has long been appreciated that the normal biological ECM, in addition to contributing to mechanical integrity, has important signaling and regulatory functions in the development, maintenance, and regeneration of tissues. ECM components, in synergy with soluble signals provided by growth factors and hormones, participate in the tissue-specific control of gene expression through a variety of transduction mechanisms [41–44]. Furthermore, the ECM is itself a dynamic structure that is actively remodeled by the cells with which it interacts [45,46]. An important future area of tissue engineering will be to develop improved scaffolds that more nearly recapitulate the biological properties of authentic ECM [47,48].

Decellularized tissues or organs can serve as sources of biological ECM for tissue engineering. The relatively high degree of evolutionary conservation of many ECM components allows the use of xenogeneic materials (often porcine). Various extracellular matrices have been utilized successfully for tissue engineering in animal models, and products incorporating decellularized heart valves, small intestinal submucosa (SIS), and urinary bladder matrix have received regulatory approval for use in human patients [49]. The use of decellularized matrices is likely to expand because they retain the complex set of molecules and three-dimensional structure of authentic ECM. The precise degree to which structural and signaling components are preserved depends on the choices of detergents and enzymes used to remove cells and the washing conditions used to clear these reagents. Despite many

advantages, there are also concerns about the use of decellularized materials. These include the potential for immunogenicity, the possible presence of infectious agents, variability among preparations, and the inability to completely specify and characterize the bioactive components of the material.

Decellularized scaffolds may serve as platforms for the engineering of whole organs [3,50]. A compelling demonstration was provided by the production of acellular hearts that retained patent vascular networks, functional valves, and normal chamber architecture [51]. When cardiomyocytes and endothelial cells were perfused back into the scaffolds and the constructs maintained in a bioreactor for several weeks, the partially reconstituted organs showed muscular contractions and detectable pump function. Similarly, whole organ decellularized liver scaffolds were produced with an intact vascular tree. Seeding of these scaffolds with endothelial cells and hepatocytes generated organoids with appropriate cellular organization, marker expression, and a degree of tissue-specific function [52]. A novel whole organ decellularization procedure designed to retain all collagen types, along with other structural proteins, proteoglycans, and ECM-bound cytokines and growth factors, yielded a liver scaffold that promoted the rapid differentiation of hepatic stem cells to mature hepatocytes and bile duct cells [53].

From a cellular perspective the heart and liver, are relatively homogeneous organs. Even so, the goal of attaining complete tissue reconstitution and function after seeding cells onto decellularized scaffolds from these organs remains to be achieved. Still greater challenges will be faced in the reconstruction of the kidney, with its multiplicity of cell types (up to 23 distinct classes) and complex physiology. As an initial step, decellularized renal scaffolds were re-implanted successfully into experimental animals, were readily reperfused, and sustained blood pressure [54].

ELECTROSPINNING

There would be significant advantages to synthetic scaffolds that closely mimic key features of the ECM, but might be manufactured more easily and reproducibly than decellularized organs. Electrospinning has enabled the production of a new generation of highly biocompatible micro- and nano-fibrous scaffolds from materials such as poly(epsilon-caprolactone), from diverse matrix proteins such as collagen, elastin, fibrinogen, and silk fibroin, from polysaccharides, and from carbon nanofibers [55–66]. Electrospun materials have fiber diameters in the range of those found in native ECM and generally display greater mechanical strength than hydrogels. The electrospun scaffolds may be assembled utilizing a variety of nano-fibrous structures and may incorporate additional important ECM components such as particular subtypes of collagen, glycosaminoglycans, and laminin, either in the spun fibers or as coatings, to promote cell adhesion, growth, and differentiation [55,64,67–69].

The use of specialized proteins such as recombinant silk fibroin offers the opportunities to design scaffolds with enhanced strength or other favorable features, for example controlled release of entrapped materials for drug or bioactive factor delivery [70,71]. The incorporation of plant materials such as wheat gluten and gliadin also may enable the cost-effective production of novel electrospun biomaterials [72,73].

It often proves problematic to introduce cells into a nanofibrillar structure in which pore spaces are considerably smaller than the diameter of a cell [74]. However, remarkably, it is possible to utilize electrospinning to incorporate living cells into a fibrous matrix. A proof-of-concept study documented that smooth muscle cells could be concurrently electrospun with an elastomeric poly(ester urethane)urea, leading to 'microintegration' of the cells in strong, flexible fibers with mechanical properties not greatly inferior to those of the synthetic polymer valone [75]. The cell population retained high viability and, when maintained in a

perfusion bioreactor, the cellular density in the electrospun fibers doubled over four days in culture. By electrospraying the smooth muscle cells concurrently with electrospinning a small-diameter conduit, the cells could be integrated efficiently into a biodegradable tubular matrix with properties favorable for a tissue-engineered blood vessel substitute [76].

BIOPRINTING

An equally surprising observation is that living cells can survive inkjet printing [77–79]. Modification of printers to add a z-axis allows the generation of three-dimensional structures. Thus, 'bioprinting' of cells together with matrix biomaterials and bioactive factors should enable the production of constructs that mimic the architectural complexity, signaling capacity, and cellular distribution of complex tissues [80–83]. Bioprinting can be applied even to highly specialized, fragile cells. For example, printed hippocampal and cortical neurons retained their characteristic phenotypes as judged by immunohistochemical staining and whole cell patch-clamping, a stringent functional test of electrical excitability [84].

Bioprinting technology for tissue engineering has developed rapidly, and will continue to do so [80,85–92]. Examples of applications under development include: directed differentiation of stem cells [93,94]; drug-screening in tissue-like contexts [95]; generation of tissue constructs with growth factor gradients [81] and tailored mechanical properties [89]; production of blood vessel substitutes [96]; and direct *in vivo* repair of tissues such cartilage and skin [97,98]. Eventually, bioprinting may prove the method of choice to generate whole organ constructs comprising multiple cell types and microenvironments.

SMART POLYMERS

Incorporation of cells by electrospinning or printing generates, in a sense, the ultimate 'smart' biomaterials. Even without cells, biomaterials can be produced that are capable of interactive behavior, both responsive to and able to modulate the local environment and cellular activities.

At the chemical level a number of groups have explored the production of biomaterials that unite the advantages of synthetic polymers with the biological activities of proteins. The notion of smart polymers initially described materials that undergo large conformational changes in response to environmental stimuli such small changes in temperature, ionic strength, or pH [99,100]. Light stimuli can activate crosslinking of polymers [90]. Ultrasound can be used to trigger nanobubble formation and facilitate DNA uptake [101]. The responses of a polymer may include precipitation or gelation, reversible adsorption on a surface, collapse of a hydrogel or surface graft, and alternation between hydrophilic and hydrophobic states [102]. In many cases the change in the state of the polymer is reversible.

Biological applications of smart polymer technology span diverse areas including bioseparation, drug delivery, reusable enzymatic catalysts, molecular switches, biosensors, regulated protein folding, microfluidics, and gene therapy [103,104]. In tissue engineering smart polymers offer promise for revolutionary improvements in scaffolds. Beyond the physical properties of polymers, a major goal is to invest smart biomaterials with specific properties of signaling proteins such as ECM components and growth factors.

One approach is to link smart polymers to proteins [103,102]. Conjugation can occur at random positions or in a site-specific manner, through engineering of the protein to introduce a reactive amino acid at a particular point in the primary sequence. If a conjugation site is introduced near the ligand-binding domain of a protein, induction of a change in conformational state of the smart polymer can serve to regulate the protein's activity [105]. This may allow selective capture and recovery of specific cells, delivery of cells to a desired location, and modulation of enzymes such as matrix metalloproteases that influence tissue remodeling.

PROTEINS AND MIMETICS

More broadly, the design of genetically modified proteins or of hybrid polymers incorporating peptides and protein domains enables the creation of a wealth of novel smart biomaterials [106]. These include: engineered mutant variants of natural proteins; semi-synthetic scaffold materials incorporating protein domains; scaffold materials linked to synthetic peptides; and engineered peptides capable of self-assembly into nanofibers.

Genetic engineering may improve on endogenous proteins for applications in tissue engineering [107]. For example, a collagen-like protein was generated by using recombinant DNA technology to introduce tandem repeats of the domain of human collagen II most critically associated with the migration of chondrocytes [108]. When coated onto a synthetic PLGA scaffold and seeded with chondrocytes, the engineered collagen was superior to wild-type collagen II in promoting artificial cartilage formation. Similarly, recombinant technology was employed to generate a series of elastin-mimetic protein triblock copolymers [109]. These varied broadly in their mechanical and viscoelastic properties, offering substantial choices for the production of novel materials for tissue engineering.

Bioactive signals can be incorporated into scaffold materials by the chemical linkage of proteins, protein domains, or synthetic peptides as tethered ligands. Integrins, transmembrane receptors that serve as adhesion molecules between cells and other cells and/or the ECM, are key targets for ligands used to modify scaffold surfaces. Numerous studies have confirmed that addition of the integrin-binding motif arginine-glycine-aspartic acid (RGD), first identified in fibronectin, enhances the binding of many types of cells to a variety of synthetic scaffolds and surfaces, including metals, polymers, potassium phosphate bone surrogates, and hydrogels [110–114]. The CS5 cell-binding domain of fibronectin also has been incorporated into scaffolds, and its activity is subject to regulation by sequence context [115]. Greater selectivity and potency in cellular binding and enhancement of growth and function can be achieved by taking advantage of additional binding motifs in concert with RGD, or independently of that tripeptide [116,117]. The integrin family comprises two-dozen heterodimeric proteins, so there remains great opportunity to expand the set of peptide binding motifs that could be utilized on tissue-engineering scaffolds, with the hope of achieving greater selectivity and control.

Even within the particular context of RGD-binding domains, scaffolds can be engineered with enhanced specificity for specific integrin heterodimers. For example, modification of a biomaterial by attachment of a fibronectin fragment containing both the RGD site and a so-called 'synergy site' (sequence proline-histidine-serine-arginine-asparagine [PHSRN]) promotes binding of cells via the integrin alpha(5)beta(1) [118]. A distinct synergy site promotes binding via a different integrin heterodimer, alpha(IIb) beta(3) [116]. Multiple chemical and biochemical approaches, including combinations of small protein fragments, peptide cyclization, peptide modification by N-methylation, linking of peptide motifs from different proteins, and use of small molecule peptide mimetics, are being explored to optimize specific cell-adhesive surfaces [119–123].

The modification of matrices with bioactive peptides and proteins can extend well beyond insertion of motifs to promote cell adhesion [124]. Cells also need to migrate in order to form remodeled tissues. Thus, the rate of degradation of scaffolds used for tissue engineering is a crucial parameter affecting successful regeneration [125]. Regulation of the degradation rate can be achieved by varying physical parameters of the scaffold. Alternatively, the scaffold can be engineered to contain target sites for proteolytic degradation [126,127]. For example, the incorporation into a crosslinked synthetic hydrogel of sequences cleaved by matrix metallopreases, known to play an important role in cell invasion, enhanced the migration of fibroblasts *in vitro* and the healing of bony defects *in vivo* [128]. Biodegradation of the synthetic matrix was efficiently coupled to tissue regeneration.

GROWTH AND ANGIOGENIC FACTORS

Factors that drive cell growth and differentiation can be added to the matrix in the form of recombinant proteins or, alternatively, expressed by regenerative cells via gene therapy. Growth factors of potential importance in tissue engineering and methods to deliver them have been reviewed [129]. Ideally, for optimized tissue formation without risk of hyperplasia, the growth factors should be presented to cells for a limited period of time and in the correct local environment. Biodegradable scaffolds produced by electrospinning or other methods are capable of sustained release of growth factors and cytokines, or small molecule drugs, over periods of weeks to months [130–135]). Biologically regulated release of growth factors from scaffolds appears particularly promising as a means to ensure that cells in regenerating neotissues receive signals at appropriate times and in physiological doses. For example, physical entrapment of recombinant bone morphogenetic protein-2 (BMP-2) in a hydrogel so that it would be released by matrix metalloproteases promoted excellent bone healing in a critical-size rat calvarial defect model [128]. Similarly, incorporation of a neurotrophic factor in a slowly degradable hydrogel promoted local extension of neurites from explanted retina, and gels were designed to release multiple neurotrophin family members at different rates [136].

The expression of recombinant growth factor domains and their immobilization to the matrix provides another means to control the presentation of biological signals in time and space. Model studies with epidermal growth factor (EGF) showed that oriented coupling to a scaffold via a protein coil domain provides an efficient means to preserve a high level of receptor-binding activity while localizing the signal to a desired scaffold region [137,138].

Using similar strategies, controlled presentation of angiogenic factors such as vascular endothelial growth factor (VEGF) should promote the well-regulated neovascularization of engineered, regenerating tissue [139,140]. Again, it is possible to covalently couple an angiogenic factor to a matrix and to regulate its release based on cellular activity and demand [141–144]). The selection of a sulfated tetrapeptide that mimics the VEGF-binding capability of heparin, a sulfated glycosaminoglycan, provides another potential tool for the construction of scaffolds able to deliver an angiogenic factor to cells in a regulated manner [145].

Spatial gradients can be generated in the presentation of growth factors within scaffold constructs [81,146–149]). By mimicking the distribution of normal developmental morphogens, graded signals from imprinted or bound factors may guide the formation of engineered complex tissues by directing both cellular migration and differentiation. For example, bioprinting of bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) gradients can direct the formation of bone and tendon by stem and progenitor cells [81,93,150–152]). Similarly, gradients of neurotrophic factors and scaffold matrix components guide neuronal differentiation and the extension of nerve processes [153,154].

Gradients of scaffold-linked growth factor-binding motifs provide a generalized means to regulate the spatial distribution of signaling molecules [155]. The introduction of sophisticated manufacturing technologies, such as solid free-form fabrication, will allow the production of tissue-engineering constructs comprising scaffolds, incorporated cells, and growth factors in precise, complex three-dimensional structures [156].

DISCOVERY OF NEW MATERIALS

One potential next stage of smart biomaterial development extends to the design or discovery of truly novel bioactive matrices, not necessarily derived from naturally occurring carbohydrate and protein structures. At one level this may entail the relatively straightforward chemical synthesis of new materials. By adapting the combinatorial library approach already well established for synthetic peptides and drug-like structures, together with even moderately high throughput biological assays, thousands of candidate scaffold materials can be generated and tested. Thus, screening of a combinatorial library derived from commercially available

monomers in the acrylate family revealed novel synthetic polymers that influenced the attachment, growth and differentiation of human embryonic stem (ES) cells in unexpected ways [157]. As understanding of stem cell biology has become more sophisticated, comparable screening strategies have led to the discovery of an increasing number of biomaterials that mimic normal microenvironments in promoting the expansion and differentiation of various stem and progenitor cell populations [158–60].

Revolutionary developments in biomaterials are likely to arise at the interface of tissue engineering with nanotechnology. Basic understanding of the three-dimensional structure of existing biological molecules is being applied to a 'bottom-up' approach to generate new, self-assembling supramolecular architectures [161–164]. In particular, self-assembling peptides offer promise because of the large variety of sequences that can be made easily by automated chemical synthesis, the potential for bioactivity, the ability to form nanofibers, and responsiveness to environmental cues [165,166].

Pioneering studies have included the design of short peptides (e.g., heptamers) based on coiled-coil motifs that reversibly assemble into nanofilaments and nanoropes, without excessive aggregation [167]. These smart peptide amphiphiles can be induced to self-assemble by changes in concentration, pH, or the level of divalent cations [168,169].

As noted with electrospinning and bioprinting, tissue engineering will be facilitated greatly if cells can be incorporated readily into the biomaterial. An early proof-of-concept study showed that it is possible to entrap viable cells during the assembly of a nanofiber matrix [170]. The captured cells retained motility and the ability to proliferate. A subsequent report showed that specialized cells such as neural progenitors can be encapsulated by peptide amphiphiles, and their subsequent growth and differentiation influenced by incorporation of cell-adhesive ligands and other bioactive molecules [171].

Self-assembling amphiphilic peptides and, most broadly, rationally designed peptide and polypeptide-based hydrogels, will continue to serve as a platform and toolbox for a wide array of new biomaterials [172,173]. For example, branched structures can be designed to present bioactive sequences such as RGD to cells via nanofiber gels or as coatings on conventional tissue-engineering scaffolds [174,175]. Other features of the ECM, such as mechanical properties, sensitivity to enzymatic remodeling, and presentation of growth factor or cytokine signaling sequences can be selected or designed through the choice of peptide units, functionalization, and incorporation of bioactive macromolecules and small molecule drugs [176,177]. Other nanomaterials, such as carbon nanotubes and DNA polyplexes can be incorporated to add additional features and activities [178].

Further opportunities exist to expand the range of peptidic biomaterials by utilizing additional chemical components such as porphyrins, which can bind to peptides and induce folding [179]. Porphyrins and similar structures also may add functionality such as oxygen storage, catalysis or photosensitization of chemical reactions, or transfer of charge or molecular excitation energy. Peptide-porphyrin complexes thus offer promise of a new generation of regulated, photoelectronically active biomaterials that will be exquisitely sensitive to environmental stimuli [180,181].

Peptide-based nanofibers may be designed to present bioactive sequences to cells at very high density, substantially exceeding that of corresponding peptide epitopes in biological ECM. For example, a pentapeptide epitope of laminin, isoleucine-lysine-valine-alanine-valine (IKVAV), known to promote neurite extension from neurons, was incorporated into peptide amphiphiles (PA) capable of self-assembly into nanofibers that form highly hydrated (>99.5 weight % water) gels [182]. When neural progenitor cells capable of differentiating into neurons or glia were encapsulated during assembly of the nanofibers, they survived over several weeks in culture. Moreover, even without the addition of neurotrophic growth factors, they displayed neuronal differentiation as exemplified by the extension of large neurites, already

obvious after one day, and by expression of βIII-tubulin. The production of neuron-like cells from the neural progenitors, whether dissociated or grown as clustered 'neurospheres,' was more rapid and robust in the IKVAV-PA gels than on laminin-coated substrates or with soluble IKVAV. By contrast, the production of cells expressing glial fibrillary acidic protein (GFAP), a marker of astrocytic differentiation, was suppressed significantly in the IKVAV-PA gels even when compared to growth on authentic laminin, which also favors neuronal over glial differentiation. The ability to direct stem or progenitor cell differentiation via a chemically synthesized biomaterial, without the need to incorporate growth factors, offers many potential advantages in product development for regenerative medicine.

Combinations of the peptide amphiphile with other matrix components, such as collagen, and growth factors provide the basis for optimized smart scaffold biomaterials that ultimately should find medical application in tissue engineering [183]. For example aligned amphiphile nanofibers used as a scaffold and delivery system for Sonic hedgehog (SHH) have proven effective in an animal model of peripheral nerve regeneration, in part through modulation of the production of endogenous neurotrophic factors [184–186]).

Bioreactors

After seeding of cells onto scaffolds, a period of growth *in vitro* often is required prior to implantation. Static cell culture conditions generally have proven sub-optimal for the development of engineered neo-tissues because of limited seeding efficiency and poor transport of nutrients, oxygen, and wastes. Bioreactors systems have been designed to overcome these difficulties and to facilitate the reproducible production of tissue-engineered constructs under tightly controlled conditions [187–192]). Future advances will come through improved understanding of the requirements for tissue development coupled with increasingly sophisticated reactor engineering.

An important parameter that must be determined for the *in vitro* maturation of a tissue-engineered construct is the optimal level of oxygen. Contrary to conventional wisdom, for some tissues or cell types it appears that low oxygen tension is important for optimal growth and specialized function. For example, in tissue engineering of cartilage, while aerobic conditions are essential for adequate tissue production [193], cultivation in bioreactors at reduced oxygen tension (e.g., 5% instead of the 20% found in room air) improved the biosynthesis of glycoasminoglycans and the expression of additional characteristic phenotypic markers and functions [194–196]).

Regulation of oxygen tension also proves critical in handling many progenitor and stem cell populations in culture, particularly in bioreactors [197–201]. Physiological (i.e., reduced compared to ambient) oxygen generally mimics the normal microenvironment or 'niche' for stem cells, and enhances their growth and stability. Optimization of oxygen tension, sometimes at a low level sometimes at a significantly higher level than for stem cell proliferation, also frequently improves the production of particular differentiated derivatives [202–206].

It has become increasingly clear that, in addition to regulating mass transport, bioreactors may be used to enhance tissue formation through mechanical stimulation. For example, pulsatile flow helps the maturation of blood vessels [207,208]. Similarly, mechanical stretch strengthens engineered muscles, of both skeletal and smooth types [209–211]. Engineering of bone, cartilage, blood vessels, and both skeletal and cardiac muscle all will continue to advance in part through more sophisticated mechanical conditioning of developing neo-tissues.

A third area of great importance is the use of bioreactors to expedite the manufacture of engineered grafts for clinical use, at levels up to whole organ constructs [187,189,205,212–216]. Key goals will be to optimize the development of neo-tissues and neo-organs, and then to standardize production in order to eliminate wasted units and control

costs. Standardization also will help to meet regulatory requirements in the context of Good Manufacturing Practice (GMP).

The direct interface between man and bioreactors represents another significant challenge in the bioreactor field. On one hand, the patient is increasingly viewed as a potential *'in vivo* bioreactor,' providing an optimal environment for cell growth and differentiation to yield neotissues [217]. There also are circumstances in which a bioreactor may serve as a bioartificial organ, attached directly to a patient's circulation. There have been longstanding efforts to develop a bioartificial liver capable of sustaining life during acute liver failure, until the patient's endogenous organ regenerates or can be replaced by orthotopic transplantation [218–221]. Most designs to date have used hollow fiber bioreactors seeded either with human hepatic lineage cell lines or xenogeneic (e.g., porcine) hepatocytes. Despite intensive efforts over more than three decades, and multiple clinical trials, no bioartificial liver assist device has yet achieved full regulatory approval. Both bioreactor design and cell sourcing require further improvements before the hurdle of demonstrating safety and efficacy in human trials will be surmounted [222–228].

The creation of a robust bioartificial pancreas to provide a physiologically responsive supply of insulin to patients with diabetes represents a comparably significant challenge for bioreactor development. Again, no design has yet proved entirely successful [229–231]. Because pancreatic islets are among the most richly vascularized tissues in the body, providing sufficient oxygen appears to be one rate-limiting factor that may be addressed by improved bioreactor design [232,233]. Furthermore, although human islets can be isolated from pancreata obtained by organ donation, they are in scarce supply. Clearly, if bioartificial organ technology continues to advance, the demand for new sources of functional human cells such as hepatocytes and pancreatic β-cells will expand dramatically [234,235].

Cell sources

Choices of new sources of cells for tissue-engineered products hinge on two fundamental dichotomies: autologous *versus* allogeneic cells; and pluripotent stem cells *versus* adult multipotent or unipotent stem and progenitor cells (Fig. 6.1). Previous clinical and commercial experience sheds light on key differences between off-the-shelf products containing allogeneic cells and personalized products containing autologous cells. To date the vast majority of human studies have focused on tissue-engineered products in which the cellular component contains or derives from adult stem and progenitor cells. However, initial clinical trials of products derived from pluripotent stem cells have begun and may herald a dominant approach of the future.

The first clinical products of tissue engineering that achieved marketing approval from the US Food and Drug Administration (FDA) were skin substitutes used for wound healing. Both Dermagraft (Shire Regenerative Medicine) and Apligraf (Organogenesis) utilize cells expanded greatly from donated human foreskins to treat many unrelated patients. The cell types used to manufacture these products are fibroblasts for Dermagraft, and fibroblasts plus epithelial cells (keratinocytes) for Apligraf. Whereas fibroblasts had been grown *in vitro* since early in the 20th century [236], the successful cultivation of human keratinocytes represented an important breakthrough for regenerative medicine [237].

Despite the inevitable histocompatibility mismatches between donor and recipient, the off-the-shelf skin substitutes lack antigen-presenting cells and are not acutely rejected [238–241]. This stands in contrast to standard organ transplantation in which immune suppressive drug therapy generally is essential for allogeneic grafts [242]. Eventually, the cells in the skin substitutes may be rejected, but the grafts buy enough time for patients' own skin cells to regenerate.

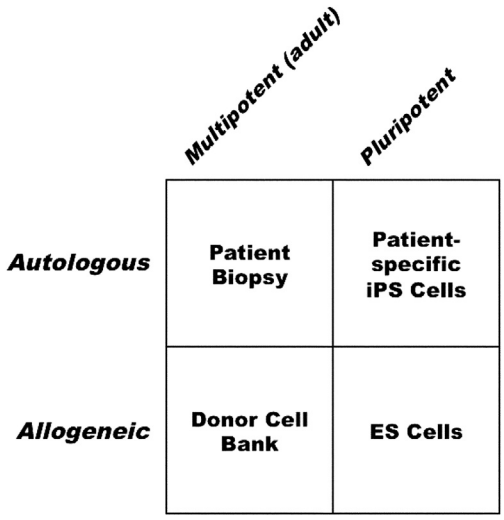

FIGURE 6.1

Four classes of stem cell sources for tissue engineering. Autologous cells are obtained in a biopsy sample harvested from the individual who will subsequently receive the engineered construct. Expansion of cells *in vitro* and regeneration and long-term maintenance of biopsy-derived cells *in vivo* often will depend on the presence of lineage-restricted stem cells in the biopsied tissue. In principle, any desired cell type also can be obtained for a patient by reprogramming to pluripotency cells from a readily obtained biopsy of cells from her/his blood, skin, hair, urine, etc. The iPS cells are then restricted to the desired lineage and used for the tissue-engineered construct. Allogeneic cells can be sourced from banked cells obtained from tissue donors. An already established example is banking of umbilical cord blood cells [501]. Pluripotent ES cell lines (or iPS cell lines) also can be used as a source to derive lineage-restricted cells for off-the-shelf products. Banking of primary donated cells and ES or iPS cell lines enables histocompatibility matching to varying levels of stringency. Strategies to achieve beneficial matching for large segments of a population from modest numbers of banked cell lines are discussed in the text.

Tissue-engineered products based on harvesting and expanding autologous cells containing stem and/or progenitor populations also have been developed successfully. Two examples, each marketed by Genzyme Biosurgery, are: Epicel, a permanent skin replacement product based on expanded keratinocytes for patients with life-threatening burns; and Carticel (Genzyme Biosurgery), a chondrocyte-based treatment for large articular cartilage lesions [243,244].

For some tissue-engineering applications, such as bladder augmentation, the ability to expand a sufficient number of autologous cells (in this case smooth muscle and urothelium) from a small biopsy is well established [245]. In other circumstances methods are not available to harvest and/or expand enough appropriate autologous cells to manufacture the needed neo-tissue or organ. Cardiomyocytes, neurons of the central nervous system, hepatocytes and other liver cells, kidney cells, osteoblasts, and insulin-producing pancreatic beta cells are examples of differentiated cell types for which new sources could enable novel therapies to address significant unmet medical needs.

Immature precursor cells present within tissue samples are essential for the expansion of cells from biopsies of skin, muscle, cartilage, or bladder to enable the engineering of the corresponding neo-tissues [246]. The extension of tissue engineering to other tissue and organ systems will depend greatly on finding sources of appropriate stem and progenitor cells. Three major stem cell sources are currently under intensive investigation:

1) ES cells, derived from discarded human embryos, or the essentially equivalent embryonic germ [EG] cells [247,248];
2) Induced pluripotent stem (iPS) cells derived by genetic reprogramming of somatic cells [249,250]; and
3) Adult tissue stem cells (so named regardless of sourcing from fetal, neonatal, pediatric, or adult donors), either autologous or allogeneic [251].

Shared features of all stem cells are their capacity self-renewal, that is, for extensive replication while maintaining 'stemness', and their ability to give rise to particular classes of differentiated cells. ES, EG and iPS cells can serve as precursors to any specialized cell type found in normal development and are termed pluripotent. Adult stem cells generally are restricted to limited sets of cell lineages. They are called multipotent or, if constrained to a single fate, unipotent. The restriction of the developmental fate of cells to a particular lineage is called commitment, and can refer to stem cells or to down-stream progenitors with lesser replicative capacity. It appears likely that multiple tissue-engineered products based on each class of stem cell source will be tested in the clinic in the coming years. The various stem cell types pose certain common challenges, and each also has specific drawbacks that must be overcome to achieve clinical utility. We will attempt to prognosticate future prospects for the three classes of stem cells in tissue-engineering applications, with particular attention to the implications of histocompatibility matching.

EMBRYONIC STEM CELLS

ES cells and EG cells will be treated together under the ES designation. ES cells indeed are quite similar to early germ cells [252]. They can self-renew apparently without limit in culture and are pluripotent. Their great degree of plasticity represents both the strongest attraction and a significant potential limitation to their use for tissue engineering. A major ongoing challenge lies in efficiently obtaining pure populations of specific desired specialized cell types from human ES cells [253–255].

Much recent work has yielded more robust methods to isolate and grow ES cells under conditions consistent with GMP, and to generate differentiated cell products that will pass stringent regulatory reviews [256,257]. While initial efforts have focused on cell therapies, the advances will have equal impact on the production of complex tissue-engineered constructs. Human ES cells are considerably more difficult to isolate and maintain stably in culture than the cell types that have previously entered clinical testing. However, they can now be derived, grown, and cryopreserved without exposure to non-human cells or proteins, in a GMP environment, and under conditions that appear to preserve genomic integrity [258–266]. The use of microcarriers and bioreactors, along with improved serum-free and xeno-free media, and possibly small molecules to inhibit spontaneous differentiation, will continue to facilitate the expansion of pluripotent stem cells to the population sizes required for product development and eventual broad medical application [267–273].

Even given clinical grade ES cells that can be produced at large scale, the manufacturing of specialized cell types derived from them for tissue-engineered products poses formidable challenges. Human tissues include more than 200 distinct cell types, and ES cells in principle can give rise to all of them. The historical approach of allowing ES cells to differentiate spontaneously after aggregation into embryoid bodies, followed by selection of a particular mature cell type, has largely been supplanted. Current strategies employ staged differentiation guided by knowledge of signaling events that regulate normal embryonic development [274]. A first step is to direct the pluripotent stem cells to one of the three germ layers, namely the ectoderm, mesoderm, or definitive endoderm, that form during the gastrulation stage of embryogenesis. For example, exposure of very early embryonic cells to the growth factor Nodal (a member of the TGF-β family) or its analog Activin A suppresses a default pathway of differentiation to ectoderm and favors the generation of mesendoderm, the immediate precursor of mesoderm and endoderm [275,276]. Fine tuning of the application of Nodal or Activin, in conjunction with other growth factors or small molecules, now allows the quantitative generation *in vitro* from ES cells of a reasonably homogeneous population with markers characteristic of definitive endoderm [277–281]. This is a key, albeit early, milestone in multi-step differentiation to cells that eventually may be used for tissue engineering of such organs as the liver and pancreas. Conversely, inhibition of Nodal/Activin signaling favors the

production of ectoderm, a precursor for ES cell-derived neural lineage cells [282,283]. The optimization of growth factor cocktails and the use of scale-up bioreactors will continue to make the initial steps of stem cell commitment to each germ layer more robust, as an important stage in the development of tissue-engineered products. This has been exemplified recently by scaled-up production of definitive endoderm [284].

Subsequent steps employed to drive differentiation to a desired lineage may be determined both by knowledge of normal development and by empirical observations, including screening for small molecule modulators. Increasingly, efforts to isolate a wide variety of specialized cells from human ES and iPS cells are approaching the goal of achieving differentiated populations in which high percentages of cells express morphological characteristics, biochemical and antigenic markers, and functional properties consistent with the desired cell types. Many of these differentiated cells have potential application in tissue engineering. Examples include: a number of classes of neurons [285–289] other cells of the nervous system including oligodendrocytes and subsets of astrocytes [290–296]; cardiomyocytes and endothelial cells [297–311]; mesenchymal stem and progenitor cells [312–316]; hepatocytes [277,317–321]; and endocrine cells of the pancreas, notably insulin-producing beta-like cells [322–326].

However, much remains to be accomplished in utilizing pluripotent stem cells as a source for future cell-based therapeutic products. Purity of differentiated cell populations and exclusion of potentially tumorigenic undifferentiated stem cells must be confirmed for every application. Furthermore, not surprisingly, the differentiated cells derived from ES cells in culture often more closely resemble those in embryonic and early fetal development than in adult or even neonatal human tissues. A recent report concludes that the gene expression profiles of ES cell-derived specialized cells retain many primitive features, and resemble those of cells in human fetuses at about six weeks of gestation. Detailed studies of various cell lineages, such as pancreatic β cells and hepatocytes, have highlighted the difficulty of achieving fully adult phenotypes from ES cells [323, 327–329]. The completion of development may actually require additional maturation for several months *in vivo*, as shown for endocrine pancreatic progenitors of insulin-producing β-like cells [330]. Therefore, while serving as useful models for human development, the immature cells derived from pluripotent stem cells may have significant limitations as components of safe and effective tissue-engineered therapies.

A particular safety concern is that undifferentiated pluripotent ES and iPS cells form teratomas *in vivo*. These tumors contain cell types representing each of the three embryonic germ layers [331]. The risk of tumorigenicity makes it essential to determine rigorously the residual level of undifferentiated stem cells in any therapeutic product derived from ES or iPS cells [332]. Assays for teratoma formation are being standardized, but currently have a lower sensitivity limit of about 100 stem cells [333]. It remains to be determined whether a small number of undifferentiated pluripotent stem cells can be introduced into humans without significant risk of tumor growth, and whether this threshold is influenced by immune suppressive drugs.

It should be possible to decrease risk of teratoma formation by introducing additional manufacturing steps to eliminate undifferentiated pluripotent stem cells from cell therapy or tissue-engineered products. For example, this might be accomplished by negative selection with antibodies to surface markers of pluripotent stem cells [334]. Another approach would be to facilitate elimination of any tumors that might actually arise in recipients of tissue-engineered products. One proposed strategy is to transduce into the stem cells a 'suicide' gene, such as one encoding a viral thymidine kinase that would serve as a fail-safe mechanism to allow eradication of any donor cell-derived tumors by treatment with ganciclovir [335]. However, the drug also would be expected to kill the therapeutically beneficial cells of the graft, unless it is placed under the control of a promoter expressed preferentially in pluripotent stem cells [336].

Despite substantial challenges, especially overcoming regulatory concerns over a first-in-class product with potential for tumorigenicity, the first ES cell-derived therapeutics entered clinical trials. Geron Corporation obtained approval to test hES cell-derived oligodendrocyte progenitor cells, thought capable of remyelinating axons and stimulating nerve process growth, in subjects with spinal cord injury [337,338]. Four patients were treated before the company elected for business reasons to drop its ES cell program.

Advanced Cell Technology has begun clinical trials of ES cell-derived retinal pigment epithelium (RPE) cells, implanted under the retina, in patients with Stargardt's macular dystrophy and dry age-related macular degeneration. The subretinal space is immune privileged, so the use of immune suppressive drugs was not considered necessary for this allogeneic cell therapy. Observations on two subjects at four months after cell transplantation were published in early 2012 [339]. Evidence for safety included absence of excess cell proliferation, ectopic tissue or tumor formation by grafted cells, or of immunological rejection. Vision apparently approved in at least one of the subjects. At the time of writing, this remains the only reported active clinical stage development program of an ES cell-derived product. Positive outcomes would undoubtedly stimulate future efforts to develop tissue-engineered products from pluripotent stem cells.

Two areas of clear medical need that might be addressed by stem cell-derived products are Parkinson's disease [340] and type 1 diabetes [234]. In each case the replacement of a specific cell type, dopamine-producing neurons for Parkinson's disease and insulin-producing pancreatic β cells for diabetes, would be potentially curative.

Significant advances have been made in the production of functional midbrain dopaminergic neurons by staged differentiation from ES cells [274,285,288,289,341,342–346]. Recent studies demonstrated the efficient grafting of these cells leading to physiological correction of symptoms in several animal models, including non-human primates, with chemical lesions that mimic the degenerative loss in Parkinson's disease [347,348]. The identification of conditions to more accurately replicate the normal developmental pathway, including the specification of the midbrain floorplate, proved crucial in generating neurons with the desired specific phenotype and a high capacity to engraft in the brain. Scale-up and the optimization of delivery should enable testing of dopaminergic cells in human subjects.

A team of scientists at Viacyte (formerly Novocell) similarly pursued the strategy of following a normal developmental sequence to produce progenitors of pancreatic endocrine cells from human ES cells [323]. They used transcription factors as stage-specific markers and induced stepwise advancement from pluripotent stem cells to definitive endoderm, primitive gut tube, posterior foregut, and pancreatic endoderm and endocrine precursors. As noted above, the progenitor cells from the final stage reached *in vitro* were able to mature further *in vivo* to yield glucose-responsive β-like cells [330,349]. Screening for small molecules that could replace growth factors or hormones in driving key steps in the differentiation sequence, e.g., the generation of definitive endoderm or pancreatic lineage specification, proved successful, and should facilitate the eventual development of cost-effective therapeutic products [350,351].

The production of ES cells and differentiation to pancreatic progenitors have been scaled-up under conditions compatible with GMP to support clinical testing [272]. It is anticipated that the grafted cells will be susceptible to acute immune attack, both by alloreactive T cells and by the autoimmune T cells that initially caused the patients' type 1 diabetes. Therefore, Viacyte proposes to deliver a tissue-engineered product with the therapeutic cells encapsulated in a semipermeable device designed to protect them from the immune system, while allowing exchange of nutrients and release of insulin to the circulation.

The need to protect grafts from the recipient's immune system is a fundamental problem for regenerative medicine and tissue engineering [34,352], aptly captured by the metaphor of 'the elephant in the room' [353]. The risk of immune rejection must be considered for any cell

therapy or tissue-engineered products based on ES cells, as these are genetically mismatched for all recipients. However, the situation appears more tractable than for classical whole organ transplantation. Tissue-engineered products generated *in vitro* will not contain the professional antigen-presenting cells and inflammatory cells that are present in donated cadaveric organs. Furthermore, undifferentiated human ES cells express very low levels of Class I Major Histocompatibility Complex (MHC) antigens, and do not express Class II MHC antigens (HLA-DR, -DQ, and -DP) [354]. Differentiated cells derived from the ES cells express higher levels of MHC Class I (human leukocyte antigens, HLA-A, -B, and -C), which can be induced further by interferon, but generally remain negative for Class II. One potential way to ensure successful allo-transplantation of these cells would be to modify ES cells genetically to knock out MHC Class I, and possibly Class II, expression [34].

Perhaps because of their relative immaturity and low MHC Class II expression, differentiated ES cell derivatives in fact display lower immunogenicity than do corresponding adult human cells, either *in vitro* or after implantation into mice with a 'humanized' immune system [355,356]. There has been a suggestion from transplantation of murine embryoid bodies in allogeneic mice that ES cells actually exert a local immunosuppressive effect [357], but this claim has been debated [358]. Others reported that various human ES cell-derived specialized cells are subject to immune rejection [359]. However, they found that transplantation tolerance could be induced readily by non-ablative treatment of the host with monoclonal antibodies to the T cell antigens CD4 and CD8. The investigators showed further that transplantation of ES cell-derived tissues across MHC Class I barriers also can occur spontaneously, and that this immune privilege depends on regulatory T cells (Treg) [360]. These observations imply that methods may be found to efficiently induce immunological tolerance in recipients of allogeneic stem cell-derived grafts [352,361–363]. Future developments in this arena could have major impact on the future of tissue engineering, as off-the-shelf therapies that could be employed without any immune suppression would lower both economic and safety barriers to widespread adoption in medical practice [14].

Another approach to enable the use of allogeneic human ES cells for tissue engineering with decreased, or zero, need for immune suppressive drugs rests on cell banking strategies. The simple expedient of deriving clinically intended ES cells only from blood group O donors obviates issues of ABO type compatibility [34]. More profoundly, while complete genetic matching can be achieved only with autologous cells or between identical twins, the establishment of GMP-grade ES cell line banks of relatively modest size, comprising from tens to under 200 lines from appropriately selected donors, would enable complete or 'beneficial' matching at the most important MHC loci (HLA-A, -B, and -DR) for the large majority of the population. Calculation of the numbers of lines that would be required depends on assumptions about the degree of matching that would be required, on the choices of cell line donors and methods of ES cell production, and on the genetic heterogeneity of various national populations [364,365]. In particular, derivation of ES cells from parthenogenetic embryos, which would be homozygous for HLA loci, or selection of rare HLA-homozygous donors from the general population to obtain pluripotent stem cell lines (more readily achieved for iPS than for ES cell banking), would significantly decrease the size of bank required to ensure matching of most potential recipients [364,365,366–369]. Stem cell line banking on a global scale seems an idealistic but potentially feasible goal for future development [370].

INDUCED PLURIPOTENT STEM (IPS) CELLS

At least superficially, the development of iPS cells offers the most direct way to ensure immune compatibility of tissue-engineered products, by allowing any individual to serve as her/his own donor. It also may enable the patient-specific correction of certain mutations that affect tissue development. The approach would be to isolate iPS cells and utilize a method such as

targeting with zinc finger nucleases to correct the genetic defect at the DNA level [371–377]. The genetically repaired cells would then be directed to an appropriate lineage for cell therapy or tissue engineering, and implanted to effectively cure the patient's condition. Clearly, such personalized therapies would demand a trade-off between perfect histocompatibility matching *versus* elevated costs and regulatory complexities [378,379].

Reprogramming of mature somatic cells to a pluripotent state was accomplished initially by the forced expression of four transcription factors common to murine [35] and human ES cells: *OCT4* and *SOX2*, together with either *KLF4* and c-*MYC* [249] or *NANOG* and *LIN28* [250]. The resulting iPS cells closely resemble ES cells in key properties such as the capacity for extensive self-renewal, ability to differentiate to many (presumably all) cell lineages derived from each of the three embryonic germ layers, and generation of teratomas *in vivo*. Initial studies on reprogramming of fibroblasts soon were extended to a variety of cell types that can be obtained with minimal invasiveness from large groups of human donors, such as peripheral blood cells, cord blood cells, keratinocytes from hair shafts, fat-derived MSCs, and cells present in urine [380–390].

Numerous developments have advanced reprogramming technology towards safer, more efficient translation to therapeutic products. Improved methods to deliver the pluripotency factors minimize the risk of inadvertent permanent genetic modification of iPS cells, particularly integration of an oncogene such as c-*MYC*, and thereby decrease the potential for tumorigenicity [391]. One approach is to transiently deliver the factors using various non-integrating viral or plasmid vector systems [392–399]. Reprogramming also can be achieved by direct delivery of either synthetic mRNA encoding the pluripotency factors or of the protein factors themselves [400,401]. Already a next generation of reprogramming methods has emerged in which drug-like compounds substitute for some of the transcription factors [402–407]. Indeed, efficient generation of iPS cells can be achieved with the single transcription factor *OCT4* and a cocktail of small molecules [408–410]. Another new method utilizes microRNA miR-302, which appears to regulate chromatin demethylation and other factors involved in global reprogramming [411–413].

For eventual clinical application, it is essential that reprogramming to generate iPS cells be carried out under GMP-compliant conditions. This has been accomplished [388,414]. Further scale-up and cell differentiation to yield clinical grade cell therapy and tissue-engineered constructs from iPS cells raises the same essential manufacturing and regulatory issues as already discussed above for ES cells. Demonstration projects with cell types including neurons and retinal pigmented epithelial cells support the feasibility of using iPS cells as a potential source of clinical grade products [415–417].

Nevertheless, questions remain about whether iPS cells are entirely equivalent to ES cells in their genomic state and pattern of gene expression, and in their potential to yield a full range of differentiated cell types [418–420]. Critical issues that must be addressed for the future therapeutic application of iPS cells center on the epigenetic mechanisms underlying the resetting of pluripotency [407,418,421,422], and the extent to which subtle variations in the genome and epigenome, which might persist through or be induced by the reprogramming process, influence the properties of iPS cells and of the mature, specialized cells to which they give rise [423–429]. As production methods improve, it is possible that best practices will become standardized and that iPS cell lines intended for clinical use will routinely meet high standards of uniformity and quality. However, it is likely that the reprogramming process itself causes some degree of genomic and epigenomic instability. If so, then to assure performance and safety it may be necessary to subject every line intended for therapeutic use to stringent, deep analysis of DNA sequence and methylation patterns.

One practical outcome that may result from more complete understanding of the mechanics of reprogramming will be the identification of preferred cell types from which to generate iPS

cells. For example, the epigenetic and gene expression signatures of peripheral blood mononuclear cells more closely resemble those of pluripotent stem cells than do those of fibroblasts, so that blood cells may be preferred starting material for the production of clinical grade iPS cell lines [430].

Furthermore, factors may be identified that protect genomic stability during reprogramming. Zscan4, a zinc finger protein that is expressed selectively in 2-cell embryos at the time when zygotic gene activation occurs, appears to have this property. The addition of Zscan4 to the 'Yamanaka' set of transcription factors decreased the DNA damage response during reprogramming of mouse somatic cells, and improved the yield of pluripotent cells [431]. Most tellingly, iPS cell lines produced using Zscan4 were of higher quality than those obtained with the standard factor set alone, as judged by significantly enhanced ability to give rise to live-borne mice derived entirely from the reprogrammed cells in a tetraploid complementation assay.

Important correlates of consistency in the genomic and epigenomic (i.e., DNA methylation and chromatin organization) patterns of reprogrammed cells will be increased predictability in their differentiation to desired lineages, and decreased likelihood of abnormal behavior, including tumorigenicity. A particular risk associated with the inadvertent introduction of changes in genomic DNA during reprogramming could be the creation of abnormal proteins and/or patterns of gene expression that might induce immune responses. This would defeat the purpose of utilizing autologous iPS cells as a putatively non-immunogenic 'self' cell source for tissue engineering. Indeed, experiments on cell transplantation in inbred C57BL/6 mice showed that iPS cells can be unexpectedly immunogenic [432]. Whereas, as expected, ES cells derived from this strain efficiently formed teratomas in syngeneic hosts, iPS cell lines derived from the same strain either failed to form tumors or formed teratomas that became strongly infiltrated by host T lymphocytes. The report's authors attributed the immune rejection of iPS cells to overexpression of multiple genes, compared to levels in ES cells. However, more encouragingly, an independent study in the same mouse strain provided no evidence for immunogenicity of differentiated skin or bone marrow tissues derived from either ES or iPS cells when transplanted to the syngeneic hosts [433]. These results are consistent with a test of immunogenicity of bovine cells derived after reprogramming by somatic cell nuclear transfer (i.e., development of an embryo after insertion of the nucleus of an adult tissue cell into an enucleated, activated egg cell — also known as cloning). Engineered constructs of multiple tissue types from developing cows cloned by this method were implanted into the animals that had served as donors of the nuclei, and there was no evidence for immune rejection [434]. Thus, the use of reprogrammed cells as a histocompatible source for tissue engineering has been established clearly in principle, but it remains possible for genetic or epigenetic aberrations to trigger unanticipated immune responses. This reinforces the importance of finding general means to induce immunological tolerance to tissue-engineered constructs [362], even if these are generated using reprogrammed autologous cells [435].

Much recent excitement in the reprogramming field has centered efforts to bypass the circuitous route of resetting cells to a pluripotent ground state and then inducing them to a desired lineage. Instead, may it be possible to achieve directed 'transdifferentiation' between cell lineages? A number of laboratories have indeed reported that fibroblasts or other adult cells can be reprogrammed directly to various specialized cell types such as neurons and neural progenitors, cardiomyocytes, endothelial cells, and hepatocytes [436–447]. Significant questions about direct lineage-to-lineage reprogramming still must be addressed. Does it generate cells that accurately mimic authentic gene expression patterns and differentiated function of the desired product, or is there carryover of residual signatures of the starting cells? Do the cells display fully adult phenotypes, or, like differentiated ES and iPS cells, do they more closely resemble relatively immature embryonic cells? Is the risk of introducing unwanted genetic or epigenetic abnormalities less or greater than in reprogramming to a pluripotent state?

The answers to these questions will clarify the value of direct cell lineage conversions for tissue engineering and regenerative medicine.

ADULT STEM CELLS

Despite the promise of ES and iPS cells, and of directly reprogrammed cell types, the challenges of controlling lineage-specific differentiation, eliminating residual pluripotent stem cells, and confirming the safety and accurate phenotypes of cell products are likely to extend the timeline for a number of tissue-engineering applications. By contrast, adult stem cells present in many tissues throughout fetal development and postnatal life are committed to restricted cell lineages, and they are not intrinsically tumorigenic [448–451]. The adult stem cells, therefore, provide a starting point for more rapid clinical development of tissue-engineered, cell-based products.

Today, by far the most commonly utilized cells for experiments in tissue engineering are mesenchymal stromal or stem cells (MSC), often isolated from bone marrow [452]. As presciently hypothesized by Caplan, MSC can give rise to a number of tissue types, mainly connective, including bone, cartilage, tendon, adipose, and some categories of muscle [453]. Essentially the same cell type can be harvested readily from fat tissue obtained by processing of suction-assisted lipectomy (liposuction) specimens [454,455]. It now appears that MSC comprise a subset of pericytes associated with vasculature throughout the body [454,455,500]. MSC have generated considerable interest for musculoskeletal and vascular tissue engineering [456–458]. For example, a large European consortium named Genostem (an acronym for 'Adult mesenchymal stem cells engineering for connective tissue disorders. From the bench to the bed side') seeks to develop MSC/scaffold combinations for bone, cartilage and tendon repair [459].

A broader use for MSC in regenerative medicine, distinct from their use as precursors of specialized connective tissues, followed from the unexpected discovery that they can be transplanted readily into allogeneic recipients [460]. The ability to escape immune rejection results from a variety of mechanisms, most notably the secretion of anti-inflammatory cytokines [461,462]. These constitute a subset of a large array of trophic growth factors, cytokines, and other molecules that collectively endow MSC with potent immunomodulatory, wound healing, and regenerative properties [463–466]. Advanced clinical trials have assessed MSC cell therapy to treat Graft versus Host Disease (GvHD) and various inflammatory or autoimmune conditions [467–469]. The first approvals for sale of a bone marrow-derived MSC product (Prochymal®, Osiris Therapeutics) were obtained for treatment of GvHD in Canada and New Zealand, and similar products from academic and commercial entities are in late stages of development and, in some cases, regulatory review around the world [470].

Cell therapy aimed primarily at immunodulation arguably falls outside of a strict definition of tissue engineering. The mechanistic contributions of MSC to regenerative therapies easily can be confounded, because this population also may give rise to cells expressing markers associated with various non-mesodermal lineages, including hepatocytes (endoderm) and neurons (ectoderm). However, the observations supporting the apparently extensive plasticity of MSC must be interpreted with caution [471,472]. 'Differentiation' events may be relatively rare, and in many cases the detailed phenotype of the resulting end cells has not been compared rigorously with authentic specialized cells of the lineage in question.

Certain cells originating in a developing fetus and isolated from amniotic fluid or chorionic villi share some properties with adult MSC, but are capable of more extensive proliferation in clonal cultures [473]. These so-called amniotic fluid-derived stem (AFS) cells could represent more primitive precursors of MSC, but also express some markers in common with ES cells, so that their precise lineage and physiological plasticity is not certain. However, like MSC the AFS cells possess immunomodulatory properties that could contribute to clinical utility [474].

Advances in MSC cell therapy for heart disease may point to an effective future role for these cells in tissue engineering, beyond the constellation of connective tissues for which the cells are direct progenitors. The delivery of either autologous or allogeneic MSC into the left ventricle wall using a transendocardial injection device has given promising indications for efficacy in the treatment of ischemic cardiomyopathy in a large animal (minipig) model and initial human trials [475–477]. A principal explanation for the beneficial action of the injected MSC appears to be trophic effects on endogenous cardiac stem and progenitor cells that can give rise to new cardiomyocytes [478,479]. The MSC also apparently contribute to reverse remodeling of damaged areas of the heart, even at long times after the initial insults [477]. Recently published data demonstrate that the combined delivery of isolated adult cardiac stem cells and MSC substantially improves outcomes in the porcine model of ischemic cardiomyopathy, as judged by infarct size and cardiac function [480]. It seems plausible that tissue-engineered constructs incorporating both stem cell types will evolve from these studies.

The long history of hematopoietic stem cell transplantation established that long-lived donor cells could permanently colonize bone marrow niches and continuously replenish the full array of mature blood cell type [481,482]. However, to date there has been remarkably little clinical experience with any other therapy based on the engraftment of a defined stem cell population and its differentiation to specialized cells that persist and function in a particular organ. Several years ago Stem Cells Inc. initiated human studies of a brain-derived neural stem cell preparation called HuCNS-SC in a handful of subjects with neurological degenerative conditions, namely, neuronal lipoid fuscinosis (Batten's Disease) and Pelizaeus-Merzbacher Disease (PMD) [483]. The cells were isolated by selection with antibodies to cell surface markers. They expand extensively as suspension aggregates ('neurospheres') in a defined culture medium containing EGF and FGF [484]. The clinical experiments provided preliminary evidence for safety of these stem cells [485]. MRI data demonstrated durable cell engraftment and suggested that donor-derived cells contributed to myelination in recipient brain. Compelling evidence for myelination was observed by transplantation of the human stem cells in mutant mice with a severe dysmyelination disorder [486]. Stem Cells Inc. recently began further clinical trials of the same neural stem cell product in subjects with spinal cord injury and dry age-related macular degeneration.

Another human adult stem cell population that gives rise to organ parenchymal cells was isolated from the liver [487]. These hepatic stem cells (HpSC) were enriched from cadaveric fetal, neonatal, or fully mature donors by selection with a monoclonal antibody to the surface marker CD326 (epithelial cell adhesion molecular, EpCAM), which constitute roughly 1 percent of cells in suspensions prepared from young or old postnatal livers. They can be maintained for more than 40 population doublings, corresponding to a potential expansion of one trillion-fold (1×10^{12}), with a doubling time of 36 to 40 hours, in a defined serum-free culture medium containing insulin and transferrin, but lacking classical growth factors [488,489]. If cultured as adherent cells on tissue culture plastic, the HpSC grow in cooperation with immature mesenchymal cells (e.g., angioblasts) that may provide elements of their normal stem cell niche [487,490,491]. Appropriate ECM molecules thought to be associated with that niche, type III collagen and/or weakly crosslinked hyaluronic acid hydrogels of appropriate mechanical stiffness, can replace the need for mesenchymal cells to assist the expansion of the stem cells [491–494]). Exposure to certain growth factors, for example EGF, and different collagens and tissue-specific matrix molecules (e.g., liver proteoglycans) induces efficient differentiation of the HpSC to either hepatocytes or cholangiocytes (bile duct cells) [53].

C.M. Habibullah and associates in Hyderabad, India initiated clinical assessment of CD326-positive hepatic stem and progenitor cells, selected from human fetal liver and used without expansion of the cell population in culture [495,496]. They found that delivery of these cells to the liver was best achieved by infusion via the hepatic artery. In a published report on

25 subjects with decompensated liver cirrhosis from various causes, at 6 months post-infusion improvements were observed in a number of objective clinical parameters, including a significant decrease in the mean Mayo End-stage Liver Disease (MELD) score ($P < .01$) [495]. It appeared important to identify patients whose initial status was not terminal, presumably to allow sufficient time (several weeks) for stem cells to differentiate to hepatocytes. Imaging studies with radiolabeled stem cells demonstrated retention of a significant fraction of the delivered cells in the liver, although, not surprisingly, a majority traveled through the circulation to distant sites such as the lung. The data were consistent with, but did not prove, differentiation of the stem cells to more mature parenchymal cells. Data from a subsequent clinical trial in over 250 subjects have not yet been published in the peer-reviewed literature. However, the results apparently were consistent with those reported for the initial clinical study, and have been submitted for regulatory review in India to support product approval.

Most remarkably, the encouraging clinical results with HpSC transplantation were achieved using allogeneic donors with no effort to match at HLA loci, and without the use of immune suppressive drugs. It is conceivable that the fetal liver-derived stem cells are particularly non-immunogenic; indeed, HpSC, like ES cells, express only low levels of MHC Class I and lack detectable MHC Class II [487]. Furthermore, it is possible that the liver is significantly immune privileged with respect to transplant rejection. Another possibility is that when HpSC are isolated by immunoselection with magnetic antibody-coated beads specific for CD326, some angioblast-like mesenchymal cells remain associated with them. Such cells, like MSC, might secrete immunomodulatory factors that could protect the HpSC and differentiated cells derived from them against immune rejection.

Future clinical trials based on HpSC may transition to cells sourced from neonatal livers obtained through organ donation. This would be facilitated by the relatively long survival of hepatic stem and progenitor cells under ischemic conditions, and the development of cryopreservation conditions for these cells [496,497]. Tissue engineering would appear a superior long-term option over simple cell therapy. One possible approach would utilize HpSC, probably in combination with endothelial progenitors such as the liver-derived angioblasts, to reconstitute whole-organ decellularized scaffolds [52,53]. A simpler strategy, more likely to be feasible in the near term, would be to deliver the HpSC in hyaluronan hydrogels, thereby minimizing escape of the cells to ectopic locations and encouraging local tissue development from cell aggregates confined in the matrix [498]. The addition of liver-specific matrix components and/or growth factors to the hydrogels might promote more rapid, robust differentiation to hepatocytes [53].

A newly discovered human stem cell population may prove to be a more readily isolated source for liver tissue engineering. Excitingly, these stem cells also offer hope for engineering of pancreatic islet-like structures to treat insulin-dependent diabetes. The cells were identified in peribiliary glands found in the extrahepatic biliary tree, ramifying ducts located between the liver and pancreas [499]. Analysis of cell surface marker and transcription factor expression *in situ*, along with the growth and differentiation potential of the cells, suggests that they include a population of more primitive endodermal stem cells than the HpSC identified within the liver. Some of these biliary tree stem cells do not express CD326, but appear to be precursors of the CD326-positive HpSC. Remarkably, these cells express detectable levels of transcription factors characteristic of both hepatic and pancreatic lineages; among the key pancreatic markers are *PDX1* and *NGN3*. A working hypothesis is that these stem cells serve as a potential source for cell turnover in both the liver and pancreas (at least the endocrine portion; their potential to differentiate to pancreatic exocrine cells has not yet been tested) throughout life.

The biliary tree stem cells proliferate extensively when cultured in the serum-free defined medium developed for HpSC. They could be induced to differentiate to either cholangiocytes or hepatocytes under conditions previously worked out for differentiation of HpSC. However, when exposed to soluble factors known to induce differentiation of pancreatic progenitors

to endocrine cells, and to ECM extracted from pancreatic islets of Langerhans, the stem cells gave rise to islet-like structures with cells expressing characteristic pancreatic hormones, most notably pro-insulin. Release of insulin was responsive to extracellular glucose.

In vivo studies supported the hypothesis that human biliary tree stem cells are multipotent for liver and pancreas lineages. Upon intrahepatic injection in immune-deficient mice, the stem cells gave rise to hepatocyte-like and cholangiocyte-like progeny. To assess endocrine pancreatic differentiation, islet-like structures were induced in culture and implanted into mouse fat pads. After several weeks the animals were treated with streptozotocin (STZ) at a concentration expect to destroy murine pancreatic β cells, but spare human ones, which are more resistant to the toxin. After the STZ treatment the mice that had received transplanted human islet-like clusters indeed became significantly less hyperglycemic than controls that had not received cell therapy. The presence of functional β-like cells derived from the biliary tree stem cells was supported by detection of human C-peptide in the circulation, and its level was regulated correctly in response to a glucose challenge [499]. Thus, it appears that biliary tree stem cells, which could be harvested from tissue frequently discarded after certain surgical procedures and should also be accessible by laparoscopy, represent a potential cell source for tissue engineering of islet-like structures for diabetes therapy.

Future challenges

The medical application of tissue engineering still lies largely ahead of us. Although a handful of products have achieved regulatory approval and entered the marketplace, many more are in the planning or proof-of-concept stage, or in relatively early clinical trials. In order to reach the large number of patients who might potentially benefit from bioengineered therapeutics, advances will be required in manufacturing and distributing complex products. This will be a fruitful area for engineers to address.

It also will be critical to develop closer partnerships among academic and industrial scientists and the regulatory agencies (e.g., the US FDA) that must assess new therapies for safety and efficacy. Products that may contain novel cellular components, biomaterials, and active growth or angiogenic factors will demand sophisticated, multifaceted review. Historically, regulatory agencies have had far greater experience with single drug entities or devices than with combination products. However, there is reason for optimism that the FDA's experiences to date with successful applications and early trials of products derived from both adult and embryonic human stem cells will pave the way to effective review of future bioengineered products.

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PART 1

The Basis of Growth and Differentiation

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CHAPTER 7

Molecular Biology of the Cell

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To a naïve observer the term 'tissue engineering' might seem a contradiction in terms. The word 'engineering' tends to conjure up a vision of making objects from hard components such as metals, plastics, concrete or silicon, which are mechanically robust and will withstand a range of environmental conditions. The components themselves are often relatively simple, and the complexity of a system emerges from the number and connectivity of the parts. By contrast, the cells of living organisms are themselves highly delicate and highly complex. Despite our knowledge of a vast amount of molecular biology detail concerning cell structure and function, their properties are still understood only in qualitative terms and so any application using cells requires a lot of craft skill as well as rational design. What follows is a very brief account of cell properties, intended for newcomers to tissue engineering who have an engineering or physical science background. It is intended to alert readers to some of the issues involved in working with cells, and to pave the way for an understanding of how cells form tissues and organs, topics dealt with in more detail in the later chapters. As it comprises very general material, it is not specifically referenced, although some further reading is provided at the end.

Cells are the basic building blocks of living organisms in the sense that they can survive in isolation. Some organisms, such as bacteria, protozoa or many algae, actually consist of single free living cells. But most cells are constituents of multicellular organisms, and although they can survive in isolation they need very carefully controlled conditions in order to do so. A typical animal cell suspended in liquid will be a sphere of the order of about 20 microns diameter (Fig. 7.1). Most cells will not grow well in suspension, and so they are usually grown attached to a substrate, where they flatten and may be quite large in horizontal dimensions but only a few microns in vertical dimension. All eukaryotic cells contain a nucleus in which is located the genetic material that ultimately controls everything that the cell is composed of and all the activities it carries out. This is surrounded by cytoplasm, which has a very complex structure and contains sub-structures called organelles which are devoted to specific biochemical functions. The outer surface of the cell is the plasma membrane, which is of crucial importance as it forms the frontier across which all materials must pass on their way in or out. The complexity of a single cell is awesome since it will contain thousands of different types of protein molecules, arranged in many very complex multimolecular aggregates comprising both hydrophobic and aqueous phases; and also many thousands of low molecular weight metabolites, including sugars, amino acids, nucleotides, fatty acids, phospholipids and many others. Although some individual steps of metabolism may be near to thermodynamic equilibrium, the cell as a whole is very far from equilibrium and is maintained in this condition by a continuous interchange of substances with the environment. Nutrients

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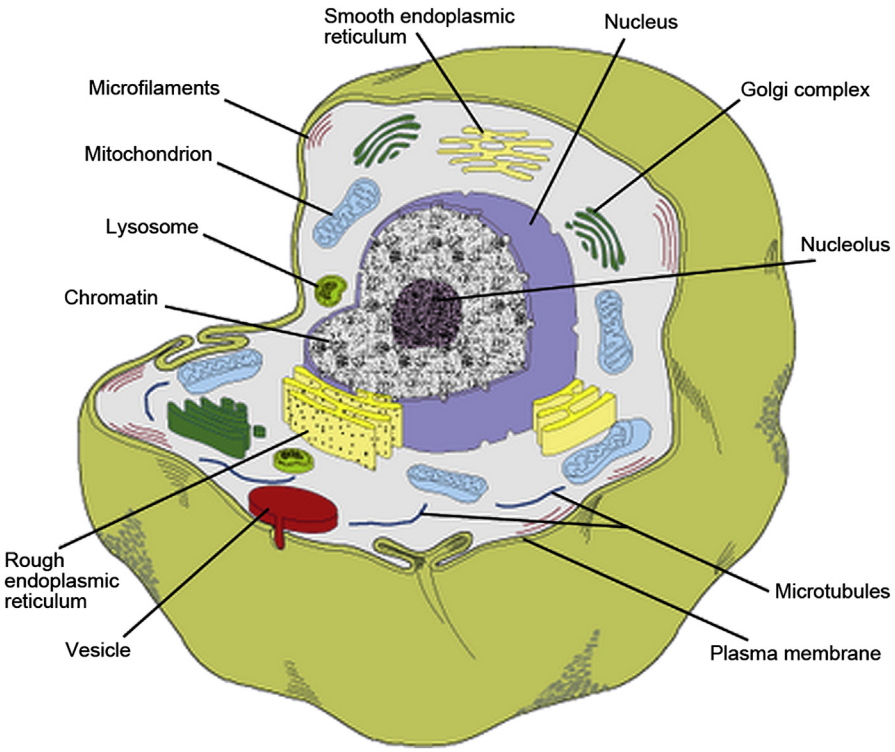


FIGURE 7.1
Structure of a generalized animal cell. *(From http://scienceblogs.com/clock/2006/11/09/cell-structure/). accessed 5-15-2013.*

are chemically transformed with release of energy which is used to maintain the structure of the cell and to synthesize the tens of thousands of different macromolecules on which its continued existence depends. Maintaining cells in a healthy state means providing them continuously with all the substances they need, in the right overall environment of substrate, temperature and osmolarity; and also continuously removing all potentially toxic waste products.

THE CELL NUCLEUS

The nucleus contains the genes that control the life of the cell. A gene is a sequence of DNA that codes for a protein, or for a non-translated RNA, and it is usually considered also to include the associated regulatory sequences as well as the coding region itself. The vast majority of eukaryotic genes are located in the nuclear chromosomes, although there are also a few genes carried in the DNA of mitochondria and chloroplasts. The genes encoding non-translated RNAs include those for ribosomal or transfer RNAs, and also for a large number of microRNAs that are probably involved in controlling the expression of protein coding genes. The total number of protein coding genes in vertebrate animals is about 25,000, and every nucleus contains all the genes, irreversible DNA modifications being confined to B and T cells of the immune system in respect of the genes encoding antibodies and T cell receptors.

The DNA is complexed into a higher order structure called chromatin by the binding of basic proteins called histones. Protein coding genes are transcribed into messenger RNA (mRNA) by the enzyme RNA polymerase II. Transcription commences at a transcription start sequence and finishes at a transcription termination sequence. Genes are usually divided into several exons, each of which codes for a part of the mature mRNA. The primary RNA transcript is extensively processed before it moves from the nucleus to the cytoplasm. It acquires a 'cap' of methyl guanosine at the 5' end, and a polyA tail at the 3' end, both of which stabilize the message by protecting it from attack by exonucleases. The DNA sequences in between the exons are called introns and the portions of the initial transcript complementary to the introns

are removed by splicing reactions catalyzed by 'snRNPs' (small nuclear ribonucleoprotein particles). It is possible for the same gene to produce several different mRNAs as a result of alternative splicing; whereby different combinations of exons are spliced together from the primary transcript. In the cytoplasm, the mature mRNA is translated into a polypeptide by the ribosomes. The mRNA still contains a 5′ leader sequence and a 3′ untranslated sequence flanking the protein coding region, and these untranslated regions may contain specific sequences responsible for translational control or intracellular localization.

Control of gene expression

There are many genes whose products are required in all tissues at all times; for example those concerned with basic cell structure, protein synthesis or metabolism. These are referred to as 'housekeeping' genes. But there are many others whose products are specific to particular cell types and indeed the various cell types differ from each other *because* they contain different repertoires of proteins. This means that the control of gene expression is central to tissue engineering. Control may be exerted at several points. Most common is control of transcription, and we often speak of genes being 'on' or 'off' in particular situations, meaning that they are or are not being transcribed. There are also many examples of translational regulation, where the mRNA exists in the cytoplasm but is not translated into protein until some condition is satisfied. Control may also be exerted at the stage of nuclear RNA processing, or indirectly via the stability of individual mRNAs or proteins.

Control of transcription depends on regulatory sequences within the DNA, and on proteins called transcription factors that interact with these sequences. The promoter region of a gene is the region to which the RNA polymerase binds, just upstream from the transcription start site. The RNA polymerase is accompanied by a set of general transcription factors, which together make up a transcription complex. In addition to the general factors required for the assembly of the complex, there are numerous specific transcription factors that bind to specific regulatory sequences that may be either adjacent to or at some distance from the promoter (Fig. 7.2). These are responsible for controlling the differences in gene expression between different types of cell.

TRANSCRIPTION FACTORS

Transcription factors are the proteins that regulate transcription. They usually contain a DNA-binding domain and a regulatory domain, which will either upregulate or repress transcription. Looping of the DNA may bring these regulatory domains into contact with the transcription complex with resulting promotion or inhibition of transcription. There are many families of transcription factors, classified by the type of DNA-binding domain they contain, such as the homeodomain or zinc-finger domain. Most are nuclear proteins, although some exist in the cytoplasm until they are activated and then enter the nucleus. Activation often occurs in response to intercellular signaling (see below). One type of transcription factor, the nuclear receptor family, is directly activated by lipid-soluble signaling molecules such as retinoic acid or glucocorticoids.

Each type of DNA-binding domain in a protein has a corresponding type of target sequence in the DNA, usually 20 nucleotides or less. The activation domains of transcription factors often contain many acidic amino acids making up an 'acid blob', which accelerates the formation of the general transcription complex. Some transcription factors recruit histone acetylases which open up the chromatin by neutralizing amino groups on the histones by acetylation, and allow access of other proteins to the DNA. Although it is normal to classify transcription factors as activators or repressors of transcription, their action is also sensitive to context, and the presence of other factors may on occasion cause an activator to function as a repressor, or vice versa.

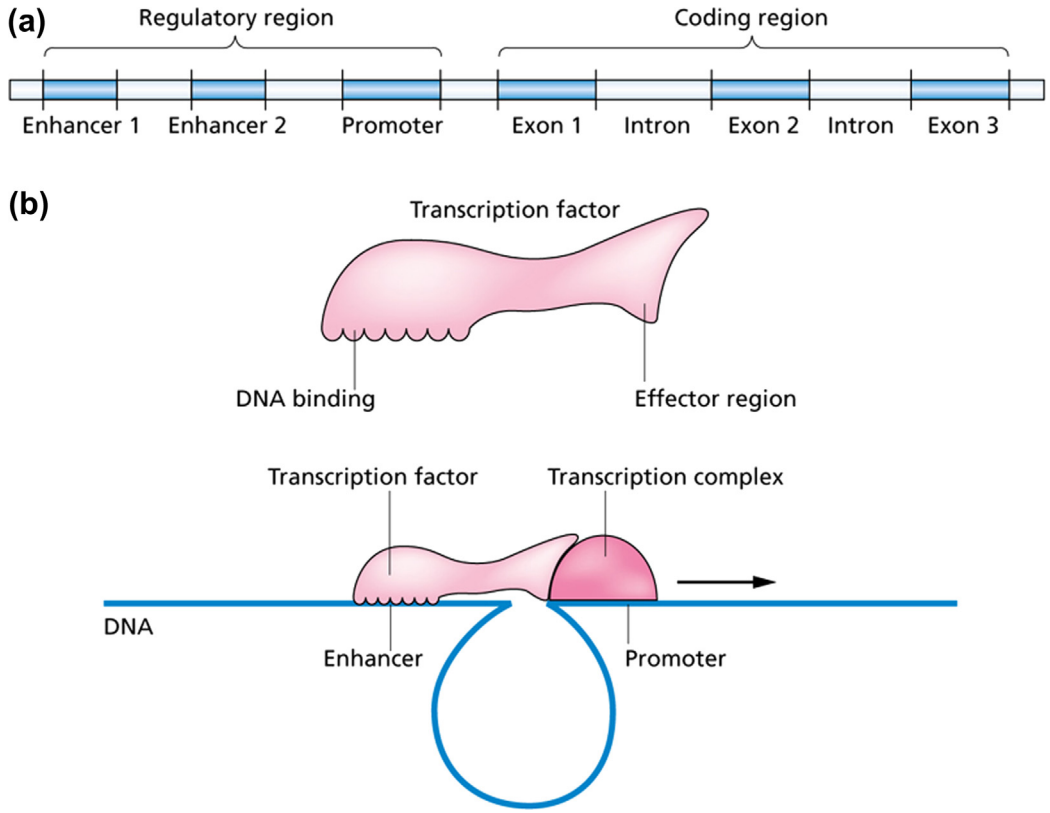


FIGURE 7.2
a) Structure of a typical gene. b) Operation of a transcription factor. *(From Slack 2012).*

OTHER CONTROLS OF GENE ACTIVITY

Some aspects of gene control are of a more stable and longer term character than that exerted by combinations of positive and negative transcription factors. To some extent this depends on the remodeling of the chromatin structure, which is still poorly understood. The chromosomal DNA is complexed with histones into nucleosomes and is coiled into a 30 nm diameter filament, which is in turn arranged into higher order structures. In much of the genome the nucleosomes are to some extent mobile, allowing access of transcription factors to the DNA. This type of chromatin is called euchromatin. In other regions the chromatin is highly condensed and inactive, then being called heterochromatin. In the extreme case of the nucleated red blood cells of non-mammalian vertebrates the entire genome is heterochromatic and inactive. Chromatin structure is regulated to some degree by protein complexes (such as the well known Polycomb and trithorax groups), which affect the expression of many genes but are not themselves transcription factors.

An important element of the chromatin remodeling is the control through acetylation of lysines on the exposed N-termini of histones. This partially neutralizes the binding of the histones to the negatively charged phosphodiester chains of DNA, so opens up the chromatin structure and enables transcription complexes to assemble on the DNA. The degree of histone acetylation is controlled, at least partly, by DNA methylation, because histone deacetylases are recruited to methylated regions and will tend to inhibit gene activity in these regions. DNA methylation occurs on cytosine residues in CG sequences of DNA. Because CG on one strand will pair with GC on the other, antiparallel, strand, potential methylation sites always lie opposite one another on the two strands. There are several DNA methyl transferase enzymes, including *de novo* methylases that methylate previously unmethylated CGs, and maintenance methylases that methylate the other CG of sites bearing a methyl group on only one strand.

Once a site is methylated, it will be preserved through subsequent rounds of DNA replication, because the hemimethylated site resulting from replication will be a substrate for the maintenance methylase.

There are many other chemical modifications of the histones in addition to acetylation, including methylation of sites within the histone polypeptides. It is probable that these modifications can also be retained on chromosomes when the DNA is replicated. So both DNA methylation and histone modifications provide means for maintaining the state of activity of genes in differentiated cells even after the original signals for activation or repression have disappeared.

THE CYTOPLASM

The cytoplasm consists partly of proteins in free solution, although it also possesses a good deal of structure that can be visualized as the cytoskeleton (see below). Generally considered to be in free solution, although probably in macromolecular aggregates, are the enzymes that carry out the central metabolic pathways. In particular the pathway called glycolysis leads to the degradation of glucose to pyruvate. Glucose is an important metabolic fuel for most cells. Mammalian blood glucose is tightly regulated at around 5–6 mM, and glucose is a component of most tissue culture media. Glycolysis leads to the production of two molecules of adenosine triphosphate (ATP) per molecule of glucose, with a further 36 molecules of ATP produced by oxidative phosphorylation, and this ATP is needed for a very wide variety of synthetic and biochemical maintenance activities. Apart from the central metabolic pathways, the cell is also engaged in the continuous synthesis and degradation of a wide variety of lipids, amino acids and nucleotides.

The cytoplasm contains many types of organelle which are structures composed of phospholipid bilayers. Phospholipids are molecules with a polar head group and a hydrophobic tail. They tend to aggregate to form sheets in which all the head groups are exposed on the surface and the hydrophobic tails associate with each other to form a hydrophobic phase. Most cell organelles are composed of membranes comprising two sheets of phospholipid molecules with their hydrophobic faces joined. The mitochondria are the organelles responsible for oxidative metabolism, as well as other metabolic processes such as the synthesis of urea. They are composed of an outer and an inner phospholipid bilayer. The oxidative degradation of sugars, amino acids and fatty acids is accompanied by the production of ATP. Pyruvate produced by glycolysis is converted to acetyl CoA, and this is oxidized to two molecules of CO_2 by the citric acid cycle, with associated production of 12 molecules of ATP in the electron transport chain of the mitochondria. Because of the importance of oxidative metabolism for ATP generation, cells need oxygen to support themselves. Tissue culture cells are usually grown in atmospheric oxygen concentration (about 20% by volume), although the optimum concentration may be somewhat lower than this since the oxygen level within an animal body is normally lower than the external atmosphere. Too much oxygen can be deleterious, because it leads to the formation of free radicals that cause damage to cells. Tissue culture systems are therefore often run at lower oxygen levels, such as 5%. The oxidation of pyruvate and acetyl CoA also results in the production of CO_2 which needs to be removed continuously to avoid acidification of the culture medium.

Among the organelles in the cytoplasm is the endoplasmic reticulum, which is a ramifying system of phospholipid membranes. The interior of the endoplasmic reticulum can communicate with the exterior medium through the exchange of membrane vesicles with the plasma membrane. Proteins that are secreted from cells, or that come to lie within the plasma membrane, are synthesized by ribosomes that lie on the cytoplasmic surface of the endoplasmic reticulum, and the products are passed through pores into the endoplasmic reticulum lumen. From here they move to the Golgi apparatus, which is another collection of internal membranes, in which carbohydrate chains are added. From there they move to the cell

surface or the exterior medium. Secretion of materials is a very important function of all cells and it needs to be remembered that their environment in tissue culture depends not only on the composition of the medium that is provided but also on what the cells themselves have been making and secreting.

The intracellular proteins are synthesized by ribosomes in the soluble cytoplasm. There is a continuous production of new protein molecules, the composition depending on the repertoire of gene expression of the cell. There is also a continuous degradation of old protein molecules, mostly in a specialized structure called the proteasome. This continuous turnover of protein requires a lot of ATP.

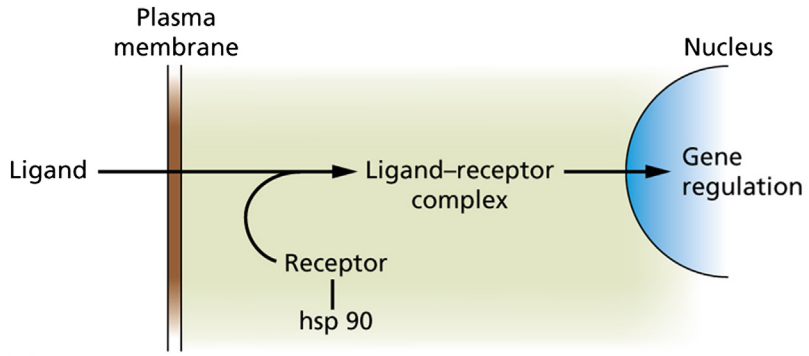
The cell surface

The plasma membrane is the frontier between the cell and its surroundings. It is a phospholipid bilayer incorporating many specialized proteins. Very few substances are able to enter and leave cells by simple diffusion; in fact this method is really only available to low molecular weight hydrophobic molecules like retinoic acid, steroids or thyroid hormones. The movement of inorganic ions across the membrane is very tightly controlled. The main control is exerted by a sodium-potassium exchanger known as the Na^+K^+ATPase, which expels sodium (Na) and imports potassium (K) ions. Because three sodium ions are expelled for every two potassium ions imported, this process generates an electric potential difference across the membrane, which is intensified by differential back diffusion of the ions. The final membrane potential is negative inside the cell and ranges from about 10 mV in red blood cells to about 70 mV in excitable cells such as neurons. This maintenance of membrane potential by the Na^+K^+ATPase accounts for a high fraction of total cellular ATP consumption. Calcium (Ca) ions are very biologically active within the cell and are normally kept at a very low intracellular concentration, about 10^{-7}M. This is about 10^4 times lower than the typical exterior concentration, which means that any damage to the plasma membrane is likely to let in a large amount of calcium which will damage the cell beyond repair.

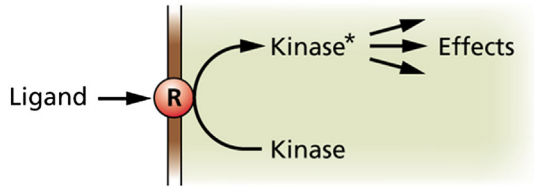
Some proteins of the plasma membrane are very hydrophobic molecules entirely contained within the lipid phase, but more usually they have hydrophilic regions projecting to the cell exterior or to the interior cytoplasm or both. These proteins have a huge range of essential functions. Some are responsible for anchoring cells to the substrate or to other cells through adhesion molecules and junctional complexes. Others, including ion transporters and carriers for a large range of nutrients, are responsible for transporting molecules across the plasma membrane. Then there are the receptors for extracellular signaling molecules (hormones, neurotransmitters and growth factors) which are critical for controlling cellular properties and behavior. Some types of receptor serve as ion channels, for example admitting a small amount of calcium when stimulated by their specific ligand. Other types of receptor are enzymes and initiate a metabolic cascade of intracellular reactions when stimulated. These reaction pathways often involve protein phosphorylation and frequently result in the activation of a transcription factor and thereby the activation of specific target genes. The repertoire of responses that a cell can show depends on which receptors it possesses, how these are coupled to signal transduction pathways, and how these pathways are coupled to gene regulation. It is sometimes called the 'competence' of the cell. The serum that is usually included in tissue culture media contains a wide range of hormones and growth factors and is itself likely to stimulate many of the cell surface receptors.

SIGNAL TRANSDUCTION

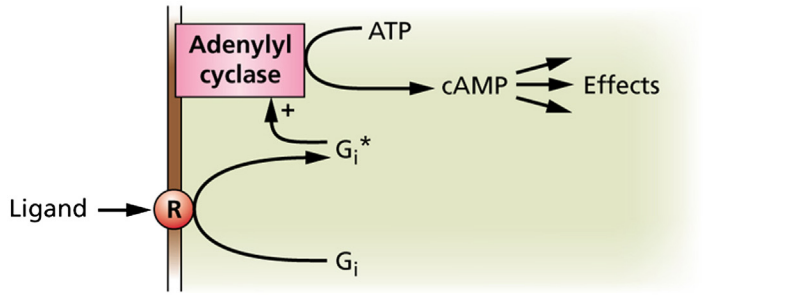
Lipid soluble molecules such as steroid hormones can enter cells by simple diffusion. Their receptors, called nuclear hormone receptors, are multidomain molecules that also function as transcription factors. Binding of the ligand activates the factor, in many cases also causing translocation to the nucleus where the receptor-ligand complex can upregulate its target genes (Fig. 7.3a).

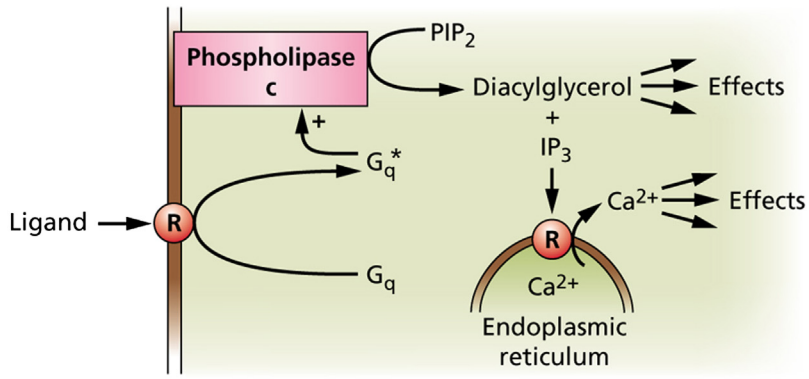


(a) Nuclear receptor

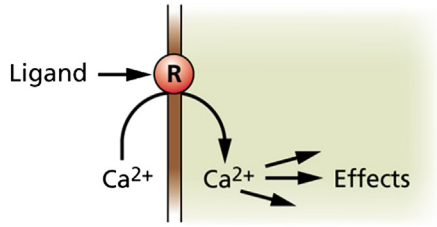


(b) Enzyme–linked receptor





(c) G protein–linked receptors



(d) Ion channel receptor

FIGURE 7.3
Different types of signal transduction. *(From Slack 2012).*

Most signaling molecules are proteins which cannot diffuse across the plasma membrane and so work by binding to specific cell surface receptors. There are three main classes of these: enzyme-linked receptors, Gprotein-linked receptors and ion channel receptors.

Enzyme-linked receptors are often tyrosine kinases or Ser/Thr kinases (Fig. 7.3b). All have a ligand-binding domain on the exterior of the cell, a single transmembrane domain, and the enzyme active site on the cytoplasmic domain. For receptor tyrosine kinases, the ligand-binding brings about dimerization of the receptor, and this results in an autophosphorylation whereby each receptor molecule phosphorylates and activates the other. The phosphorylated receptors can then activate a variety of targets. Many of these are transcription factors that are activated by phosphorylation and move to the nucleus where they upregulate their target genes. In other cases there is a cascade of kinases that activate each other down the chain, culminating in the activation of a transcription factor. Roughly speaking, each class of factors has its own associated receptors and a specific signal transduction pathway; however, different receptors may be linked to the same signal transduction pathway, or one receptor may feed into more than one pathway. The effect of one pathway upon the others is often called 'cross talk'. The significance of cross talk can be hard to assess from biochemical analysis alone, but is easier using genetic experiments in which individual components are mutated to inactivity and the overall effect on the cellular behavior can be assessed.

There are several classes of Gprotein-linked receptor (Fig. 7.3c). The best known are seven-pass membrane proteins, meaning that they are composed of a single polypeptide chain crossing the membrane seven times. These are associated with trimeric G proteins composed of α, β and γ subunits. When the ligand binds, the activated receptor causes exchange of guanosine diphosphate (GDP) bound to the α subunit for guanosine triphosphate (GTP), then the activated α subunit is released and can interact with other membrane components. The most common target is adenylyl cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) which phosphorylates various further target molecules affecting both intracellular metabolism and gene expression.

Another large group of Gprotein-linked receptors use a different trimeric G protein to activate the inositol phospholipid pathway (Fig. 7.3c). Here the G protein activates phospholipase Cβ which breaks down phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). The DAG activates an important membrane-bound kinase, protein kinase C. Like protein kinase A, this has a large variety of possible targets in different contexts and can cause both metabolic responses and changes in gene expression. The IP_3 binds to an IP_3 receptor (IP_3R) in the endoplasmic reticulum and opens Ca channels which admit Ca ions into the cytoplasm. Normally cytoplasmic calcium is kept at a very low concentration of around 10^{-7}M. An increase caused either by opening of an ion channel in the plasma membrane, or as a result of IP_3 action, can have a wide range of effects on diverse target molecules.

Ion channel receptors (Fig. 7.3d) are also very important. They open on stimulation to allow passage of Na, K, Cl (chloride) or Ca ions. As mentioned above, Na and K ions are critical to the electrical excitability of nerve or muscle, and Ca ions are very potent, having a variety of effects on cell structure and function at low concentration.

GROWTH AND DEATH

Tissue engineering inevitably involves the growth of cells in culture, so the essentials of cell multiplication need to be understood. A typical animal cell cycle is shown in Fig. 7.4 and some typical patterns of cell division in Fig. 7.5. The cell cycle is conventionally described as consisting of four phases. M indicates the phase of mitosis, S indicates the phase of DNA replication, and G1 and G2 are the intervening phases. For growing cells, the increase in mass

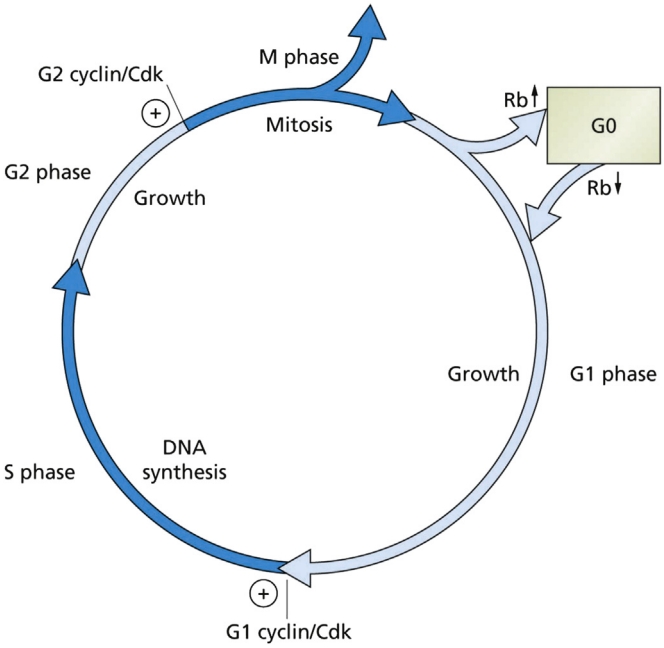


FIGURE 7.4
The cell cycle, with phases of growth, DNA replication and division. *(From Slack 2012).*

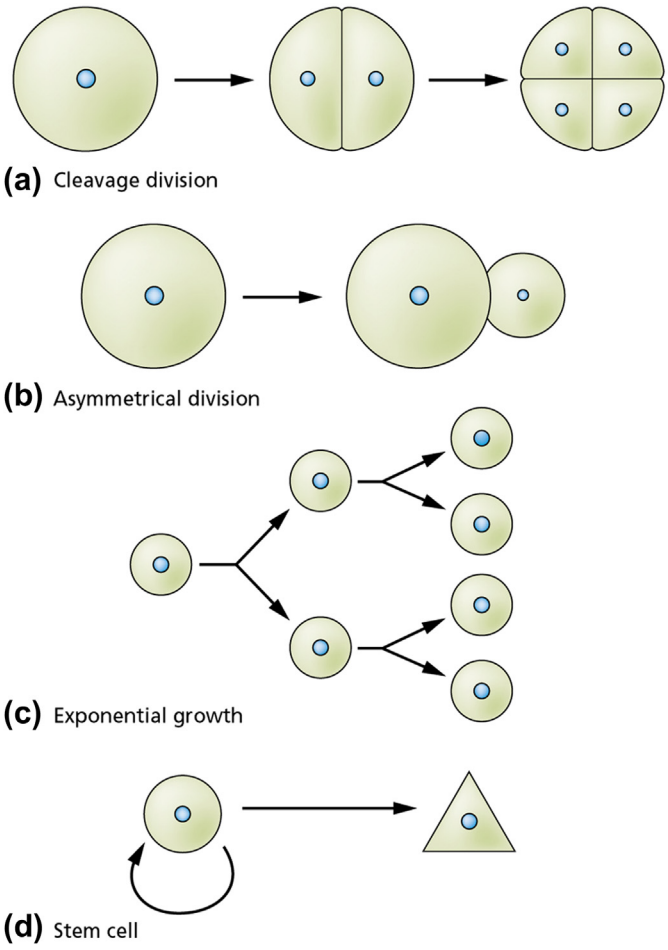


FIGURE 7.5
Types of cell division. a) Cleavage as found in early embryos. b) Asymmetrical division also found in some early embryos. c) Exponential growth found in tissue culture. d) Stem cell division found in renewal tissues in animals. *(From Slack 2012).*

is continuous around the cycle and so is the synthesis of most of the cell's proteins. Normally the cell cycle is coordinated with the growth of mass. If it were not, cells would increase or decrease in size with each division. There are various internal controls built into the cycle, for example to ensure that mitosis does not start before DNA replication is completed. These controls operate at checkpoints around the cycle at which the process stops unless the appropriate conditions are fulfilled.

Control of the cell cycle depends on a metabolic oscillator comprising a number of proteins called cyclins and a number of cyclin-dependent protein kinases (Cdks). In order to pass the M checkpoint and enter mitosis, a complex of cyclin and Cdk (called M-phase promoting factor, MPF) has to be activated. This phosphorylates and thereby activates the various components required for mitosis (nuclear breakdown, spindle formation, chromosome condensation). Exit from M phase requires the inactivation of MPF, via the destruction of cyclin. Passage of the G1 checkpoint depends on a similar process operated by a different set of cyclins and Cdks, whose active complexes phosphorylate and activate the enzymes of DNA replication. This is also the point at which the cell size is assessed.

The cell cycle of G1, S, G2 and M phases is universal although there are some modifications in special circumstances. The rapid cleavage cycles of early development have short or absent G1 and G2 phases and there is no size check, the cells halving in volume with each division. The meiotic cycles require the same active MPF complex to get through the two nuclear divisions, but there is no S phase in between.

In the mature organism most cells are quiescent unless they are stimulated by growth factors. In the absence of growth factors cells enter a state called G0, in which the Cdks and cyclins are absent. Restitution of growth factors induces the resynthesis of these proteins and the resumption of the cycle starting from the G1 checkpoint. One factor maintaining the G0 state is a protein called Rb (retinoblastoma protein). This becomes phosphorylated, and hence deactivated, in the presence of growth factors. In the absence of Rb, a transcription factor called E2F becomes active and initiates a cascade of gene expression culminating in the resynthesis of cyclins, Cdks and other components needed to initiate S phase.

Cells often have the capability for exponential growth in tissue culture (Fig. 7.5c) but this is very rarely found in animals. Although some differentiated cell types can go on dividing, there is a general tendency for differentiation to be accompanied by a slow down or cessation of division. In postembryonic life most cell division is found among stem cells and their immediate progeny called transit amplifying cells. Stem cells are cells that can both reproduce themselves and generate differentiated progeny for their particular tissue type (Fig. 7.5d). This does not necessarily mean that every division of a stem cell has to be an asymmetrical one, but over a period of time half the progeny will go to renewal and half to differentiation. The term 'stem cell' is also used for embryonic stem cells (ES cells) of early mammalian embryos. These are early embryo-type cells that can be grown in culture and are capable of repopulating embryos and contributing to all tissue types.

Asymmetric cell divisions necessarily involve the segregation of different cytoplasmic determinants to the two daughter cells, evoking different patterns of gene activity in their nuclei and thus bringing about different pathways of development. The asymmetry often arises from auto-segregation of a self-organizing protein complex called the PAR (partitioning defective) complex.

CYTOSKELETON

The cytoskeleton is important for three distinct reasons. Firstly, the orientation of cell division may be important. Secondly, animal cells move around a lot, either as individuals or as part of moving cell sheets. Thirdly, the shape of cells is an essential part of their ability to carry out their functions. All of these activities are functions of the cytoskeleton.

The main components of the cytoskeleton are microfilaments, microtubules and intermediate filaments. Microtubules and microfilaments are universal constituents of eukaryotic cells, while intermediate filaments are found only in animals. Microfilaments are made of actin, microtubule of tubulin, and intermediate filaments are composed of different proteins in different cell types: cytokeratins in epithelial cells, vimentin in mesenchymal cells, neurofilament proteins in neurons, and glial fibrilliary acidic protein (GFAP) in glial cells.

Microtubules

Microtubules (Fig. 7.6) are hollow tubes of 25 nm diameter composed of tubulin. Tubulin is a generic name for a family of globular proteins which exist in solution as heterodimers of α- and β-type subunits and is one of the more abundant cytoplasmic proteins. The microtubules are polarized structures with a minus end anchored to the centrosome and a free plus end at which tubulin monomers are added or removed. Microtubules are not contractile but exert their effects through length changes based on polymerization and depolymerization. They are very dynamic, either growing by addition of tubulin monomers, or retracting by loss of monomers, and individual tubules can grow and shrink over a few minutes. The monomers contain GTP bound to the β subunit and in a growing plus end this stabilizes the tubule. But if the rate of growth slows down, hydrolysis of GTP to GDP will catch up with the addition of monomers. The conversion of bound GTP to GDP renders the plus end of the tubule unstable and it will then start to depolymerize. The drugs colchicine and colcemid bind to monomeric tubulin and prevent polymerization and among other effects this causes the disassembly of the mitotic spindle. Because these drugs cause cells to become arrested in mitosis they are often used in studies of cell division kinetics.

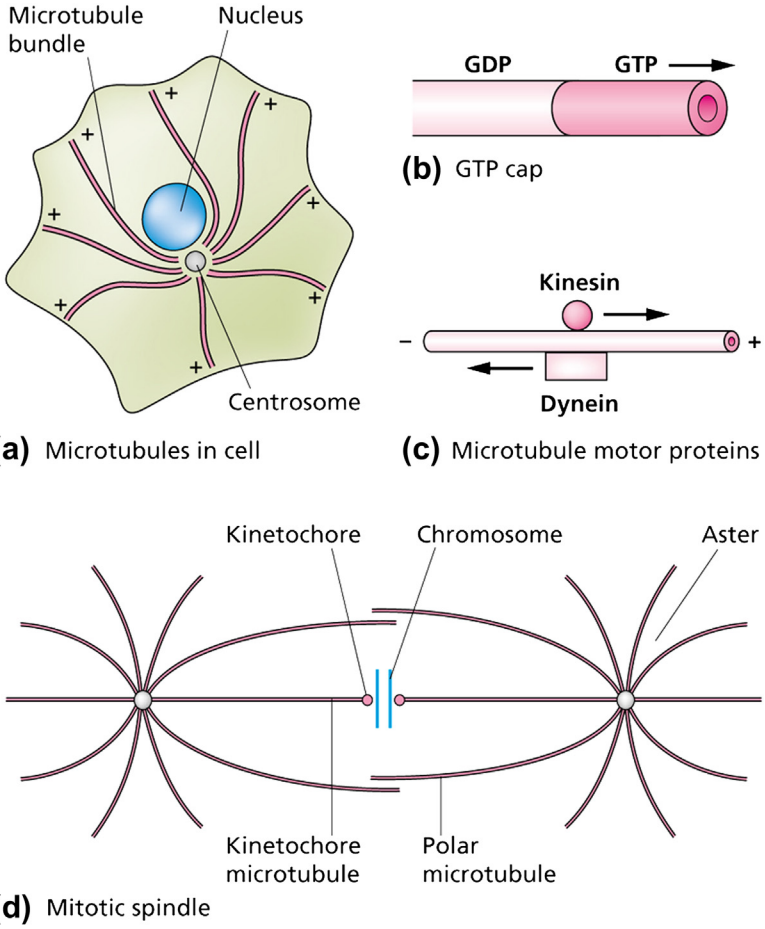


FIGURE 7.6
Microtubules. a) Arrangement in cell. b) The GTP cap. c) Motor proteins move along the tubules. d) Structure of the cell division spindle. *(From Slack 2012).*

The shape and polarity of cells can be controlled by locating capping proteins to particular parts of the cell cortex which bind the free plus ends of the microtubules and stabilize them. The positioning of structures within the cell also depends largely on microtubules. There exist special motor proteins that can move along the tubules, powered by hydrolysis of ATP, and thereby transport other molecules to particular locations within the cell. The kinesins move towards the plus ends of the tubules while the dyneins move towards the minus ends.

Microtubules are prominent during cell division. The minus ends of the tubules originate in the centrosome, which is a microtubule-organizing center able to initiate the assembly of new tubules. In mitotic prophase the centrosome divides and each of the radiating sets of microtubules becomes known as an aster. The two asters move to the opposite sides of the nucleus to become the two poles of the mitotic spindle. The spindle contains two types of microtubules. The polar microtubules meet each other near the center and become linked by plus-directed motor proteins. These tend to drive the poles apart. Each chromosome has a special site called a kinetochore which binds another group of microtubules called kinetochore microtubules. At anaphase the kinetochores of homologous chromosomes separate. The polar microtubules continue to elongate while the kinetochore microtubules shorten by loss of tubulin from both ends and draw the chromosome sets into the opposite poles of the spindle.

Microfilaments

Microfilaments (Fig. 7.6) are polymers of actin, which is the most abundant protein in most animal cells. In vertebrates there are several different gene products of which α actin is found in muscle and β/γ actins in the cytoskeleton of non-muscle cells. For all actin types the monomeric soluble form is called G-actin. Actin filaments have an inert minus end, and a growing plus end to which new monomers are added. G-actin contains ATP and this becomes hydrolyzed to adenosine diphosphate (ADP) shortly after addition to the filament. As with tubules, a rapidly growing filament will bear an ATP cap which stabilizes the plus end. Microfilaments are often found to undergo 'treadmilling' such that monomers are continuously added to the plus end and removed from the minus end while leaving the filament at the same overall length. Microfilament polymerization is prevented by a group of drugs called cytochalasins, and existing filaments are stabilized by another group called phalloidins. Like microtubules, microfilaments have associated motor proteins that will actively migrate along the fiber. The most abundant of these is myosin II, which moves toward the plus end of microfilaments, the process being driven by the hydrolysis of ATP. To bring about contraction of a filament bundle, the myosin is assembled as short bipolar filaments with motile centers at both ends. If neighboring actin filaments are arranged with opposite orientation then the motor activity of the myosin will draw the filaments past each other leading to a contraction of the filament bundle.

Microfilaments can be arranged in various different ways depending on the nature of the accessory proteins with which they are associated. Contractile assemblies contain microfilaments in antiparallel orientation associated with myosin. These are found in the contractile ring which is responsible for cell division, and in the stress fibers by which fibroblasts exert traction on their substratum. Parallel bundles are found in filopodia and other projections from the cell. Gels composed of short randomly orientated filaments are found in the cortical region of the cell.

Small GTPases

There are three well known GTPases that activate cell movement in response to extracellular signals: Rho, Rac and Cdc42. They are activated by numerous tyrosine kinase-, G-coupled-, and cytokine type receptors. Activation involves exchange of GDP for GTP and many downstream proteins can interact with the activated forms. Rho normally activates the

assembly of stress fibers. Rac activates the formation of lamellipodia and ruffles. Cdc42 activates formation of filopodia. In addition all three promote the formation of focal adhesions, which are integrin-containing junctions to the extracellular matrix. These proteins can also affect gene activity through the kinase cascade signal transduction pathways.

CELL ADHESION MOLECULES

Organisms are not just bags of cells, rather each tissue has a definite cellular composition and microarchitecture. This is determined partly by the cell surface molecules by which cells interact with each other, and partly by the components of the extracellular matrix (ECM). Virtually all proteins on the cell surface or in the ECM are glycoproteins, containing oligosaccharide groups added before secretion from the cell. These carbohydrate groups often have rather little effect on the biological activity of the protein but they may affect its physical properties and stability.

Cells are attached to each other by adhesion molecules (Fig. 7.7). Among these are the cadherins, which stick cells together in the presence of Ca, the cell adhesion molecules (CAMs), that do not require Ca, and the integrins that attach cells to the extracellular matrix. When cells come together they often form gap junctions at the region of contact. These consist of small pores joining the cytosol of the two cells. The pores, or connexons, are assembled from proteins called connexions and can pass molecules up to about 1000 molecular weight by passive diffusion.

Cadherins are a family of single-pass transmembrane glycoproteins which can adhere tightly to similar molecules on other cells in the presence of calcium. They are the main factors attaching embryonic cells together, which is why embryonic tissues can often be caused to disaggregate simply by removal of calcium. The cytoplasmic tail of cadherins is anchored to actin bundles in the cytoskeleton by a complex including proteins called catenins. One of these, β-catenin, is also a component of the important Wnt signaling pathway, providing a link between cell signaling and cell association. Cadherins were first named for the tissues in which they were originally found, so E-cadherin occurs mainly in epithelia and N-cadherin occurs mainly in neural tissue.

The immunoglobulin superfamily is made up from single-pass transmembrane glycoproteins with a number of disulfide-bonded loops on the extracellular region, similar to the loops

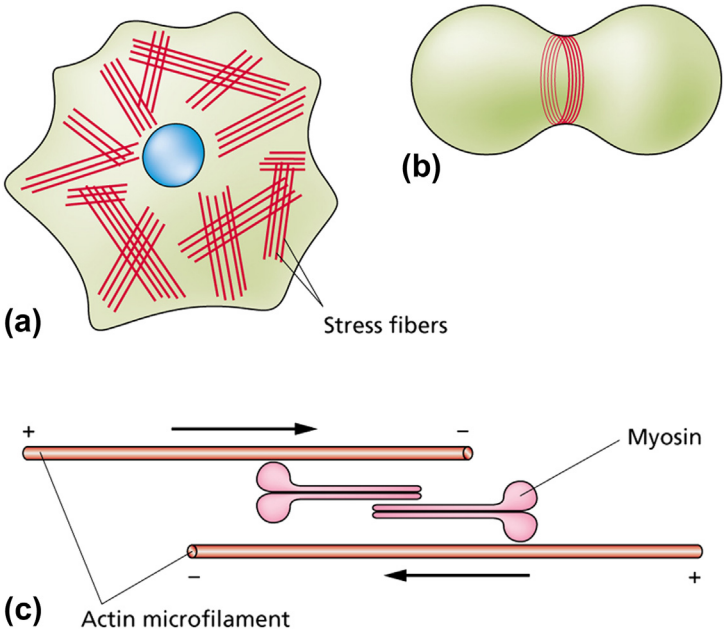


FIGURE 7.7
Microfilaments. a) Arrangement in cell. b) Role in cell division. c) Contraction achieved by movement of myosin along microfilament. *(From Slack 2012).*

found in antibody molecules. They also bind to similar molecules on other cells, but unlike the cadherins they do not need calcium to do so. The neural cell adhesion molecule (N-CAM) is composed of a large family of different proteins formed by alternative splicing. It is most prevalent in the nervous system but also occurs elsewhere. It may carry a large amount of polysialic acid on the extracellular domain, and this can inhibit cell attachment because of the repulsion between the concentrations of negative charge on the two cells.

The integrins are cell surface glycoproteins that interact mainly with components of the extracellular matrix. They are heterodimers of α and β subunits, and require either magnesium or calcium for binding. There are numerous different α and β chain types and so there is a very large number of potential heterodimers. Integrins are attached by their cytoplasmic domains to microfilament bundles, so, like cadherins, they provide a link between the outside world and the cytoskeleton. They are also thought on occasion to be responsible for the activation of signal transduction pathways and new gene transcription following exposure to particular extracellular matrix components.

EXTRACELLULAR MATRIX

Glycosaminoglycans (GAGs) are unbranched polysaccharides composed of repeating disaccharides of an amino sugar and a uronic acid, usually substituted with some sulfate groups. GAGs are constituents of proteoglycans, which have a protein core to which the GAG chains are added in the Golgi apparatus before secretion. One molecule of a proteoglycan may carry more than one type of GAG chain. GAGs have a high negative charge and a small amount can immobilize a large amount of water into a gel. Important GAGs, each of which have different component disaccharides, are heparan sulfate, chondroitin sulfate and keratan sulfate. Heparan sulfate, closely related to the anti-coagulant heparin, is particularly important for cell signaling as it is required to present various growth factors, such as the fibroblast growth factors (FGFs), to their receptors. Hyaluronic acid differs from other GAGs because it occurs free, and not as a constituent of a proteoglycan. It consists of repeating disaccharides of glucuronic acid and N-acetyl glucosamine, and is not sulfated. It is synthesized by enzymes at the cell surface and is abundant in early embryos.

Collagens are the most abundant proteins by weight in most animals. The polypeptides, called α chains, are rich in proline and glycine. Before secretion, three α chains become twisted around each other to form a stiff triple helical structure. In the extracellular matrix, the triple helices become aggregated together to form the collagen fibrils often visible in the electron microscope. There are many types of collagen, which may be composed of similar or of different α chains in the triple helix. Type I collagen is the most abundant and is a major constituent of most extracellular material. Type II collagen is found in cartilage and in the notochord of vertebrate embryos. Type IV collagen is a major constituent of the basal lamina underlying epithelial tissues. Collagen helices may become covalently crosslinked through their lysine residues, and this contributes to the changing mechanical properties of tissues with age.

Elastin is another extracellular protein with extensive intermolecular cross-linking. It confers the elasticity on tissues in which it is abundant, and also has some cell signaling functions. Fibronectin is composed of a large disulfide-bonded dimer. The polypeptides contain regions responsible for binding to collagen, to heparan sulfate, and to integrins on the cell surface. These latter, cell-binding domains are characterized by the presence of the amino acid sequence Arg-Gly-Asp (= RGD). There are many different forms of fibronectin produced by alternative splicing. Laminin is a large extracellular glycoprotein, found particularly in basal laminae. It is composed of three disulfide-bonded polypeptides joined in a cross shape. It carries domains for binding to type IV collagen, heparan sulfate and another matrix glycoprotein, entactin.

CULTURE MEDIA

Mammalian cells will only remain in good condition very close to the normal body temperature, so good temperature control is essential. Because water can pass across the plasma membranes of animal cells, the medium must match the osmolarity of the cell interior, otherwise cells will swell or shrink due to osmotic pressure difference. Mammalian cells media generally have a total osmolarity about 350 mOsm. The pH needs to be tightly controlled, 7.4 being normal. The pH control is usually achieved with bicarbonate-CO_2 buffers (2.2 gm/l bicarbonate and 5% CO_2 being a common combination). These give better results with most animal cells than other buffers, perhaps because bicarbonate is also a type of nutrient. The medium must contain a variety of components: salts, amino acids, sugars, plus low levels of specific hormones and growth factors required for the particular cell type in question. Because of the complexity of tissue culture media they are rarely optimized for a given purpose by varying every one of the components. Usually changes are incremental and the result of a 'gardening' type of approach rather than a systematic one.

The requirement for hormones and growth factors is usually met by including some animal serum, often 10% fetal calf serum. This is long standing practice but has two substantial disadvantages. Serum can never be completely characterized and there are often differences between batches of serum that can be critical for experimental results. Also there is currently a drive to remove serum from the preparation of cells that are intended for implantation into human patients. This is because of the perceived possibility, actually very remote, of transmitting animal diseases to patients.

Assuming cells are kept in a near optimal medium, they can in principle grow exponentially, with a constant doubling time. Indeed it is possible to grow many types of tissue culture cell in exponential cultures rather like microorganisms. In order to keep them growing at maximal rate they need to have their medium renewed regularly and to be subcultured and replated at lower density whenever they approach confluence, that is approach a density at which they cover all the available surface. Subculturing is usually carried out by treatment with the enzyme trypsin, which degrades much of the extracellular and cell surface protein and makes the cells drop off the substrate and become roughly spherical bodies in suspension. The trypsin is diluted out and the cells are transferred at lower density into new flasks. The cells take an hour or two to resynthesize their surface molecules and can then adhere to the new substrate and carry on growing.

CELLS IN TISSUES AND ORGANS

Cell types

On the basis of light microscopy it is estimated that there are about 210 different types of differentiated cell in the mammalian body. This number is certainly an underestimate since there are many subtypes of cells that cannot be seen via the light microscope, particularly the different types of neuron in the nervous system and different types of lymphocyte in the immune system. Cells types are different from one another because they are expressing different subsets of genes and hence contain different proteins. The products of a relatively small number of genes may dominate the appearance of a differentiated cell, for example the proteins of the contractile apparatus of skeletal muscle are very abundant. However a typical cell will express many thousands of genes, and its character will also depend on the genes that are not expressed. It is possible to control cell differentiation to some extent. There are special culture media that are favorable for differentiation of particular cell types, such as adipocytes, muscle or bone. In some cases differentiated cells can continue to grow in pure culture. But in many cases differentiation causes slowing or cessation of cell division.

From a morphological point of view most cells can be regarded as epithelial or mesenchymal (Fig. 7.8). These terms relate to cell shape and behavior rather than to embryonic origin. An

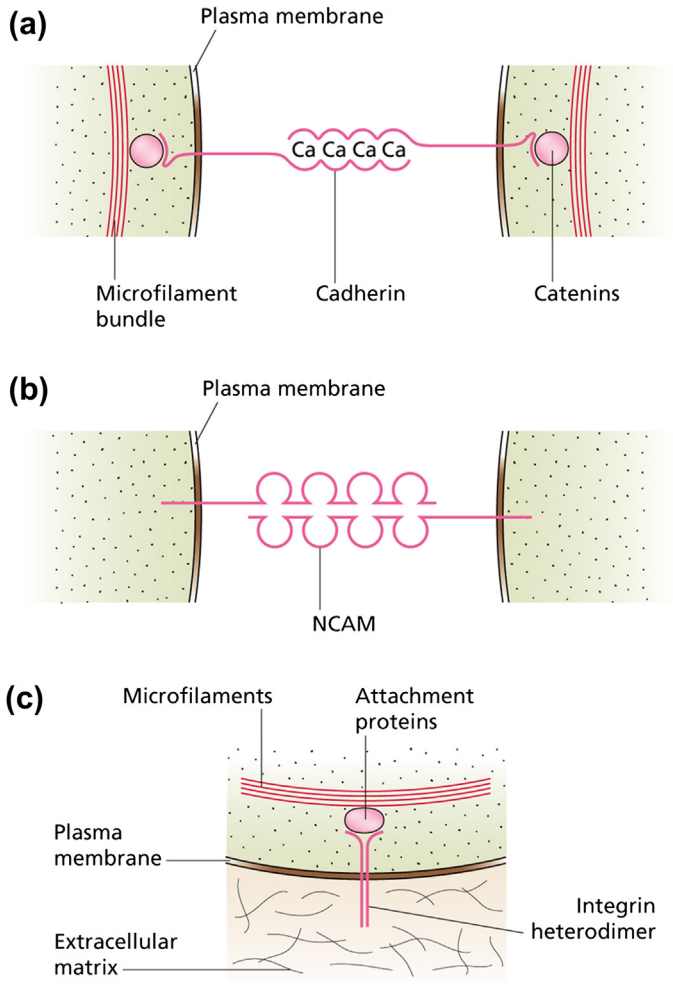


FIGURE 7.8
Cell adhesion molecules. a) Calcium dependent system. b) Calcium independent system. c) Adhesion to the extracellular matrix. *(From Slack 2012).*

epithelium is a sheet of cells, arranged on a basement membrane, each cell joined to its neighbors by specialized junctions, and showing a distinct apical-basal polarity. Mesenchyme is a descriptive term for scattered stellate-shaped cells embedded in loose extracellular matrix. It fills up much of the space within the embryo and later forms fibroblasts, adipose tissue, smooth muscle and skeletal tissues.

Tissues

From a developmental biology standpoint, a tissue is the set of cell types arising from one sort of progenitor or stem cell. For example, the intestinal epithelium is a tissue. It is structured into crypts, containing undifferentiated cells, and villi covered with differentiated cells. There are four mature cell types: absorptive, goblet, Paneth and enteroendocrine cells. The stem cells are located at the crypt base and produce progenitor cells, called transit amplifying cells, which divide a few times before differentiating into one of the four mature cell types. The tissue comprises the stem cells, the transit amplifying cells, and the differentiated cells. This is an example of a renewal tissue, in which there is continuous production and replacement of differentiated cells. Not all tissues are renewal tissues although most display a small degree of renewal or the ability to replace the constituent cell types following damage. For example the central nervous system arises from the neuroepithelium of the early embryo and contains a variety of types of neuron together with glial cells, especially astrocytes and oligodendrocytes. There is only replacement of neurons in two regions of the brain and turnover of glial

populations is slow. But because it arises from a clearly identified progenitor cell population, the central nervous system can be regarded as one tissue.

In cases where differentiated cells are derived from different stem or progenitor cell populations, they cannot be considered as a single tissue. For example, the thyroid gland contains many follicles of thyroglobulin-producing epithelial cells. It also contains endocrine cells producing the hormone calcitonin. However, the latter do not arise from the same endodermal bud as the rest of the thyroid. Instead they arise from the neural crest of the embryo. Likewise, many tissues contain macrophages (histiocytes) derived from the hematopoietic stem cells of the bone marrow. These cells are properly considered to belong to the blood/immune tissue rather than the epithelia or connective tissues in which they reside.

Organs

Organs are the familiar structures in the body which are each associated with a particular function. They are typically composed of several tissue layers and arise from multiple developmental origins. For example, the stomach has the function of preliminary food digestion. The lining of the stomach is one tissue, the gastric epithelium, derived from gastric stem cells. The outer layers consist of smooth muscle and connective tissues, blood vessels, nerve fibers, and cells of the blood and immune system. Together these make up a discrete and integrated body part. A single named muscle contains many muscle fibers, which are postmitotic multinucleate cells. It also contains muscle satellite cells, which are stem cells that can regenerate fibers. The fibers and the satellite cells are one tissue, arising from the myotome of the somites of the embryo. Surrounding the bundles of fibers are connective tissue sheaths composed of fibroblasts and extracellular matrix derived from lateral plate mesoderm. Again, there are always blood vessels, nerve fibers and cells of the blood and immune system. A muscle is one organ, composed of several tissues, each consisting of more than one cell type (Fig. 7.9).

Organs often have an epithelial and mesenchymal component. The epithelium is usually the functional part, for example the epithelial lining of the various segments of the gut have specific properties of protection, absorption or secretion, while the underlying mesenchyme provides mechanical support, growth factors, and physiological response in terms of muscular movements.

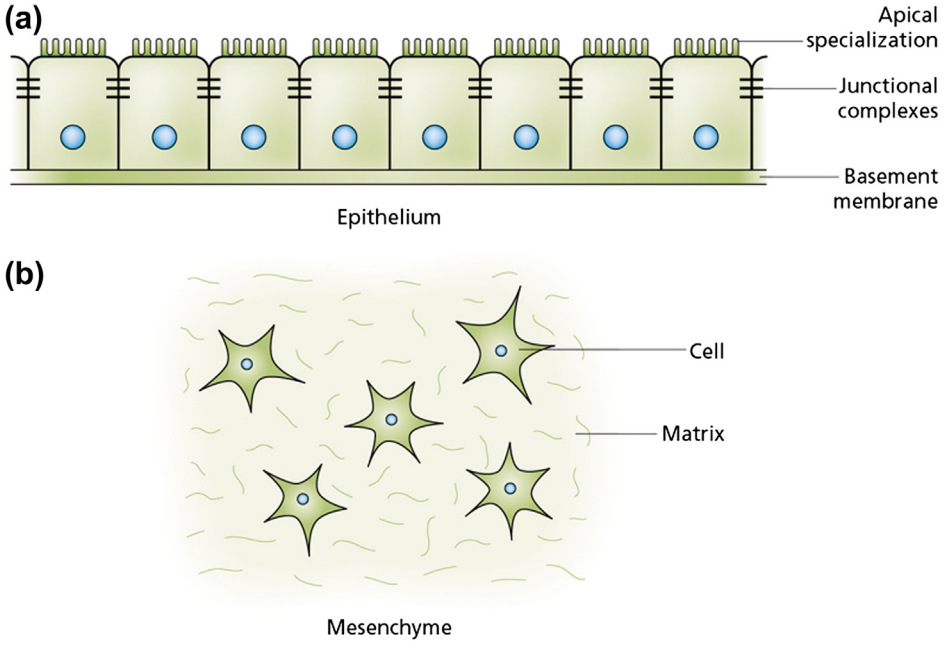


FIGURE 7.9
Most tissues are composed of epithelial (a) and mesenchymal (b) components. *From Slack (2012).*

With the exception of the kidney, all other organs draw their epithelium and mesenchyme from different germ layers of the embryo. The implication of this for tissue engineering is that it will probably be necessary to assemble structures from separate epithelial and mesenchymal cells, designed such that each population can support the other by secretion of appropriate factors.

The final consideration is that cells need a continuous supply of nutrients and oxygen and continuous removal of waste products. *In vivo* this is achieved by means of the blood vascular system which culminates in capillary beds of enormous density such that all cells are within a few cell diameters of the blood. For tissue engineering the lesson is clear: it is possible to grow large avascular structures only so long as they are two dimensional: for example large sheets of epidermis a few cells thick can be grown *in vitro* and used successfully for skin grafting. But any tissue more than a fraction of a millimeter thickness will need to be provided with some sort of vascular system.

Tissue engineering need not attempt to copy everything that is found in the normal body. However it is necessary to be aware of the constraints provided by the molecular biology of the cell. Factors to be considered include:

- How to keep cells in the desired state by providing the correct substrate and medium.
- How to create engineered structures containing two or more cell types of different origins which can sustain one another.
- How to provide a vascular system capable of delivering nutrients and removing waste products.
- How to control cell division to achieve homeostasis rather than uncontrolled growth or regression.

Further Reading

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CHAPTER 8

Molecular Organization of Cells

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INTRODUCTION

Multicellular tissues exist in one of two types of cellular arrangements; epithelial or mesenchymal. Epithelial cells adhere tightly to each other at their lateral surfaces and to an organized ECM (extracellular matrix) at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface. Mesenchymal cells, in contrast, are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional ECM (see Fig. 8.1). The conversion of epithelial cells into mesenchymal cells, an 'epithelial-mesenchymal transition' (EMT), is central to many aspects of embryonic morphogenesis, adult tissue repair, as well as a number of disease states [1,2]. The reverse process, whereby mesenchymal cells coalesce into an epithelium is a 'mesenchymal-epithelial transition' (MET). Understanding the molecules that regulate this transition between epithelial and mesenchymal states offers important insights into how cells and tissues are organized.

The early embryo is structured as one or more epithelia. An EMT allows the rearrangements of cells to create additional morphological features. Well-studied examples of EMTs during embryonic development include gastrulation in *Drosophila* [3], the emigration of primary mesenchyme cells (PMCs) in sea urchin embryos [4], and gastrulation in amniotes (reptiles, birds, and mammals) at the primitive streak [5]. EMTs also occur later in vertebrate development, such as the emigration of neural crest cells from the neural tube, the formation of the sclerotome from epithelial somites, and during palate fusion [1]. The reverse process of MET is likewise crucial to development, and examples include the condensation of mesenchymal cells to form the notochord and somites, kidney tubule formation from nephrogenic mesenchyme, and the creation of heart valves from cardiac mesenchyme [1]. In the adult organism, EMTs and METs occur during wound healing and tissue remodeling. The conversion of neoplastic epithelial cells into invasive cancer cells has long been considered an EMT process [1]. However, there are also examples of tumor cells that have functional cell-cell adhesion junctions, yet are still migratory and invasive as a group. This 'collective migration' also occurs during development [2]. Hence, there is debate about whether an EMT model accurately describes all epithelial metastatic cancers. Similarly, the fibrosis of cardiac, kidney, lens, and liver epithelial tissue has also long been categorized as an EMT event [1]. However, recent research into the kidney *in vivo* shows that the myofibroblasts induced following kidney injury are derived from mesenchymal pericytes, rather than the proximal epithelial cells [6]. Therefore, the origin of the cells that contribute to fibrotic tissue scarring (epithelial or otherwise) may need to be carefully re-examined.

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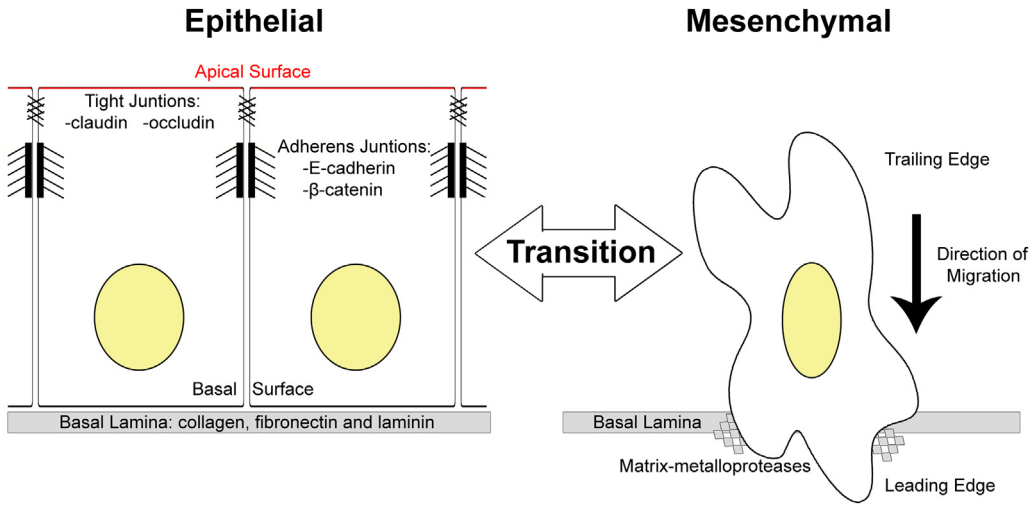

FIGURE 8.1
Epithelial vs. Mesenchymal. Epithelial cells adhere together by tight junctions and adherens junctions localized near the apical surface. Epithelial cells also have a basal surface that rests on a basal lamina (ECM). Mesenchymal cells in contrast do not have well-defined cell-cell adhesion complexes, have front-end/back-end polarity instead of apical/basal polarity, and mesenchymal cells are characterized by their ability to invade the basal lamina.

The focus of this chapter is on the molecules that regulate the organization of cells into epithelium or mesenchyme. We will first discuss the cellular changes that occur during an EMT, including changes in cell-cell and cell-ECM adhesions, changes in cell polarity, and the stimulation of invasive cell motility. Then we will consider the molecules and mechanisms that control the EMT or MET, including the structural molecules, transcription factors and signaling pathways that regulate EMTs.

MOLECULES THAT ORGANIZE CELLS

The conversion of an epithelial sheet into individual migratory cells and back again requires the coordinated changes of many distinct families of molecules.

Changes in cell-cell adhesion

Epithelial cells are held together by specialized cell-cell junctions, including adherens junctions, desmosomes, and tight junctions. These junctions are localized in the lateral domain near the apical surface and establish the apical polarity of the epithelium. In order for an epithelial sheet to produce individual mesenchymal cells, cell-cell adhesions must be disrupted. The principle transmembrane proteins that mediate cell-cell adhesions are members of the cadherin superfamily. E-cadherin and N-cadherin are classical cadherins that interact homotypically through their extracellular IgG domains with like-cadherins on adjacent cells. Cadherins are important mediators of cell-cell adhesion. For example, misexpression of E-cadherin is sufficient to promote cell-cell adhesion and assembly of adherens junctions in fibroblasts [5]. In epithelial cancers (carcinomas), E-cadherin acts as a tumor suppressor [7]. In a mouse model for β-cell pancreatic cancer, the loss of E-cadherin is the rate-limiting step for transformed epithelial cells to become invasive [8]. Although the loss of cadherin-mediated cell-cell adhesion is necessary for an EMT, the loss of cadherins is not always sufficient to generate a complete EMT *in vivo*. For example, the neural tube epithelium in mice expresses N-cadherin, but in the N-cadherin knockout mouse an EMT is not induced in the neural tube [9]. Hence, cadherins are essential for maintaining epithelial integrity, and the loss of cell-cell adhesion due to the reduction of cadherin function is an important step for an EMT.

One characteristic of an EMT is 'cadherin switching'. Often, epithelia that express E-cadherin will downregulate E-cadherin expression at the time of the EMT, and express different cadherins such as N-cadherin [2]. Cadherin switching may promote motility. For instance, in mammary epithelial cell lines, the misexpression of N-cadherin is sufficient for increased cell motility, and blocking N-cadherin expression results in less motility. However, the misexpression of N-cadherin does not result in the complete loss of epithelial morphology [10]. Hence, cadherin switching may be necessary for cell motility, but cadherin switching alone is not sufficient to bring about a complete EMT.

There are several ways that cadherin expression and function are regulated. Transcription factors that are central to most EMTs such as Snail-1, Snail-2, Zeb1, Zeb2, Twist, and E2A, all bind to E-boxes on the *E-cadherin* promoter and repress the transcription of *E-cadherin* [11]. Post-transcriptionally, the E-cadherin protein is ubiquitinated by the E3-ligase, Hakai, which targets E-cadherin to the proteasome [7]. E-cadherin turnover at the membrane is regulated by either caveolae-dependent endocytosis or clathrin-dependent endocytosis [12], and p120-catenin prevents endocytosis of E-cadherin at the membrane [13]. E-cadherin function can also be disrupted by matrix metalloproteases, which degrade the extracellular domain of E-cadherin [14]. Some or all of these mechanisms may occur during an EMT to disrupt cell-cell adhesion.

In summary, cell-cell adhesion is maintained principally by cadherins, and changes in cadherin expression are typical of an EMT.

Changes in cell-ECM adhesion

Altering the way that a cell interacts with the ECM is also important in EMTs. For example, at the time that sea urchin PMCs ingress, the cells have increased adhesiveness for ECM [4]. Cell-ECM adhesion is mediated principally by integrins. Integrins are transmembrane proteins composed of two non-covalently linked subunits, α and β, that bind to ECM components such as fibronectin, laminin, and collagen. The cytoplasmic domain of integrins links to the cytoskeleton and interacts with signaling molecules. Changes in integrin function are required for many EMTs, including neural crest emigration [15], mouse primitive streak formation [5], and cancer metastasis [16]. However, the misexpression of integrin subunits is not sufficient to bring about a full EMT *in vitro* [17] or *in vivo* [18].

The presence and function of integrins is modulated in several ways. For example, the promoter of the *integrin β6* gene is activated by the transcription factor Ets-1 during colon carcinoma metastasis [19]. Most integrins can also cycle between 'On' (high affinity) or 'Off' (low affinity) states. This 'inside-out' regulation of integrin adhesion occurs at the integrin cytoplasmic tail. In addition to integrin activation, the 'clustering' of integrins on the cell surface also affects the overall strength of integrin-ECM interactions. The increased adhesiveness of integrins due to clustering, known as avidity, can be activated by chemokines, and is dependent on RhoA and phosphatidylinositol 3' kinase (PI3K) activity [20].

In summary, changes in ECM adhesion are required for an EMT. Cell-ECM adhesions are maintained by integrins, and integrins have varying degrees of adhesiveness dependent upon the presence, activity, or avidity of the integrin subunits.

Changes in cell polarity and stimulation of cell motility

Cellular polarity is defined by the distinct arrangement of cytoskeletal elements and organelles in epithelial verses mesenchymal cells. Epithelial polarity is characterized by cell-cell junctions found near the apical-lateral domain (non-adhesive surface), and a basal lamina opposite of the apical surface (adhesive surface). Mesenchymal cells in contrast do not have apical/basal polarity, but rather front-end/back-end polarity, with actin-rich lamellipodia and Golgi localized at the leading edge [5]. Molecules that establish cell polarity include Cdc42, PAK1,

PI3K, PTEN, Rac, Rho, and the PAR proteins [21]. Changes in cell polarity help to promote an EMT. In mammary epithelial cells, the activated TGF-β receptor II causes Par6 to activate the E3 ubiquitin ligase Smurf1, and Smurf1 then targets RhoA to the proteasome. The reduction of RhoA activity results in the loss of cell-cell adhesion and epithelial cell polarity [22].

In order for mesenchymal cells to leave the epithelium, they must become motile. Many of the same polarity (Crumbs, PAR, and Scribble complexes), structural (actin, microtubules), and regulatory molecules (Cdc42, Rac1, RhoA) that govern epithelial polarity are also central to cell motility [23]. Cell motility mechanisms also vary depending on whether the environment is 2D or 3D [24]. Many mesenchymal cells express the intermediate filament vimentin, and vimentin may be responsible for several aspects of the EMT phenotype [25].

In short, a wide variety of structural, polarity, and regulatory molecules must be re-assigned as cells transition between epithelial polarity and mesenchymal migration.

Invasion of the basal lamina

In most EMTs the emerging mesenchymal cells must penetrate a basal lamina which consists of ECM components such as collagen type IV, fibronectin and laminin. The basal lamina functions to stabilize the epithelium and is a barrier to migratory cells [26]. One mechanism that mesenchymal cells use to breach the basal lamina is to produce enzymes that degrade it. Plasminogen activator is one protease associated with a number of EMTs, including neural crest emigration [26] and the formation of cardiac cushion cells during heart morphogenesis [27]. The type II serine protease, TMPRSS4, also promotes an EMT and metastasis when overexpressed *in vitro* and *in vivo* [1]. Matrix metalloproteases (MMPs) are also important for many EMTs. When MMP-2 activity is blocked in the neural crest EMT, neural crest emigration is inhibited, but not neural crest motility [28]. In mouse mammary cells, MMP-3 overexpression is sufficient to induce an EMT *in vitro* and *in vivo* [29]. Misexpressing MMP-3 in cultured cells induces an alternatively spliced form of Rac1 (Rac1b), which then causes an increase in reactive oxygen species (ROS) intracellularly, and Snail-1 expression. Either Rac1b activity or ROS are necessary and sufficient for an MMP-3-induced EMT [30]. Hence, a number of extracellular proteases are important to bring about an EMT.

While epithelial cells undergoing an EMT do eventually lose cell-cell adhesion, apical-basal polarity, and gain invasive motility, the EMT program is not necessarily ordered or linear. For example, in a study where neural crest cells were labeled with cell adhesion or polarity markers and individual live cells were observed undergoing the EMT in slice culture, neural crest cells changed epithelial polarity either before or after the complete loss of cell-cell adhesion, or lost cell-cell adhesions either before or after cell migration commenced [31]. Therefore, while an EMT does consist of several distinct phases, these steps may occur in different orders or combinations, some of which (e.g., the complete loss of cell-cell adhesion) may not always be necessary.

In summary, changes in a wide range of molecules are needed for an EMT as epithelial cells lose cell-cell adhesion, change cellular polarity, and gain invasive cell motility.

THE EMT TRANSCRIPTIONAL PROGRAM

At the foundation of every EMT or MET program are the transcription factors that regulate the gene expression required for these cellular transitions. While many of the transcription factors that regulate EMTs have been identified, the complex regulatory networks are still incomplete. Here we review the transcription factors that are known to promote the various phases of an EMT. Then we examine how these EMT transcription factors themselves are regulated at the promoter and post-transcriptional levels.

Transcription factors that regulate EMTs

The Snail family of zinc finger transcription factors, including Snail-1 and Snail-2 (formerly Snail and Slug) are direct regulators of cell-cell adhesion and motility during EMTs [11,32]. The knockout of *Snail-1* in mice is lethal early in gestation, and the presumptive primitive streak cells that normally undergo an EMT still retain apical/basal polarity, adherens junctions, and express E-cadherin mRNA [33]. Snail-1 misexpression is sufficient for breast cancer recurrence in a mouse model *in vivo*, and high levels of *Snail-1* predict the relapse of human breast cancer [1]. Snail-2 is necessary for the chicken primitive streak and neural crest EMTs [34]. One way that Snail-1 or Snail-2 causes a decrease in cell-cell adhesion is by repressing the *E-cadherin* promoter [11]. This repression requires the mSin3A co-repressor complex, histone deacetylases, and components of the Polycomb 2 complex [2]. Snail-1 is also a transcriptional repressor of the tight junction genes *Claudin* and *Occludin* [11] and the polarity gene *Crumbs3* [1]. The misexpression of Snail-1 and Snail-2 further leads to the transcription of proteins important for cell motility such as fibronectin, vimentin, and RhoB [11]. Further, Snail-1 promotes invasion across the basal lamina. The misexpression of Snail-1 represses laminin (basement membrane) production and indirectly upregulates *mmp-9* transcription [1]. Snail and Twist also make cancer cells more resistant to senescence, chemotherapy, apoptosis, and endow cancer cells with 'stem cell' properties [1]. Hence, Snail-1 or Snail-2 are necessary and sufficient for bringing about many of the steps of an EMT, including loss of cell-cell adhesion, changes in cell polarity, gain of cell motility, invasion of the basal lamina, and increased proliferation and survival.

Other zinc finger transcription factors important for EMTs are zinc finger E-box-binding homeobox 1 (Zeb1, also known as δEF1), and Zeb2 (also known as Smad-interacting protein-1, Sip1). Both Zeb1 and Zeb2 bind to the *E-cadherin* promoter and repress transcription [11]. Zeb1 can also bind to and repress the transcription of the polarity proteins Crumbs3, Pals1-associated tight junction proteins (PATJ), and lethal giant larvae 2 (Lgl2) [1]. Zeb2 is structurally similar to Zeb1, and Zeb2 overexpression is sufficient to downregulate E-cadherin, dissociate adherens junctions, and increase motility in MDCK cells [35].

The Lymphoid Enhancer-binding Factor/ T Cell Factor (LEF/TCF) transcription factors also play an important role in EMTs. For instance, the misexpression of Lef-1 in cultured colon cancer cells reversibly causes the loss of cell-cell adhesion [36]. LEF/TCF transcription factors directly activate genes that regulate cell motility, such as the L1 adhesion molecule [37], and the *fibronectin* gene [38]. LEF/TCF transcription factors also upregulate genes required for basal lamina invasion, including *mmp-3* and *mmp-7* [39].

Other transcription factors that have a role in promoting EMTs are the class I basic helix-loop-helix factors E2-2A and E2-2B [40], the forkhead box transcription factor FOXC2, the homeobox protein Goosecoid, and the homeoprotein Six1[1].

To summarize, transcription factors that regulate an EMT often do so by directly repressing cell adhesion and epithelial polarity molecules, and by upregulating genes required for cell motility and basal lamina invasion.

Regulation at the promoter level

Given the importance of the Snail, Zeb and LEF/TCF transcription factors in orchestrating the various phases of an EMT, it is essential to understand the upstream events that regulate these EMT-promoting transcription factors.

The activation of *Snail-1* transcription in *Drosophila* requires the transcription factors Dorsal (NF-κB) and Twist [11]. The human *Snail-1* promoter also has functional NF-κB sites [41] and blocking NF-κB reduces *Snail-1* transcription [42]. Additionally, a region of the *Snail-1* promoter is responsive to integrin-linked kinase (ILK) [11], and ILK can activate Snail-1

expression via poly-ADP-ribose polymerase (PARP) [43]. In mouse mammary epithelial cells, high mobility group protein A2 (HMGA2) and Smads activate *Snail-1* expression, and subsequently *Snail-2*, *Twist*, and *Id2* transcription [44]. For *Snail-2* expression, myocardin-related transcription factors (MRTFs) interact with Smads to induce *Snail-2* and MRTFs may play a role in metastasis and fibrosis [1]. There are also several *Snail-1* transcriptional repressors. In breast cancer cell lines, metastasis-associated protein 3 (MTA3) binds directly to and represses the transcription of *Snail-1* in combination with the Mi-2/NuRD complex [11], as also does lysine-specific demethylase (LSD1) [2]. The Ajuba LIM proteins (Ajuba, LIMD1 and WTIP) are additional transcriptional corepressors of the Snail family [1].

The transcription of LEF/TCF genes such as *Lef-1* is activated by Smads [5]. The misexpression of Snail-1 results in the transcription of *δEF-1* and *Lef-1* through a yet unknown mechanism [11].

Post-transcriptional regulation of EMT transcription factors

The activity of EMT transcription factors is also regulated post-transcriptionally, where alternative splicing, translational control, protein stability (targeting to the proteasome) and nuclear localization can all regulate an EMT.

One newly discovered layer of EMT regulation is the epithelial or mesenchymal specific expression of alternatively spliced transcripts. For example, epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) are two RNA-binding proteins that regulate the epithelial-specific expression of many molecules that are important to an EMT such as Rho regulators, integrins, and collagen [45]. Blocking ESRP1 and 2 expression changes the pattern of alternatively spliced transcripts and causes cultured epithelial cells to upregulate vimentin and fibronectin, to reduce E-cadherin at cell-cell contacts, and to increase protease activity [45].

Non-coding RNAs are also emerging as important regulators EMTs. In a breast cancer model, Myc activates the expression of microRNA-9 (miR-9), and miR-9 directly binds to and represses the *E-cadherin* promoter [2]. Members of the miR-200 family repress the translation of *Zeb1*, and the expression of these miR-200 family members are repressed by Snail-1. Additionally, *Zeb2* transcription can be activated by naturally occurring RNA antisense transcripts [1]. It is not yet known if there are non-coding RNAs that regulate Snail family members. However, the Y-box binding protein-1 (YB-1) is important for the selective activation of *Snail-1* translation [2].

Protein stability is another layer of EMT control. Snail-1 is phosphorylated by GSK-3β and targeted for destruction [1]. Therefore, the inhibition of GSK-3β activity by Wnt signaling may have multiple roles in an EMT, leading to the stabilization of both β-catenin and Snail-1. Some proteins that prevent GSK-3β-mediated phosphorylation (and thus promote Snail-1 activation) are lysyl-oxidase-like proteins LOXL2[1], and ILK [5]. A Snail-1 specific phosphatase (Snail-1 activator) is C-terminal domain phosphatase (SCP) [1]. Snail-2 is targeted for degradation by the direct action of p53 and the ubiquitin ligase Mdm2 [2].

In addition to protein translation and stability, the function of Snail-1 also depends upon nuclear localization mediated by Snail-1's nuclear localization sequence. The phosphorylation of human Snail-1 by p21-activated kinase 1 (Pak1) promotes the nuclear localization of Snail-1 (and therefore Snail-1 activation) in breast cancer cells [32]. In zebrafish, LIV-1 promotes the translocation of Snail-1 into the nucleus [1]. Snail-1 also contains a nuclear export sequence (NES) that is dependent on the calreticulin (CalR) nuclear export pathway [46]. This NES sequence is activated by the phosphorylation of the same lysine residues targeted by GSK-3β, which suggests a mechanism whereby phosphorylation of Snail-1 by GSK-3β results in the export of Snail-1 from the nucleus and subsequent degradation.

LEF/TCF activity is also regulated by other proteins. β-catenin is required as a co-factor for LEF/TCF-mediated activation of transcription, and Lef-1 can also associate with co-factor Smads to

activate the transcription of additional EMT genes [5]. In colon cancer cells, Thymosin β4 stabilizes ILK activity [1].

In summary, EMT transcription factors such as Snail-1, Zeb1 and Lef-1 are regulated by a variety of mechanisms, both at the transcriptional level, and post-transcriptional level by alternative splicing, non-coding RNA translation control, protein degradation, nuclear localization, and co-factors such as β-catenin.

MOLECULAR CONTROL OF THE EMT

The initiation of an EMT or MET is a tightly regulated event during development and tissue repair because deregulation of cellular organization is disastrous to the organism. A variety of external and internal signaling mechanisms coordinate the complex events of the EMT, and these same signaling pathways are often disrupted or reactivated during disease. EMTs or METs can be induced by either diffusible signaling molecules or ECM components. Below we discuss the role of signaling molecules and ECM in triggering an EMT, and then present a summary model for EMT induction.

Ligand-receptor signaling

During development, five main ligand-receptor signaling pathways are employed, namely TGF-β, Wnt, RTK, Notch, and Hedgehog. These pathways, among others, all have a role in triggering EMTs. While the activation of a single signaling pathway can be sufficient for an EMT, in most cases an EMT or MET is initiated by multiple signaling pathways acting in concert.

TGF-β PATHWAY

The transforming growth factor-beta (TGF-β) superfamily includes TGF-β, activin, and bone morphogenetic protein (BMP) families. These ligands operate through receptor serine/threonine kinases to activate a variety of signaling molecules including Smads, MAPK, PI3K and ILK. Most of the EMTs studied to date are induced in part, or solely, by TGF-β superfamily members [47]. During embryonic heart development, TGF-β2 and TGF-β3 have sequential and necessary roles in activating the endocardium to invade the cardiac jelly and form the endocardial cushions [48]. In the avian neural crest, BMP4 induces *Snail-2* expression [32]. In the EMT that transforms epithelial tissue into metastatic cancer cells, TGF-β acts as a tumor suppressor during early stages of tumor development, but as a tumor/EMT inducer at later stages [7,47]. TGF-β-signaling may combine with other signaling pathways to induce an EMT. For example, in cultured breast cancer cells, activated Ras and TGF-β induce an irreversible EMT [49], and in pig thyroid epithelial cells, TGF-β and epidermal growth factor (EGF) synergistically stimulate the EMT [50].

One outcome of TGF-β signaling is to immediately change epithelial cell polarity. In a TGF-β-induced EMT of mammary epithelial cells, TGF-βR II directly phosphorylates the polarity protein, Par6, leading to the dissolution of tight junctions [22]. TGF-β signaling also regulates gene expression through the phosphorylation and activation of Smads. Smads are important co-factors in the stimulation of an EMT. For example, Smad3 is necessary for a TGF-β-induced EMT in lens and kidney tissue *in vivo* [51]. Smad3/4 also complex with Snail-1 and co-represses the promoters of cell-cell adhesion molecules [1]. Further, TGF-βR I directly binds to and activates PI3K [52], which in turn activates ILK and downstream pathways.

ILK is emerging as an important positive regulator of EMTs [53]. ILK interacts directly with growth factor receptors (TGF-β, Wnt or RTK), integrins, the actin skeleton, PI3K, and focal adhesion complexes. ILK directly phosphorylates Akt and GSK-3β, and results in the subsequent activation of transcription factors such as AP-1, NF-κB, and Lef-1. Overexpression of ILK in cultured cells causes the suppression of GSK-3β activity [54], translocation of

β-catenin to the nucleus, activation of Lef-1/β-catenin transcription factors, and the down-regulation of E-cadherin [55]. Inhibition of ILK in cultured colon cancer cells leads to the stabilization of GSK-3β activity, decreased nuclear β-catenin localization, the suppression of *Lef-1* and *Snail-1* transcription, and reduced invasive behavior of colon cancer cells [56]. ILK activity also results in Lef-1-mediated transcriptional upregulation of MMPs [39]. Hence, ILK (inducible by TGF-β signaling) is capable of orchestrating most of the major events in an EMT, including the loss of cell-cell adhesion and invasion across the basal lamina.

WNT PATHWAY

Many EMTs or METs are also regulated by Wnt signaling. Wnts signal through seven-pass transmembrane proteins of the Frizzled family, which activates G-proteins, PI3K, inhibits GSK-3β and promotes nuclear β-catenin signaling. For example, during zebrafish gastrulation, Wnt11 activates the GTPase Rab5c, which results in the endocytosis of E-cadherin [57]. Wnt6 signaling is sufficient for increased transcription of *Snail-2* in the avian neural crest [58]. Snail-1 expression increases Wnt signaling [59], which suggests a positive feedback loop.

One of the downstream signaling molecules activated by Wnt signaling is β-catenin. β-catenin is a structural component of adherens junctions. Nuclear β-catenin is also a limiting factor for the activation of LEF/TCF transcription factors. β-catenin is pivotal for regulating most EMTs. Interfering with nuclear β-catenin signaling blocks the ingression of sea urchin PMCs [60], and in β-catenin mouse knockouts, the primitive streak EMT does not occur, and no mesoderm is formed [61]. β-catenin is also necessary for the EMT that occurs during cardiac cushion development [62]. In breast cancer, β-catenin expression is highly correlated with metastasis and poor survival [63], and blocking β-catenin function in tumor cells inhibits invasion *in vitro* [64]. It is unclear if β-catenin overexpression alone is sufficient for all EMTs. If β-catenin is misexpressed in cultured cells, it causes apoptosis [36]. However, the misexpression of a stabilized form of β-catenin in mouse epithelial cells *in vivo* results in metastatic skin tumors [65].

SIGNALING BY RTK LIGANDS

The receptor tyrosine kinase (RTK) family of receptors and the growth factors that activate them also regulate EMTs or METs. Ligand binding promotes RTK dimerization and activation of the intracellular kinase domains by auto-phosphorylation of tyrosine residues. These phosphotyrosines act as docking sites for intracellular signaling molecules, which can activate signaling cascades such as Ras/MAPK, PI3K/Akt, JAK/STAT, or ILK. Below we cite some examples of RTK signaling in EMTs and METs.

Hepatocyte growth factor (HGF, also known as scatter factor) acts through the RTK c-met. HGF is important for the MET in the developing kidney [66]. HGF signaling is required for the EMT that produces myoblasts (limb muscle precursors) from somite tissue in the mouse [7]. In epithelial cells, HGF causes an EMT through MAPK and early growth response factor-1 (Egr-1) signaling [1].

Fibroblast growth factor (FGF) signaling regulates mouse primitive streak formation [67]. FGF signaling also stimulates cell motility and activates MMPs [68,69].

EGF promotes the endocytosis of E-cadherin [70]. EGF can also increase Snail-1 activity via the inactivation of GSK3-β [71] and EGF promotes increased *Twist* expression through a JAK/STAT3 pathway [72].

Insulin growth factor (IGF) signaling induces an EMT in breast cancer cell lines through the activation of Akt2 and suppression of Akt1 [73]. In prostate cancer cells, IGF-1 promotes Zeb-1 expression [74]. In fibroblast cells, constitutively activated IGF-IR increases NF-κB activity and Snail-1 levels [75]. In several cultured epithelial cell lines, IGFR1 is associated with the complex of E-cadherin and β-catenin, and the ligand IGF-II causes the redistribution of

β-catenin from the membrane to the nucleus, activation of the transcription factor TCF-3, and a subsequent EMT [76].

Another RTK known for its role in EMTs is the ErbB2/HER-2/Neu receptor, whose ligand is heregulin/neuregulin. Overexpression of HER-2 occurs in 25% of human breast cancers, and the misexpression of HER-2 in mouse mammary tissue *in vivo* is sufficient to cause metastatic breast cancer [77]. Herceptin® (antibody against the HER-2 receptor) treatment is effective in reducing the recurrence of HER-2-positive metastatic breast cancers. HER-2 signaling activates *Snail-1* expression in breast cancer through an unknown mechanism [78]. The RTK Axl is also required for breast cancer carcinoma invasiveness [79].

Vascular endothelial growth factor (VEGF) signaling promotes Snail-1 activity by suppression of GSK3-β [80], and results in increased levels of *Snail-1*, *Snail-2*, and *Twist* [81]. Snail-1 can also activate the expression of VEGF [82]. In summary, RTK signaling is important for many EMTs.

NOTCH PATHWAY

The Notch signaling family also regulates EMTs. When the Notch receptor is activated by its ligand Delta, an intracellular portion of the Notch receptor ligand is cleaved and transported to the nucleus where it regulates target genes. Notch1 is required for cardiac endothelial cells to undergo an EMT to make cardiac cushions, and the role of Notch may be to make cells competent to respond to TGF-β2 [83]. In the avian neural crest EMT, Notch signaling is required for the induction and/or maintenance of *BMP4* expression [84]. Similarly, Notch signaling is required for the TGF-β-induced EMT of epithelial cell lines [85], and Notch promotes *Snail-2* expression in cardiac cushion cells [86] and cultured cells [87].

HEDGEHOG PATHWAY

The hedgehog pathway is also involved in EMTs. Metastatic prostate cancer cells express high levels of hedgehog and *Snail-1*. If prostate cancer cell lines are treated with the hedgehog pathway inhibitor, cyclopamine, levels of *Snail-1* are decreased. If the hedgehog-activated transcription factor, Gli, is misexpressed, *Snail-1* expression increases [88].

Additional signaling pathways

Other signaling pathways that activate EMTs include inflammatory signaling molecules, lipid hormones, ROS species, and hypoxia. Interleukin-6 (inflammatory and immune response) can promote Snail-1 expression in breast cancer cells [89], and Snail-1 in turn can activate Il-6 expression [90], providing a link between inflammation and EMTs [91]. The lipid hormone prostaglandin E2 (PGE2) induces Zeb1 and Snail activity in lung cancer cells [92], and Snail-1 can also induce PGE2 expression [93]. ROS species can also activate EMTs by PKC and MAPK signaling [2]. Hypoxia is important for initiating EMTs during development [94] and disease [91], often through hypoxia-inducible factor-1 (HIF-1), which directly activates *Twist* expression [95]. Hypoxia also activates lysyl oxidases (LOX), which stabilize Snail-1 expression [1] by inhibiting GSK-3β activity [96].

In addition to diffusible signaling molecules, extracellular matrix molecules also regulate EMTs or METs. This was first dramatically demonstrated when lens or thyroid epithelium was embedded in collagen gels, and then promptly underwent an EMT [5]. Integrin signaling appears to be important in this process [97], and involves ILK mediated activation of NF-κB, Snail-1, and Lef-1. Other ECM components that regulate EMTs include hyaluronan [98], the gamma-2 chain of laminin 5 [99], periostin, and podoplanin [1]. In summary, a variety of diffusible signals and ECM components can stimulate EMTs or METs.

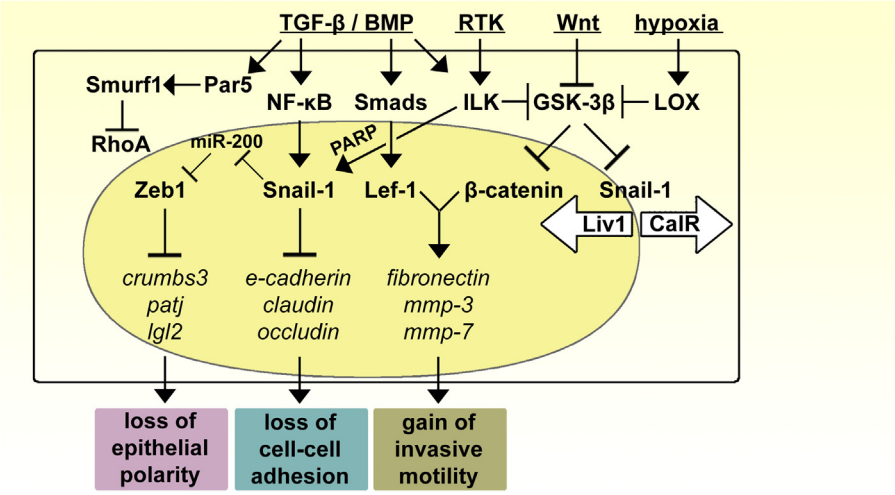

FIGURE 8.2
Induction of an EMT. This figure summarizes some of the important molecular pathways that bring about an EMT. Many of the signaling pathways converge on the activation of Snail-1 and nuclear β-catenin signaling to change gene expression, which results in the loss of epithelial cell polarity, the loss of cell-cell adhesion, and increased invasive cell motility.

A model for EMT induction

Many of the experimental studies on EMT mechanisms focus on one small part of the entire EMT process, and while great progress has been made in discovering EMT pathways, the entire EMT signaling network is still incomplete. The diagram (see Fig. 8.2) summarizes many of the various signaling mechanisms, although in actuality only a few of the inductive pathways will be utilized for a particular EMT. From experimental evidence to date, it appears that many of the EMT signaling pathways converge on ILK, the inhibition of GSK-3β, and stimulation of nuclear β-catenin signaling to activate Snail and LEF/TCF transcription factors. Snail, Zeb and LEF/TCF transcription factors then act on a variety of targets to suppress cell-cell adhesion, induce changes in cell polarity, stimulate cell motility, and promote invasion of the basal lamina.

CONCLUSION

Over the past 30 years since the term 'EMT' was coined [100], important insights have been made in this rapidly expanding field of research. EMT and MET events occur during development, tissue repair, and disease, and many molecules that regulate the various EMTs or METs have been characterized, thanks in large part to the advent of cell culture models. However, the EMT regulatory network as a whole is still incomplete. The improved understanding of EMT and MET pathways in the future will lead to novel therapeutic targets for the treatment of disease and more effective strategies for tissue engineering.

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CHAPTER 9

The Dynamics of Cell-ECM Interactions, with Implications for Tissue Engineering

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INTRODUCTION

Historical background

In the first part of last century, the extracellular matrix (ECM) was thought to serve only as a structural support for tissues. However, in 1966, Hauschka and Konigsberg [1] showed that interstitial collagen promotes the conversion of myoblasts to myotubes, and, shortly thereafter it was shown that both collagen and glycosaminoglycans are crucial for salivary gland morphogenesis. Based upon these and other findings, in 1977, Hay put forth the idea that the ECM is an important component in embryonic inductions, a concept which suggested the presence of binding sites (receptors) for specific matrix molecules on the surface of cells [2]. The stage was then set for further investigations into the mechanisms by which ECM molecules influence cell behavior. Bissell and colleagues [3] proposed the model of 'dynamic reciprocity'. In this model, ECM molecules interact with receptors on the surface of cells which then transmit signals across the cell membrane to molecules in the cytoplasm; these signals initiate a cascade of events through the cytoskeleton into the nucleus, resulting in the expression of specific genes, whose products, in turn, affect the ECM in various ways. Through the years, it has become clear that cell-ECM interactions can directly regulate cell adhesion, migration, growth, differentiation, and apoptosis, can also modulate the activities of cytokines and growth factors, and directly or indirectly activate intracellular signaling.

ECM composition

The extracellular microenvironment contains both traditional structural matrix components, such as hyaluronic acid, proteoglycans, collagens, glycosaminoglycans, and elastins, and non-structural matricellular proteins, including secreted protein acidic and rich in cysteine (SPARC), tenascin, osteopontin, and thrombospondins. The distribution and organization of these molecules is not static, but varies from tissue to tissue and, during development, from stage to stage, which has significant implications for tissue function. For example, mesenchymal cells are immersed in an interstitial matrix that confers specific biomechanical and

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functional properties to connective tissue [4], whereas epithelial and endothelial cells contact a specialized matrix, the basement membrane, via their basal surfaces only, conferring mechanical strength and specific physiological properties to the epithelia [5]. This diversity of composition, organization, and distribution of ECM results not only from differential gene expression of the various molecules in specific tissues, but also from the existence of differential splicing and post-translational modifications of those molecules. For example, alternative splicing may change the binding potential of proteins to other matrix molecules or to their receptors, variations in glycosylation can lead to changes in cell adhesion and migration, and proteolytic cleavage can generate fragments with diverse biological functions [6–8].

The ECM harbors many non-matrix proteins, including growth factors, cytokines, and matrix-degrading enzymes and their inhibitors. Tissue-specific and developmental-stage-specific variation in matrix composition may determine the presence and localization of associated growth factors and cytokines. ECM molecules can influence the local concentration and biological activity of growth factors and cytokines by regulating their diffusion from their sites of origin, by protecting them from degradation, by presenting them more efficiently to their receptors, and by altering levels of their receptors [9–13]. Growth factor binding to intact ECM molecules or matrix fragments may also have inhibitory effects on growth factor signaling, by decreasing growth factor synthesis, impairing the interaction of these factors with their receptors, and/or decreasing levels of their receptors [7,12,14].

ECM/growth factor interactions can also involve the ability of specific domains of ECM molecules (e.g., laminin-332, tenascin-C, thrombospondin, and decorin) to activate growth factor receptors [15]. The EGF-like repeats of laminin and tenascin-C bind and activate the EGFR [16–18]. In the case of laminin, the EGF-like repeats interact with EGFR following their release by MMP-mediated proteolysis whereas tenascin-C repeats are thought to bind EGFR in the context of the full-length protein [17,18]. Decorin also binds and activates EGFR, although this binding occurs via leucine-rich repeats rather than EGF-like repeats [19]. In contrast, the EGF-like repeats of thrombospondin 1 appear to activate the EGFR in an indirect, MMP9-dependent manner, likely via MMP9-mediated release of heparin-binding EGF (HB-EGF) [20]. The ability of ECM molecules to activate growth factor receptors may facilitate a stable signaling environment for the associated cells due to the inability for the ligand to either diffuse or be internalized, thus serving as a long-term pro-migratory and/or pro-proliferative signal [15].

Receptors for ECM molecules

Integrins, a family of heterodimeric transmembrane proteins composed of α and β subunits, were the first ECM receptors to be identified. At least 18 α and 8 β subunits have been identified that pair in various combinations to yield 24 separate heterodimers, many of which recognize specific sequences on the ECM molecules (Fig. 9.1 [21]). Some integrin heterodimers exhibit a high degree of ligand specificity, while others are able to interact with a variety of epitopes (Fig. 9.1), facilitating plasticity and redundancy in specific systems [22]. Although the α and β subunits of integrins are unrelated, there is 40–50% homology within each subunit with the highest divergence in the intracellular domain of the α subunit. Apart from integrin β_4, the integrins have large extracellular domains and very small intracellular domains. Despite the small size of the cytoplasmic domains, integrins are able to bind to a variety of intracellular proteins, facilitating their interactions with the cytoskeleton and with signal transduction pathways [23].

Transmembrane proteoglycans, including syndecans, a receptor for hyaluronan mediated motility (RHAMM), and CD44, can also serve as receptors for ECM molecules, including collagen, fibronectin, laminin, and hyaluronan [21]. Syndecans 1–4, for example, mediate cell-ECM interactions via chondroitin- and heparan sulfate glycosaminoglycans, whose

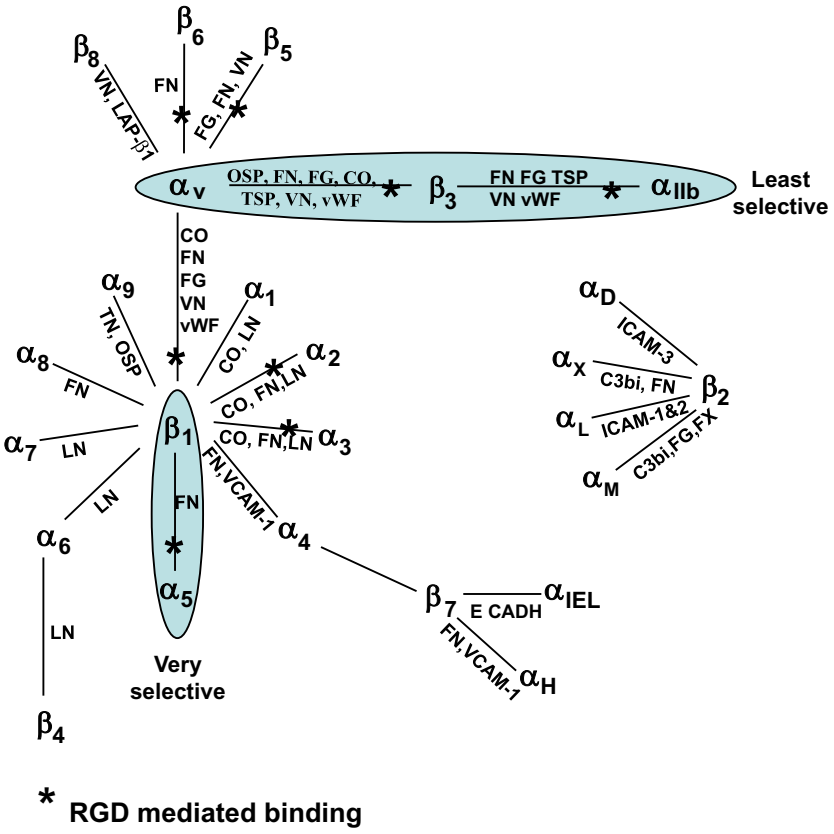

FIGURE 9.1

Members of the integrin family of ECM receptors and their respective ligands. These heterodimeric receptors are composed of one α and one β subunit, and are capable of binding a variety of ligands, including Ig superfamily cell adhesion molecules, complement factors, and clotting factors in addition to ECM molecules. Cell-cell adhesion is largely mediated through integrin heterodimers containing the β_2 subunits, while cell-matrix adhesion is mediated primarily via integrin heterodimers containing the β_1 and β_3 subunits. In general, the β_1 integrins interact with ligands found in the connective tissue matrix, including laminin, fibronectin, and collagen, whereas the β_3 integrins interact with vascular ligands, including thrombospondin, vitronectin, fibrinogen, and von Willebrand factor. Abbreviations: CO, collagens; C3bi, complement component; FG, fibrinogen; FN, fibronectin; FX, Factor X; ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; ICAM-3, intercellular adhesion molecule-3; LN, laminin; OSP, osteopontin; TN, tenascin; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule-1; VN, vitronectin; vWF, von Willebrand factor.

composition varies based upon the type of syndecan and the tissue in which it is expressed. These differential glycosaminoglycan modifications alter the binding capacity of particular ligands, such as fibronectin and tenascin [24]. The short cytoplasmic domains of syndecans can interact with signaling proteins and the cytoskeleton, and thereby induce signal transduction upon binding to their ECM ligands, resulting in changes in cell adhesion and migration [24,25]. In addition to syndecan's function as a matrix receptor, the protein core can bind and activate some integrins directly, and activate other integrins indirectly, by facilitating their interactions with the matrix.

Another proteoglycan receptor, CD44, undergoes tissue-specific splicing and glycosylation to yield multiple isoforms [26]. CD44 has multiple ligands, including collagen IV, collagen XIV, fibronectin, osteopontin, and laminin among others, in addition to its primary ligand, hyaluronan [27]. CD44 can also interact with another hyaluronan receptor, RHAMM [27]. RHAMM, which is not an integral membrane protein, must bind transmembrane protein(s), such as CD44, integrins, and/or receptor tyrosine kinases, to transmit the signal from hyaluronan to intracellular signaling proteins [27,28]. Hyaluronan is present in most tissues in a high molecular weight, native form, but can be cleaved by various enzymes to generate lower molecular weight fragments; this cleavage is frequently associated with tissue damage. The native and cleaved forms of hyaluronan elicit different cellular responses, potentially due to differential receptor selectivity. CD44 binds more stably to high molecular weight hyaluronan than low molecular weight hyaluronan fragments [29,30]. In contrast, hyaluronan fragments, but not the native high molecular weight form, bind and activate toll-like receptors (TLRs), strongly suggesting that the fragments function as 'danger signals' that sense tissue damage and induce inflammatory responses [26,31].

Additional extracellular matrix receptors have also been identified, including the elastin/laminin receptor (ELR), CD36, annexin II, and receptor tyrosine kinases. The ELR is a complex

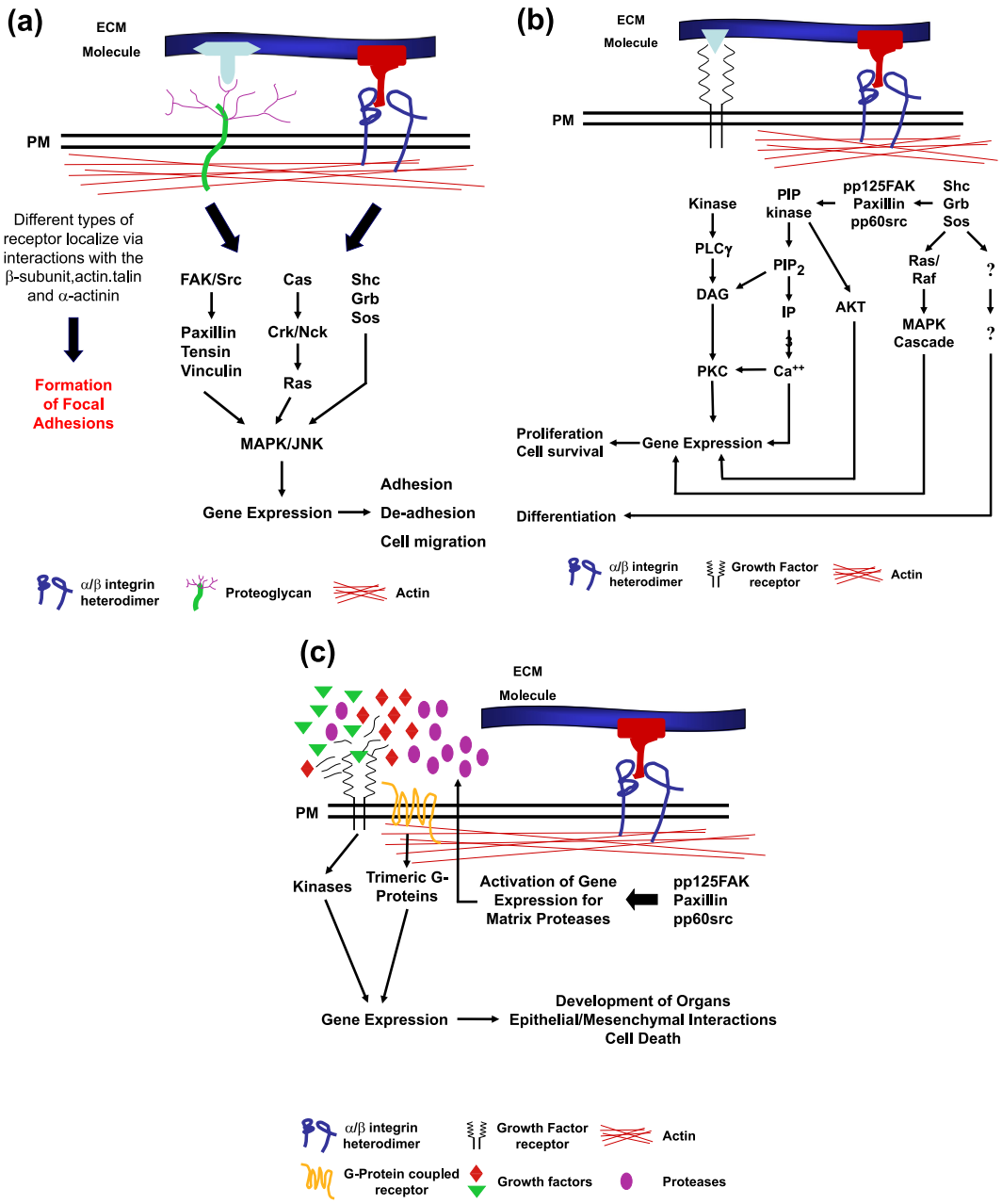

FIGURE 9.2

Schematic diagram of cell-ECM interactions present during healing and regenerative responses. Type I (a), type II (b) and type III (c). Such interactions between the ECM receptors and their respective ligands initiate signal transduction cascades culminating in a variety of cellular events important in repair and regeneration, including changes in cellular adhesion and migration and altered rates of proliferation and apoptosis. The presence and/or extent of such changes may influence the balance of repair and regenerative responses to favor one outcome over another; thus, interventions that alter ECM signaling events may shift this balance to favor tissue regeneration and thus decrease scarring.

of three proteins, including neuraminidase 1, cathepsin A, and the elastin-binding protein (EBP). The EBP binds specifically to the GXXPG sequence found in elastin, fibrillin, laminin, and fragments of these matrix molecules, and is critical for elastin deposition [32,33]. The ELR can also bind to the YIGSR sequence in the β1 chain of laminin-111 [34]. Elastin-derived peptides generated by proteases activated in response to tissue injury can promote proliferation and/or migration of fibroblasts, epithelial/endothelial cells, and monocytes downstream of the ELR [32]. These effects suggest that elastin-derived peptides are able to

promote wound healing; indeed, these peptides enhance the healing of burn wounds when used in conjunction with more conventional treatments [35].

CD36 can also function as a matrix receptor, despite its better-known role as a scavenger receptor, binding thrombospondin, collagen I, and collagen V [36]. In endothelial cells, thrombospondin binding to CD36 induces apoptosis, and is thus anti-angiogenic *in vivo* [37,38]. Another cell surface receptor, annexin II, is known to interact with alternative splice variants of tenascin-C, and mediates the cellular responses to these various forms of tenascin-C [39]. Tyrosine kinase receptors, including the EGFR and the discoidin domain receptors DDR1 and DDR2, can also function as matrix receptors. The EGFR can be activated by EGF-like domains or by MMP-mediated release of HB-EGF (see above), while DDR1 and DDR2 serve as collagen receptors [40]. Unlike most other receptor tyrosine kinases, which are activated by dimerization, DDR1 and DDR2 exist as constitutive homodimers, suggesting an alternative mechanism of activation [40]. Upon ligand binding, the DDRs undergo autophosphorylation and induce multiple downstream signaling pathways that ultimately alter cell adhesion and migration [40]. Several studies have suggested various roles for the DDR receptors in ECM remodeling. In smooth muscle cells, over-expression of DDR reduces the expression of several matrix molecules and receptors, including collagen, syndecan-1, and integrin α_3, while increasing MMP activity, resulting in degradation of collagen and elastin [41]. However, inhibition of DDR signaling in fibroblasts decreased collagen synthesis, suggesting that DDR may exert cell-type-specific effects on matrix deposition and remodeling [42,43].

Below, we will first discuss selected examples that illustrate the dynamics of cell-ECM interactions during development and wound healing, as well as the potential mechanisms involved in the signal transduction pathways initiated by these interactions. Finally, we will discuss the implications of cell-ECM interactions in tissue engineering.

CELL-ECM INTERACTIONS

Multiple biological processes, including those relevant to development and wound healing, require both interactions between cells and their environment and modulation of such interactions. During development, the cellular crosstalk with the surrounding extracellular matrix promotes the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotypes during embryogenesis. Similarly, during wound healing these interactions contribute to the processes of clot formation, inflammation, granulation tissue development, and remodeling. As outlined below, the current body of research in the fields of both embryogenesis and wound healing implicates multiple cellular behaviors, including cell adhesion/de-adhesion, migration, proliferation, differentiation and apoptosis, in these critical events.

Development

ADHESION AND MIGRATION

Today, there is a vast body of experimental evidence that demonstrates the direct participation of ECM in cell adhesion and migration, but some of the most compelling experiments came from studies in gastrulation, migration of neural crest cells (NCC), angiogenesis, and epithelial organ formation. Cell interactions with fibronectin are important during gastrulation; inhibition of fibronectin-integrin interactions in amphibian embryos by the introduction of blocking antibodies or RGD-containing peptides into their blastocoel cavities, which compete with integrins for ECM binding and disrupt normal cell movement leading to abnormal development [44]. Similarly, introduction of recombinant fibronectin lacking the RGD motif perturbs amphibian gastrulation [45]. These effects are not unique to fibronectin, as they can also be introduced by manipulation of other molecules, such as hyaluronan and heparan sulfate proteoglycans. Inhibition of hyaluronan synthesis in zebrafish embryos

interferes with cell movements in gastrulation, potentially due to a defect in Rac1 activation, as expression of constitutively active Rac rescued the observed migratory defects [46]. In the case of HSPG, degradation of HSPG by injection of the blastocoel with heparinitase interferes with gastrulation [47]. More recent studies suggest that the interaction between HSPG and fibronectin is critical for gastrulation. Binding of fibronectin to HSPG causes a conformational change in fibronectin, exposing growth factor binding sites that then bind PDGF-AA, generating a stable PDGF gradient that promotes the directional cell migration that is critical for gastrulation [48]. HSPGs are also necessary for FGF signaling during gastrulation, as inhibition of HSPG synthesis alters FGF localization, inhibits FGF signaling, and arrests embryonic development at gastrulation [49,50].

Cell-matrix interactions are also important for the migration of NCC, which develop in the dorsal portion of the neural tube just after closure of the tube, de-adhering from each other and then migrating extensively throughout the embryo in ECM-filled spaces, giving rise to a variety of phenotypes. The importance of cell-ECM interactions in NCC migration is supported by studies performed in the white mutant of Mexican axolotl embryos. The NCC that give rise to pigment cells fail to emigrate from the neural tube in these embryos, but when microcarriers containing subepidermal ECM from normal embryos are implanted into the appropriate area in these mutants, the NCC pigment cell precursors emigrate normally [51]. Laminin and fibronectin, the latter of which appears between chick NCC just prior to their emigration from the neural tube [52], play particularly critical roles in this process. Inhibition of fibronectin, laminin-111 (laminin-1), laminin-411 (laminin-8), or their integrin receptors using function-blocking antibodies, competing peptides, or antisense RNA prevents NCC migration, while exogenous laminin or fibronectin are sufficient to induce premature NCC migration [51,53]. More recent studies have identified different subsets of NCC, which may exhibit different responses to specific matrix molecules. For example, cranial NCC do not migrate in response to fibronectin, but do migrate on laminin [54,55]. Matrix remodeling may also contribute to NCC migration, as the matrix-remodeling protease MMP-9 is also required for NCC de-adhesion and migration, while exogenous MMP-9 induced premature de-adhesion and migration [56]. The role of MMP-9 in de-adhesion may be related to its ability to cleave the cell-cell adhesion molecule N-cadherin, while its role in migration may involve laminin degradation [56]. Proteoglycans also participate in the NCC migration process. Aggrecan, a proteoglycan that predominates in the notochord and cartilage tissue, inhibits NCC migration and may thus delimit NCC migration pathways [51]. The chondroitin sulfate proteoglycan versican may play a similar inhibitory role in NCC migration [57]. In contrast, syndecan-4 is not required for cell migration, but is necessary for directional migration. In conjunction with a non-canonical Wnt pathway, syndecan-4 inhibits Rac at the rear of the cell, restricting Rac activity to the leading edge of the migrating cell and facilitating directional migration [58].

Endothelial cell interactions with ECM molecules and the type and conformation of the matrix are also crucial in cell adhesion and migration during angiogenesis, a process in which new blood vessels form from pre-existing vessels [59]. Early indications of the role of ECM in angiogenesis were observed when human umbilical vein endothelial cells (HUVEC) were cultured on matrigel, a matrix synthesized by Engelbreth-Holm-Swarm (EHS) tumors. This specialized matrix has many of the properties of basement membrane; it consists of large amounts of laminin, as well as collagen IV, entactin/nidogen, and proteoglycans. When HUVEC are cultured on matrigel for 12 hours, they migrate and form tube-like structures. In contrast, when these cells are cultured with collagen I, they only form tube-like structures after they are maintained *inside* the gels for one week, at which time the cells have secreted their own basement membrane molecules [60]. The observation that tube formation occurs more rapidly on matrigel than within collagen gels strongly suggested an important role for one or more of the matrix molecules present within the basement membrane in the development of the capillary-like endothelial tubes. Indeed, laminin-111, the predominant matrix

molecule in matrigel, was later shown to participate in endothelial tube formation and angiogenesis. Purified or recombinant laminin-111 induce cell adhesion, migration, and tube formation *in vitro* and angiogenesis *in vivo*, while a mutation in laminin $\alpha 1$ leads to defective vessel formation [61]. Furthermore, studies in integrin α_7-deficient mice suggest that the laminin receptor integrin $\alpha_7\beta_1$ is necessary for appropriate pericyte interactions with blood vessels, and is thus important in vessel maturation and stability [62].

Recent studies have used knockout mice to implicate a number of ECM molecules in addition to laminin-111 in developmental blood vessel formation and maturation, including laminin $\alpha 4$, fibronectin, collagen IV, and the HSPG perlecan [59,63]. Mice deficient in laminin $\alpha 4$, fibronectin, or collagen IV exhibit defects in basement membrane formation, a process known to be critical in vessel maturation and stability, while the basement membranes in mice lacking perlecan form normally but have decreased ability to withstand mechanical stress [63,64]. Several integrin knockouts also exhibit defects in angiogenesis and vessel maturation. Like fibronectin, the fibronectin receptor integrin $\alpha_5\beta_1$ is necessary for angiogenesis, as shown by substantial angiogenesis defects in integrin α_5 and integrin β_1 endothelial cell-specific knockouts [62]. Abnormal pericyte-blood vessel interactions in integrin α_4 −deficient animals suggest the importance of integrin $\alpha_4\beta_1$ in this process, although in this case, the binding of this integrin to VCAM may be more important than its binding to fibronectin [61].

Some of the ECM effects on angiogenesis involve matrix cooperation with growth factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). VEGF and PDGF interact with HSPG, which can sequester them until their release by proteases, limit their diffusion, and/or function as co-receptors to promote receptor binding and activation [9]. Binding of VEGF to HSPG appears to be important in its localization during development. In mouse embryos, sole expression of a VEGF isoform that lacks the heparin-binding domain, VEGF121, increases diffusion of VEGF from the site of secretion and decreases blood vessel branching. This altered vascular patterning appears to result from impaired endothelial cell migration, as shown by decreased filopodia formation in the migrating 'tip cell' of nascent sprouts [65]. In embryos solely expressing the HSPG-binding VEGF isoform, VEGF188, branching of the blood vessels and filopodia formation by endothelial tip cells were increased when compared with wild type [65]. HSPG are also important for vessel stability and maturation, as HSPG are required for PDGF-BB-induced signaling, which, in turn, is important for pericyte migration and interaction with nascent blood vessels [65].

In contrast to the pro-angiogenic activities of many matrix molecules, fragments generated by their proteolysis can exert anti-angiogenic effects. The YIGSR peptide derived from laminin $\beta 1$, which binds the ELR rather than an integrin, prevents endothelial cell migration and angiogenesis [61]. YIGSR binding to the ELR promotes laminin cleavage by cathepsin-B [34]. This cleavage could reveal cryptic binding sites on laminin, altering its ability to bind specific receptors, leading to changes in cell signaling that, in turn, inhibit angiogenesis [61]. Another possibility is that the YIGSR peptides exert anti-angiogenic properties due to competition for receptor binding with the intact laminin present *in vivo*. Indeed, if YIGSR peptides can successfully compete with laminin, the displacement of the YIGSR sequence in the intact laminin molecule by soluble YIGSR peptides would alter the presentation of the ligand to its receptor, resulting in changes in mechanical resistance that alter signaling events downstream of the receptor, yielding different cellular responses. A similar hypothesis has recently been proposed for the interactions of integrins with soluble versus intact ligands [66]. Many soluble integrin ligands generated through proteolytic activity are anti-angiogenic, such as tumstatin and arresten (derived from collagen IV), endostatin (derived from collagen XVIII), and endorepellin (derived from perlecan), although their contribution to developmental angiogenesis remains unclear [7]. Although the mechanisms generating different cellular outcomes are not completely understood, the fact that soluble and intact ECM receptor ligands may, at times, lead to alternative outcomes is likely of importance *in vivo*

following matrix degradation. During angiogenesis, endothelial cell migration and invasion into surrounding tissues is accompanied by the activation of matrix-degrading enzymes, which then cleave the matrix and release both matrix-bound growth factors as well as ECM fragments, providing additional angiogenic or anti-angiogenic cues to further influence the process [10]. As such, matrix molecules that initially facilitate angiogenesis may be proteolytically cleaved to create anti-angiogenic matrix fragments, preventing additional blood vessel formation and/or resulting in vessel maturation. Thus, the temporal and spatial production and cleavage of matrix molecules may have important consequences for tissue homeostasis.

PROLIFERATION

Certain cell-ECM interactions modulate cell proliferation, with some matrix molecules inducing and others inhibiting proliferation. Tenascin-C stimulates cell proliferation *in vitro* via its EGF-like repeats, which bind and activate the EGFR [17]. Similarly, the EGF-like repeats of laminin stimulate proliferation of a variety of different cell lines, likely via EGFR activation [18]. Some of the ECM effects on cell proliferation involve matrix cooperation with growth factors. As mentioned above, under migration/adhesion, binding of growth factors to matrix molecules can affect their interactions with their receptors, limit their diffusion and/or sequester them until protease-mediated release. Several matrix-associated growth factors, including HB-EGF and TGF−β, can regulate cell proliferation during development.

Several studies have suggested that HSPG-associated molecules may regulate the proliferation of vascular smooth muscle cells (VSMC). Conditioned medium of aortic endothelial cells inhibits FGF-induced VSMC proliferation an effect that is abolished by pre-treatment with heparinase but not with proteases [67]. One possible explanation for this result is that heparan-type molecules may directly inhibit aortic VSMC proliferation. However, it is also possible that heparinase treatment may release pro-proliferative molecules interacting with heparin or heparan sulfate, thus allowing these factors to interact with their receptors and either promote proliferation or block any anti-proliferative effects. One such mitogenic heparin-binding ECM molecule is HB-EGF, which is known to promote VSMC proliferation *in vitro*, in cultured cells [68]. HB-EGF appears to regulate proliferation of additional mesenchymal cell types during development; a mouse embryo expressing of a mutant HB-EGF that could not be cleaved and released by HSPG showed defects resembling the HB-EGF knockout, suggesting that HB-EGF release is necessary for its function [69]. In contrast, embryos expressing a soluble version of HB-EGF that could not bind HSPG showed abnormal proliferation of multiple cell types, suggesting a role for soluble, and thus active, HB-EGF in regulating proliferation of these cell types during development [69].

Another heparin-binding ECM molecule that could induce VSMC proliferation is thrombospondin, which is known to exert its mitogenic activities on VSMC via its amino terminal heparin-binding domain [70,71]. Heparin blocks both thrombospondin binding to smooth muscle cells and its mitogenic effects [70]. These results suggest that interactions between heparin and thrombospondin may interfere with thrombospondin-induced smooth muscle cell proliferation, and that the observed increases in VSMC proliferation following heparinase treatment mentioned previously may result, at least in part, from the removal of such inhibitory interactions.

The effects of heparin on VSMC may also result from its regulation of TGF-β, an inhibitor of VSMC proliferation; heparin increases TGF-β activation, and heparin-mediated anti-proliferative effects are blocked by addition of a TGF-β antibody [71]. As such, heparinase treatment may prevent TGF-β activation, abolishing the anti-proliferative effects. To complicate matters further, thrombospondin can promote TGF-β activation as well [9], although the importance of thrombospondin binding to HSPG in TGF-β activation is unclear. However, if heparin's effects are exclusively mediated by the inhibition of HB-EGF or thrombospondin and/or activation of TGF-β, one would expect that treatment of the endothelial cell

conditioned medium with proteases should also eliminate the anti-proliferative effect. As the protease treatment does not prevent these effects, it is likely that heparin-like molecules also have a direct anti-proliferative effect [67].

TGFβ also cooperates with the ECM in the early developmental stages of the mammary gland during puberty [72]. During this period, inductive events take place between the epithelium and the surrounding mesenchyme that are mediated by the basement membrane (basal lamina and closely associated ECM molecules) and which play an important role in epithelial proliferation during branching of the gland. Endogenous TGFβ produced by the ductal epithelium and surrounding mesenchyme forms complexes with mature periductal ECM [73]. This TGF-β may participate in stabilizing the epithelium by stimulating expression of matrix molecules, by inhibiting cell proliferation, and/or by inhibiting matrix-degrading enzymes. In support of this possibility, the mammary gland epithelial cells in transgenic mice expressing a kinase-deficient TGF-β receptor showed excessive proliferation, whereas elevation of TGF-β decreased cell proliferation and increased matrix deposition [72]. In the branching areas, TGFβ is absent from newly synthesized ECM; thus, its inhibitory effects on epithelial cell proliferation and on production of matrix-degrading enzymes do not occur, allowing the basement membrane to undergo remodeling.

Along with the *in vivo* studies described above, *in vitro* studies using cultured cells suggest that various matrix molecules inhibit cell proliferation. For example, normal human breast cells do not growth arrest when cultured on plastic, but do so if grown in a basement membrane matrix [74]. More recent studies have shown that laminin-111 is particularly important in this inhibition of proliferation, as cells plated on this matrix molecule also stop dividing [75]. Laminin appears to suppress breast epithelial cell proliferation via expression of Id-2, a transcriptional regulator that binds and inhibits basic helix-loop-helix (HLH) transcription factors, leading to growth arrest of this cell type [76].

DIFFERENTIATION

Matrix molecules are critical in regulating the differentiation of keratinocytes, hepatocytes, and mammary gland epithelium. Keratinocytes form the stratified epidermal layers of the skin. The basal layer is highly proliferative, does not express the markers for terminal differentiation, and is the only cell layer in contact with the basement membrane. As these cells divide, the daughter cells lose contact with the basement membrane, move up to the suprabasal layers, and begin to express differentiation markers such as involucrin and keratins 1 and 10 [77]. This suggests that physical interaction with the basement membrane represses differentiation of the basal keratinocytes. Early studies implicated fibronectin and β_1 integrins in preventing differentiation; however, the later studies showed that the keratinocytes of a conditional integrin β1 skin knockout mouse and those of mice deficient lacking various β_1-associated α subunits do not undergo premature terminal differentiation. Additional studies investigating the importance of basement membrane components and basal keratinocyte integrins have found that these matrix-integrin interactions are critical in cell adhesion (and migration during wound healing), but do not appear to play an important role in differentiation [77]. These results suggest that further studies are necessary to better understand the contribution of the basement membrane in differentiation.

In the mouse mammary gland, the basement membrane and its individual components, in conjunction with lactogenic hormones, are responsible for the induction of the differentiated phenotype of the epithelial cells. When mid-pregnant mammary epithelial cells are cultured on plastic, they do not express mammary-specific genes. However, when the same cells are plated and maintained on basement membrane components (EHS), they form alveolar-like structures and exhibit the fully differentiated phenotype with expression of the genes encoding milk proteins, such as β-casein [78]. Function-blocking antibodies and conditional knockouts found that laminin-111 is the ECM molecule present in EHS ultimately responsible for the

observed differentiation, and that integrins β_1 and α_6 are critical in maintaining the differentiated state [78]. One role of laminin-111 in this process is in establishing mammary epithelial cell polarity, which re-distributes the prolactin receptor to the apical surface of the epithelium and facilitates ligand binding, receptor activation, and signaling necessary to induce expression of β-casein [79]. ECM molecules also regulate expression of another milk protein, the whey acidic protein (WAP). EHS decreases the production of TGF-α by mammary gland epithelial cells; this increases WAP expression, which is otherwise inhibited by TGF-α [80]. WAP may then participate in the maintenance of mammary epithelial differentiation by inhibiting the activity of laminin-degrading enzymes [81].

APOPTOSIS

Programmed cell death occurs during embryogenesis of higher vertebrates in areas undergoing remodeling, such as in the development of the digits, palate, nervous system, in the positive selection of thymocytes in the thymus, during mammary gland involution, and during angiogenesis. For example, intact basement membrane molecules suppress apoptosis of mammary epithelial cells, whereas matrix fragments are thought to induce apoptosis during the involution of the mammary gland [78]. The numerous alveoli that produce milk during lactation regress and are resorbed during involution due to enzymatic degradation of alveolar basement membrane and programmed cell death [82,83]. During this involution, apoptosis appears to proceed in two distinct phases, an early phase characterized by increased levels of pro-inflammatory and apoptosis-associated proteins, including several members of the tumor necrosis factor (TNF) and TNF receptor superfamilies and caspases, including caspase 1 [84], a protein known to be important in promoting mammary epithelial cell apoptosis [85]. This is then followed by a later apoptotic phase in which cell-ECM interactions are altered due to matrix degradation, preventing pro-survival integrin signaling and resulting in apoptosis [86]. The importance of integrin signaling is underscored by experiments in which mammary epithelial cells undergo apoptosis when an antibody is used to disrupt interactions between α_1 integrin and its ECM ligands [85]. This later phase of matrix degradation and apoptosis may be regulated, at least in part, by decreased production of WAP, a milk protein that can inhibit the activity of proteases that cleave laminin [81]. Decreased production of WAP by dying or de-differentiated cells could relieve WAP-mediated protease inhibition, promoting protease activation and matrix degradation. This, in turn, would decrease the interaction of intact matrix molecules with integrins, decreasing pro-survival signaling; it is also possible that integrins could then interact with soluble matrix fragments, which is known to induce apoptosis in endothelial cells (see below) [66].

In endothelial cells, $\alpha_v\beta_3$ integrin interactions with ECM play a crucial role in their survival during embryonic angiogenesis. Disruption of these interactions with an antibody to $\alpha_v\beta_3$ inhibits the development of new blood vessels in the chorioallantoic membrane (CAM) by inducing endothelial cell apoptosis [87]. In contrast, $\alpha_v\beta_3$ binding to tumstatin, a proteolytic fragment of collagen IV, induces endothelial cell apoptosis, preventing angiogenesis and/or promoting vessel regression [62]. This interaction may promote apoptosis by interfering with normal integrin-ECM binding, thus removing a critical survival signal. Tumstatin may also promote apoptosis through a separate mechanism, such as via the recruitment and activation of caspase 8, as has been suggested previously for such soluble ligands [66]. Taken together, these findings suggest that disruption of cell-ECM interactions may lead to an increase in the expression or activation of pro-apoptotic molecules, and may also lead to the removal of pro-survival signals, which then directly or indirectly cause apoptosis.

Wound healing

ADHESION AND MIGRATION

Early in the wound healing process, blood components and tissue factors are released into the wounded area in response to tissue damage, promoting both the coagulation cascade and

platelet adhesion and activation, resulting in the formation of a clot consisting of platelets, cross-linked fibrin, fibronectin, and vitronectin, along with lesser amounts of SPARC, tenascin, and thrombospondin [88]. Activated platelets, along with degranulating mast cells, release a number of cytokines and growth factors important in initiating the next phase of wound healing, the inflammatory response, and in regulating the wound healing process. The fibrin-fibronectin clot has functions in addition to hemostasis, sequestering cytokines and growth factors while providing a temporary extracellular matrix that facilitates the adhesion and migration of multiple cell types, particularly leukocytes, into the wounded area [89].

Leukocyte adhesion, migration, and secretion of inflammatory mediators are regulated by their interactions with various ECM molecules [90]. After initial interactions between neutrophil and macrophage integrins with non-matrix molecules ICAM and VCAM, these leukocytes interact with chemoattractants associated with HSPG, matrix molecules in the endothelial basement membrane, and the fibrin-based provisional matrix [88]. HSPG binding to chemokines can create a stable gradient to promote leukocyte chemotaxis into the injured area. This HSPG chemokine binding is critical for appropriate leukocyte recruitment, as shown by their defective recruitment by mutant chemokines lacking the ability to bind glycosaminoglycans, in mice deficient in heparan sulfate biosynthesis, or in mice lacking syndecan-4 [91,92]. Leukocyte adhesion to and migration through the basement membrane and provisional matrix are also mediated by matrix-receptor interactions. Neutrophils bind several matrix molecules, including fibronectin, vitronectin, laminin-511 (laminin 10), and the small leucine-rich proteoglycan (SLRP) lumican, and actively secrete laminin-411 (laminin 8) [93,94]. Both laminin-411 and lumican are necessary for neutrophil extravasation, the former via integrin $\alpha_6\beta_1$ and the latter via β_1 integrin(s) [93,94]. A proteolytic fragment of laminin-511 also promotes neutrophil migration, suggesting a potential role of proteases in this process as well [93].

Interaction with matrix molecules in the provisional matrix can also affect inflammatory cell adhesion, migration, and behavior. Integrin $\alpha_M\beta_2$ expressed by various inflammatory cells interacts with fibrin, urokinase plasminogen activator (uPA), and thrombospondin 4, inducing inflammatory cell adhesion and migration [95]. Binding of $\alpha_M\beta_2$ to uPA also promotes plasmin activation and thus fibrin degradation and removal of the provisional matrix [95]. Interactions between integrin $\alpha_M\beta_2$ and its ligands also promote pro-inflammatory cytokine production; in monocytes, fibrin binding induces expression of pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α, MIP-1, MIP-2, and MCP-1, and thrombospondin-4 binding in neutrophils induces secretion of the chemokine IL-8 and the respiratory burst [95,96]. Similarly, tissue macrophages release pro-inflammatory cytokines after TLR4 binding to low molecular weight hyaluronic acid. Due to the ability of matrix-leukocyte interactions to regulate the inflammatory process, the types of ECM molecules present in the injured area may greatly affect the inflammatory phase of wound healing.

During the re-epithelialization phase of cutaneous wound healing, keratinocytes migrate beneath the fibrin-rich provisional matrix, likely due, at least in part, to the fact that these cells do not express the fibrin-interacting integrin $\alpha_V\beta_3$ [89]. The keratinocytes do express multiple receptors for fibronectin, collagen, tenascin, and vitronectin, including the integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_5\beta_4$, and α_v, and these receptor-matrix interactions, along with the activity of several proteases, promote keratinocyte migration and subsequent wound closure [88]. Keratinocyte migration requires the synthesis and deposition of laminin-332 and its interaction with integrin $\alpha_3\beta_1$, initiating signaling leading to Rac activation and protrusion formation [97]. Protease activity is also important in keratinocyte migration, likely through the release of HB-EGF from HSPG, which then induces keratinocyte migration through the EGFR [88].

Interactions between epithelial cells and ECM are also critical in the closure of other types of epithelial wounds. After wounding, retinal pigment epithelial cells exhibit a sequential pattern

of ECM molecule deposition that is critical in the epithelial cell adhesion and migration associated with wound closure. Within 24 hours of wounding, these epithelial cells secrete fibronectin, followed shortly by laminin and collagen IV; if the cell adhesion to these ECM molecules is blocked with either cyclic peptides or specific antibodies, the epithelial cells fail to migrate and close the wound, underscoring the importance of such interactions in wound closure [98,99].

During later stages of wound healing, macrophages and fibroblasts in the injured area deposit embryonic-type cellular fibronectin, which is important in the generation of the granulation tissue, a temporary connective tissue consisting of multiple types of ECM molecules and newly-formed blood vessels [88]. The cellular fibronectin provides a substrate for the migration of endothelial cells into the granulation tissue, thus forming the wound vasculature, and also facilitates the recruitment of fibroblasts, myofibroblasts, and lymphocytes stimulated by a variety of chemotactic cytokines, or chemokines, that are produced by tissue fibroblasts and macrophages [88,100]. Many chemokines have been characterized in multiple species, including humans, other mammals, and even birds, and have been grouped into a large superfamily which is further subdivided based upon the position of the N-terminal cysteine residues [101]. These chemokines, along with cell-ECM interactions, are critical in the adhesion and chemotaxis/migration of the cells that ultimately enter the wounded area and generate the granulation tissue [88,100].

One prototypical chemokine, IL-8, has several functions that are important in wound healing. Many of these functions have been elucidated in studies performed in the chick model system using chicken IL-8 (cIL-8/cCAF) [102]. After wounding, fibroblasts in the injured area produce large quantities of cIL-8, most likely resulting from their stimulation by thrombin, a coagulation enzyme activated upon wounding that is known to induce fibroblasts to express and secrete cIL-8. The initial rapid increase in cIL-8 generates a gradient that chemoattracts neutrophils [102]. These cells, in turn, produce monocyte chemoattractant protein, a potent chemoattractant for monocytes, which differentiate into macrophages in the wound environment. In addition, thrombin is able to induce IL-8 expression in cultured macrophages [103]; this, along with the additional IL-8 secreted from the endothelial cells of the wound vasculature and bound to various matrix components of the granulation tissue, further increases the presence of IL-8 in the granulation tissue. Indeed, IL-8 participates in granulation tissue formation by stimulating angiogenesis and matrix deposition [100,102,104]; therefore IL-8 not only functions in the inflammatory phase of wound healing by serving as a leukocyte chemoattractant, but also plays an important role in granulation tissue formation.

Angiogenesis relies heavily upon cell-ECM interactions, as described in detail earlier (under 'Development'). The localization of the matrix molecules and the proteases that degrade them both have critical roles in this process. In some cases, interaction of an angiogenic factor or its receptor with a matrix molecule is important in ligand-receptor interactions and/or downstream signaling, whereas in others, matrix fragments signal differently from the parent matrix molecule [105]. As mentioned earlier, many growth factors, including VEGF, bind HSPG, which can alter their diffusion and presentation to their receptors [9]. In addition to HSPG, VEGF-VEGFR signaling is heavily influenced by the presence of specific matrix molecules and integrins. Integrin $\alpha_v\beta_3$ is important in VEGFR2 activation and VEGF-induced angiogenesis, and a $\alpha_v\beta_3$ ligand, vitronectin, promotes the interaction of this integrin with VEGFR2 and enhances VEGF-induced VEGFR2 signaling [106]. Fibronectin simultaneously binds VEGF and integrin $\alpha_5\beta_1$, promoting VEGF-induced endothelial cell migration [106]. VEGF can also bind integrin $\alpha_9\beta_1$ directly, promoting VEGFR2-mediated signaling and endothelial cell migration [107]. In contrast, collagen I binding to β_1 integrins inhibits signaling downstream of VEGFR2 [107]. Taken together, these results underscore the importance of the microenvironment in regulation of VEGF-induced angiogenesis.

During angiogenesis, protease activity is needed for the endothelial cells to degrade and then migrate through the basement membrane and surrounding connective tissue [105]. These proteases can regulate endothelial cell adhesion and migration during angiogenesis by releasing matrix-bound factors, as mentioned earlier, and also by generating functional matrix fragments and exposing previously concealed matricryptic sites [7]. Some of these 'matricryptins' promote cell migration and angiogenesis. For example, a matricryptic site in collagen IV exposed by MMP-9-mediated proteolysis changes integrin binding from $\alpha_1\beta_1$ to $\alpha_v\beta_3$, and blocking this site with an antibody or inhibiting integrin $\alpha_v\beta_3$ prevents endothelial cell adhesion and migration *in vitro* and angiogenesis *in vivo* [107]. Many other matricryptins inhibit angiogenesis. For example, endostatin and tumstatin, matricryptins derived from collagen XVIII and collagen IV, respectively, inhibit VEGF-induced endothelial cell migration. These molecules may exert their inhibitory effects by binding VEGFR2 (endostatin) and/or integrin $\alpha_v\beta_3$ (endostatin and tumstatin), both of which are essential for VEGF-induced cell migration and angiogenesis, thereby blocking signaling downstream of the individual receptor(s) and the substantial crosstalk that occurs between them [62].

PROLIFERATION

After wounding, the keratinocytes alter their proliferation and migration in order to close the wound, a process known as re-epithelialization. As this process occurs, the cells at the edge of the wound migrate, whereas the cells away from the wound proliferate in order to provide the additional cells needed to cover the wounded area. The proliferative state of these latter keratinocytes may be sustained by interactions with the ECM of the remaining basement membrane. Indeed, during the remodeling of normal skin, the proliferation of the basal layer of keratinocytes needed to replace the upper keratinocyte layers requires the presence of fibronectin in the epithelial basal lamina (see above). In addition, ECM derived from the basement membrane, when present in a dermal wound model, can maintain keratinocytes in a proliferative state for several days. It is likely that laminins-511 and -521 (laminins 10 and 11), in addition to fibronectin, participate in keratinocyte proliferation, as previous data indicates that these molecules can promote proliferation *in vitro* [108]. Further studies have suggested important roles for integrins in regulating keratinocyte proliferation, although the functions of specific integrins remain unclear. For example, expression of integrin α_9 is increased upon wounding, and keratinocytes in mice lacking epithelial integrin α_9 or β_1 exhibit reduced proliferation, suggesting a potential role for integrin $\alpha_9\beta_1$ in keratinocyte proliferation; however, re-epithelialization is not substantially affected in these animals [77]. In contrast, integrin $\alpha_v\beta_8$ is expressed specifically in non-proliferating suprabasal keratinocytes and not in the proliferating basal cells, suggesting a possible role in inhibiting keratinocyte proliferation [77].

While re-epithelialization progresses, the granulation tissue begins to form. This tissue is composed of ECM molecules, including embryonic fibronectin, type III collagen, type I collagen, and hyaluronic acid, along with multiple cell types, such as monocytes/macrophages, lymphocytes, fibroblasts, myofibroblasts, and the endothelial cells of the wound vasculature. Growth factors released by these cells and platelets cooperate with the aforementioned surrounding ECM molecules to provide pro-proliferative signals to the granulation tissue fibroblasts and endothelial cells. Endothelial cells proliferate during angiogenesis, a process that is dependent upon growth factors, such as FGFs and VEGFs, and their interactions with matrix molecules. Signaling induced by both FGFs and VEGFs is enhanced by HSPG, as discussed earlier. In addition, VEGF binding to fibronectin or tenascin enhances its effect on endothelial cell proliferation [9]. Furthermore, some growth factors appear to promote proliferation only when specific ECM molecules are present, as is seen in the fibronectin requirement for TGF-β1-mediated fibroblast proliferation [109]. In contrast, SPARC and thrombospondin inhibit proliferation induced by VEGF and bFGF, indicating that interactions between growth factors and ECM can also be inhibitory [14,110]. While ECM-growth factor

interactions can significantly impact cell proliferation, specific ECM molecules, such as laminin, also affect proliferation directly [61]. Previous studies suggest that the proliferative ability of laminin is mediated by its EGF-like domains, implicating EGFR activation in its pro-proliferative effects [21]. In addition, certain ECM molecules and/or proteolytic fragments can inhibit proliferation; SPARC and decorin, as well as peptides derived from SPARC, decorin, collagen IV (tumstatin), and collagen XVIII (endostatin) are anti-angiogenic due to their inhibitory effects on endothelial cell proliferation [7,14].

DIFFERENTIATION

As the granulation tissue forms, some of the fibroblasts within the wounded area differentiate into myofibroblasts, cells that express the protein α-smooth muscle actin (αSMA) and thus function similarly to smooth muscle cells; they also secrete a number of matrix molecules, including fibronectin and collagen I [111]. Myofibroblast differentiation is influenced by various matrix molecules, such as heparin, which decreases fibroblast proliferation while stimulating αSMA expression *in vitro* [112]. The effects of heparin on myofibroblast differentiation and αSMA expression likely result from the ability of heparin and heparan sulfate proteoglycans to interact with cytokines and/or growth factors like TGF-β1, which then modulate myofibroblast differentiation [113]. TGF-β1-induced differentiation also requires the ED-A-containing form of fibronectin and the binding of the ED-A domain to integrin $\alpha_4\beta_7$ [111,114]. Maintenance of myofibroblast differentiation is mediated by hyaluronan, as shown by reduced differentiation when hyaluronan synthesis is inhibited [115]. Interstitial collagens, in conjunction with mechanical tension, also participate in the differentiation process. Fibroblasts cultured on relaxed collagen gels fail to differentiate, whereas fibroblasts grown on stiffened collagen matrices exhibit myofibroblast characteristics [116]. In addition, increased myofibroblast differentiation is observed when splints are used to increase mechanical tension in wounds, suggesting a role for mechanical tension in myofibroblast differentiation *in vivo* [111]. Tensile stress may regulate cell signaling and cell-matrix interactions by revealing cryptic sites in intact matrix molecules; for example, the ED-A domain of fibronectin, which is necessary for TGF-β-induced myofibroblast differentiation, could be exposed by mechanical stress [116]. Tensile stress exerted on the cell by the matrix may also induce the formation of stress fibers that exert intracellular tension on the integrin, changing its conformation and strengthening its adhesion to the matrix, promoting downstream signaling [117].

APOPTOSIS

Late in the wound healing process, the granulation tissue undergoes remodeling to form scar tissue. This remodeling phase is characterized by decreased tissue cellularity due to the disappearance of multiple cell types, including fibroblasts, myofibroblasts, endothelial cells, and pericytes, and by the accumulation of ECM molecules, particularly interstitial collagens. The observed reduction in cell numbers during the remodeling phase occurs due to apoptosis; many of these apoptotic cells are endothelial cells and myofibroblasts, as shown by studies using *in situ* DNA fragment end-labeling in conjunction with transmission electron microscopy [118]. Myofibroblast apoptosis is regulated by mechanical tension; using a splint to maintain tension in a healing wound inhibits myofibroblast apoptosis, whereas the release of tension in this model promotes apoptosis [116]. Apoptosis of fibroblasts and myofibroblasts may be important in preventing excessive scarring and facilitating the resolution of wound healing. Indeed, there is a decreased apoptosis of these cells in keloids and hypertrophic scars, leading to increased matrix deposition and scarring [111]. In keloids, reduced apoptosis may result from p53 mutations or growth factor receptor over-expression [119–121]. In hypertrophic scars, however, the reduced apoptosis may result from increased expression of tissue transglutaminase, resulting in enhanced matrix degradation and diminished collagen contraction [122].

SIGNAL TRANSDUCTION EVENTS DURING CELL-ECM INTERACTIONS

As discussed above, ECM molecules are capable of interacting with a variety of receptors. Such interactions activate signal transduction pathways within the cell, altering levels of both gene expression and protein activation, thus ultimately changing outcomes in cell adhesion, migration, proliferation, differentiation, and death. The signaling pathways linked to these specific outcomes have been studied for many of the ligand-receptor interactions, particularly those involving integrins [10]. In the case of integrins, it is important to remember that these receptors can participate in both 'outside-in' and 'inside-out' signaling. Outside-in signaling occurs when an extracellular ligand binds the receptor and initiates intracellular signaling, and in inside-out signaling, intracellular signaling increases the affinity of the receptor for its ligand. Binding of the receptor to the ligand, in turn, initiates outside-in signaling. Unless otherwise indicated, the signaling events discussed below refer to outside-in signaling.

Based upon the many studies that investigate matrix-induced signaling, we postulate the existence of three categories of cell-ECM interactions, namely type I interactions that are involved in adhesion and migration, type II interactions involved in proliferation, differentiation, and survival, and type III interactions involved in apoptosis and epithelial-mesenchymal transition.

Type I interactions

These are generally mediated by integrin and proteoglycan receptors, and are important in the adhesion/de-adhesion processes that accompany cell migration. These interactions are exemplified by fibronectin-mediated cell migration, which occurs when this matrix molecule simultaneously binds integrins and proteoglycan receptors, the latter via its heparin-binding domain [123]. These receptors then co-localize and interact at cell adhesion sites, where the microfilaments interact with the cytoplasmic domain of integrin β_1 through the structural proteins talin and α-actinin and the signaling molecule paxillin, which interacts with focal adhesion tyrosine kinase (FAK) [124]. Binding of an integrin to its ligand triggers auto-phosphorylation of FAK on tyrosine 397; this then serves as the binding site for the SH2 domain of the c-Src tyrosine kinase. This kinase subsequently phosphorylates multiple proteins present in the focal adhesion plaques, including FAK itself at position 925, which increases its activity, and paxillin, tensin, vinculin, and $p130^{cas}$ [125]. FAK PY925 binds the Growth factor receptor-bound protein 2 (Grb2)/Son of Sevenless (Sos) complex, thus promoting the activation of Ras GTPase and the MAP kinase cascade, which may be involved in cell adhesion/de-adhesion and migration events [126]. Paxillin may also participate in integrin-mediated signaling and motility, as evidenced by the reduced migration and decreased phosphorylation/activation of various signaling molecules observed in paxillin-deficient fibroblasts, and the impaired migration seen in cells over-expressing a paxillin mutant unable to be phosphorylated by FAK/Src [127]. Both phosphorylated paxillin and p130Cas bind Crk, an adaptor protein bound to DOCK180, a guanine nucleotide exchange factor (GEF) that activates Rac1, while paxillin is also able to recruit another Rac1/Cdc42 GEF, β-PIX, thereby inducing lamellipodia formation and cell migration [125,128]. Src-mediated signaling also activates another Rac GEF, Vav2, along with a Rho GTPase activating protein (GAP) that inhibits RhoA. Interestingly, integrin-induced Src can phosphorylate and trans-activate the EGFR, leading to additional pro-migratory signaling [128].

The contribution of tensin to cell adhesion and motility is poorly understood, although it is known to interact with the cytoskeleton and various phosphorylated signaling molecules via its SH2 domain; therefore, tensin may facilitate various signaling events downstream of integrin ligation [129]. In cancer cells, tensin-1 interacts with a RhoGAP, and tensin mutations that blocked RhoGAP interaction decreased levels of Rho-GTP and reduced cell migration, suggesting a potential role in normal integrin-mediated signaling [130].

To this point, we have discussed the roles of FAK and Src in outside-in, traditional receptor-mediated signal transduction. However, these kinases can participate in inside-out signaling as well; FAK/Src signaling stimulates integrin activation, cell adhesion to matrix molecules, and focal adhesion strengthening [131]. Integrin activation and matrix binding then initiates outside-in signaling, promoting FAK/Src activity, suggesting a positive feedback loop of integrins and FAK/Src kinases that may facilitate directional migration.

Matrix receptors other than integrins, such as proteoglycans and DDR1, are also important in cell adhesion and migration. Syndecans can function as co-receptors, associating with matrix molecules and integrins to promote cell adhesion and migration [24]. Syndecan-4, for example, can bind the heparin-binding domain of fibronectin and the fibronectin receptor integrin $\alpha_5\beta_1$, leading to activation of PKC and downstream activation of Rac and inactivation of RhoA, promoting migration [24,128,132]. Following fibronectin binding, syndecan-4-induced PKC and RhoG promote integrin $\alpha_5\beta_1$ internalization, thus inhibiting integrin-mediated signaling [25]. Proteoglycan receptors other than syndecans induce cell migration following ligand binding. For example, hyaluronan binding to CD44 induces phosphorylation of its intracellular domain, resulting in fibroblast migration, as inhibition of CD44 with blocking antibodies or by expression of phosphorylation-deficient CD44 blocked hyaluronan-induced fibroblast migration [26]. RHAMM, another hyaluronan receptor, facilitates fibroblast migration by regulating ERK1/2 activation downstream of CD44, and in smooth muscle cells, hyaluronan induces cell migration downstream of RHAMM via PI3K and Rac [26].

The roles of DDR1/2 in cell migration are less clear. DDR1 activation by collagen inhibits epithelial cell adhesion and migration induced by integrin ligation, likely through inhibition of Cdc42 and recruitment of the phosphatase SHP-2 [40]. In contrast, collagen-induced fibroblast migration depends upon DDR2 and downstream activation of MMP-2 and Erk1/2 [133].

Type II interactions

These involve processes in which the matrix-receptor interactions, in conjunction with growth factor or cytokine receptors, affect proliferation, survival, differentiation, and/or maintenance of the differentiated phenotype. These cooperative effects may occur in a direct manner, for example, by the direct interaction of EGF-like repeats present in certain ECM molecules with the EGF receptor, thereby promoting cell proliferation [15,17]. Indirect cooperative effects are better understood at this time, particularly with regards to the anchorage dependence of cell growth. S-phase entry, even when growth factors are present, requires the interaction of cells with a substrate, while detachment of cells from matrix promotes anoikis, a type of apoptosis, underscoring the critical role of cell-ECM adhesion in cell survival and proliferation [124]. The pro-survival function of matrix interactions is mediated by Fak signaling. In fibroblasts, Fak/Src-induced p130CAS activity leads to the activation of Rac, as mentioned above, which then promotes cell survival and proliferation via the JNK pathway [125,134]. In epithelial cells, Fak/Src complexes promote cell survival through PI3K-induced Akt activation [135,136]. Signaling downstream of Akt then increases levels of anti-apoptotic Bcl family members while decreasing levels of pro-apoptotic Bcl family members, resulting in cell survival [135]. In addition to promoting cell survival, integrin signaling can promote cell proliferation, either alone or in conjunction with growth factor signaling. Integrin-induced activation of the Rac/JNK pathway can induce cell proliferation by stimulating expression of cyclin D and by promoting the degradation of p21, a cell cycle inhibitor [137,138]. ERK1/2, also MAP kinases, can be activated by integrin ligation, which promotes the activation of Fyn and its binding to the Shc adaptor protein, which then recruits Grb2/Sos and activates the Ras/ERK pathway, resulting in the phosphorylation of the transcription factor Elk-1 and the activation of genes important in cell cycle progression [124,137].

Integrin signaling can also induce cell proliferation in conjunction with growth factor receptor signaling [9]. Angiogenesis induced by the growth factors bFGF and VEGF requires the presence and activation of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively [59]. In both cases, integrin signaling and FAK are necessary for activation of Erk1/2, but Erk1/2 activation by bFGF/$\alpha_v\beta_3$ is dependent upon Pak-1-induced Raf phosphorylation but not Ras, while Erk1/2 activation by VEGF/$\alpha_v\beta_5$ requires Ras [139]. Furthermore, cell-ECM interactions are critical for the efficient and prolonged activation of MAPK by growth factors, likely participating in MAPK-induced proliferation [140]. Cell-matrix interactions can also facilitate proliferation through transactivation of growth factor receptors, as in fibronectin-induced, Src-mediated EGFR activation [124]. This EGFR transactivation is required for fibronectin-induced Erk activation, regulating genes involved in cell cycle progression, including Rb and cyclin D; however, fibronectin-induced EGFR transactivation is not sufficient for cell division, but requires growth factor-induced signaling to induce expression of genes necessary for S phase [141].

Cell-matrix interactions can also regulate growth factor receptor signaling through integrin or matrix binding to growth factors or their receptors and by modulating downstream signaling [22]. Multiple VEGF isoforms bind and activate integrin $\alpha_9\beta_1$, which, along with VEGFR2, is necessary for VEGF-induced paxillin and Erk phosphorylation and angiogenesis [142]. Similarly, integrin $\alpha_v\beta_3$ interacts with both IGF-1 and FGF-1 and is required for proliferation induced by both growth factors [124]. Several growth factor receptors interact with integrins, including VEGFR2 and EGFR, and integrin-mediated adhesion can regulate growth factor-induced proliferation [124]. Growth factor signaling, in turn, can activate associated integrins and promote matrix adhesion. For example, VEGFR2 signaling induces phosphorylation of integrin β_3, leading to activation of Fak and p38 as well as VEGFR2-integrin binding and enhanced VEGFR2 signaling [106].

As mentioned earlier, matrix molecules themselves can interact with growth factors and/or their receptors [9]. Fibronectin and collage interact with VEGF; binding to fibronectin increases VEGFR activation and endothelial cell proliferation, whereas binding to collagen activates a phosphatase and thus decreases VEGFR activity [106]. HSPG can interact with many growth factors, including FGF and VEGF, limiting their diffusion, sequestering them until enzyme-mediated release, or promoting their receptor binding. FGF and VEGF binding to HSPG facilitates binding to their respective receptor and activates mitogenic signaling [64]. Matrix molecules can also bind growth factor receptors and activate them directly; for example, the EGF-like domains of laminin and tenascin-C bind and activate the EGFR, which may promote cell proliferation [9]. In contrast, decorin binding to the EGFR promotes EGFR internalization, and thus inhibits mitogenic signaling downstream of this receptor [64].

Activation of several non-integrin matrix receptors, including CD44, RHAMM, and DDR2, are associated with cell survival and proliferation. Hyaluronan promotes Erk activation and fibroblast proliferation through RHAMM and endothelial cell proliferation through CD44 [26,27]. Low molecular weight hyaluronan fragments also activate CD44, leading to Erk activation, cyclin expression, and proliferation in smooth muscle cells [143]. In addition, the collagen receptor DDR2 promotes fibroblast and chondrocyte proliferation, although the signaling that underlies this mitogenic effect has not been elucidated [133].

Similarly, cellular differentiation also relies upon cell interactions with ECM molecules, hormones, and growth factors. For example, the binding of laminin to integrin $\alpha_2\beta_1$ in endothelial cells promotes the formation of capillary-like structures [61], whereas the binding of fibronectin to integrin $\alpha_5\beta_1$ in these cells leads to cell proliferation [124]. Additional signaling molecules are required to generate the capillary-like tubes; one such molecule is integrin-linked kinase (ILK), which, when over-expressed, rescues capillary-like tube formation in the absence of ECM molecules [144], while expression of a dominant negative version of ILK blocks tube formation even when ECM and VEGF are present [145]. Once nascent vessels are formed, smooth muscle cells called pericytes are recruited to stabilize the

endothelium and promote synthesis of the basement membrane. Pericyte differentiation relies upon cell-matrix interactions, as shown by impaired differentiation and vessel stabilization/maturation in a mural cell-specific integrin β_1 knockout [146]. TGF-β-induced myofibroblast differentiation depends upon integrin $\alpha_4\beta_7$ binding to the EDA domain of fibronectin and downstream activation of FAK, PI3K, and associated signaling pathways [8].

Non-integrin receptors such as CD44 also regulate cellular differentiation. Hyaluronan induces keratinocyte differentiation *in vitro* in a CD44-dependent manner, and CD44 deficiency inhibits keratinocyte differentiation *in vivo*, suggesting a role for hyaluronan in this process [147]. Similarly, TGF-β-induced myofibroblast differentiation requires hyaluronan [26].

Type III interactions

Type III interactions primarily involve processes leading to apoptosis and epithelial-to-mesenchymal transitions (Fig. 2 III). Normally adherent cells that fail to interact with matrix molecules undergo anoikis, a form of cell death. Anoikis results from a lack of pro-survival Fak/Src-induced PI3K/Akt or Erk signaling, coupled with pro-apoptotic signaling that inhibits anti-apoptotic Bcl family members, increases expression/activity of pro-apoptotic Bcl family members, and recruits and activates caspase 8 [147]. The mechanism involved in caspase 8 recruitment and activation by unligated integrins is unclear; one possibility is that caspase 8 is normally phosphorylated and inhibited by Src, and that the absence of integrin-activated Src signaling relieves caspase 8 inhibition [148]. In many cases, integrins that do interact with ligands can still promote apoptosis. For example, CCN1 binding to syndecan-4 and integrin $\alpha_6\beta_1$ induces expression of Bax, which promotes cytochrome C release from the mitochondria and subsequent caspase 9 activation, leading to fibroblast apoptosis [149]. In endothelial cells, thrombospondin interaction with CD36 induces a pro-apoptotic pathway culminating in Fas ligand (FasL) expression; FasL then binds Fas, a death domain receptor that induces apoptosis [110].

Alterations in the ligand presentation by ECM can also regulate apoptosis. Studies have suggested that integrin ligation by soluble, rather than intact, ligands can function as integrin antagonists and promote apoptosis rather than survival or proliferation [66]. Many of these soluble ligands can be created by matrix degradation during tissue remodeling. Endostatin, derived from collagen XVIII, and tumstatin, derived from collagen IV, promote endothelial cell apoptosis by interacting with $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, respectively [150]. The apoptosis stimulated by soluble ligands or other antagonists appears to occur via the recruitment and activation of caspase 8 by clustered integrins and decreased expression of pro-survival Bcl family members, without any requirement for death receptors [150].

Although these three categories may not be exhaustive of the general types of cell-ECM interactions that occur during development and wound healing, they encapsulate the major interactions documented to date. Each category has its place in many developmental and repair events, and they may operate in sequence. A compelling example of the latter is the epithelial-to-mesenchymal transition and morphogenesis of the NCC system [52]. These cells originate in the neural epithelium that occupies the crest of the neural folds. After the delamination event that separates the neural epithelium from the epidermal ectoderm [151], the folds fuse to form the tube. At this time, the NCC occupy the dorsal-most portion of the tube, they are not covered by basal lamina, and the subepidermal space above them contains large amounts of fibronectin [152]. Just before the NCC emigrate from the neural tube, fibronectin appears between them, they separate from each other, and migrate away carrying fibronectin on their surfaces [153]. During the period of emigration at any particular level of the neural tube, basal lamina is deposited progressively toward the crest from the sides of the tube [152,154]. NCC emigration terminates as deposition reaches the crest of the tube. The NCC then follow specific migration pathways throughout the embryo, arriving at a wide

variety of locations where they differentiate into many different phenotypes in response to external cues [51].

The appearance of fibronectin between the NCC just before emigration must be the result of secretion by the adjacent cells or introduction from the epithelial cells after loss of cell-cell adhesions. In keeping with the cell-ECM interaction mechanism of type III, either alternative could initiate a positive feedback loop and release the NCC, leading to emigration: Enzymatic degradation of the stabilizing domain of fibronectin above the tube could cause enhanced secretion of specific enzymes by the NCC in response to the effect of the cell-binding domain acting alone, thus severing the cell adhesions and producing additional fibronectin fragments containing the cell-binding domain. These fragments, in turn, would bind to adjacent cells and stimulate further enzymatic secretion that would be self-perpetuating. NCC emigration occurs in an anterior-to-posterior wave, thus, once enzymatic activity were initiated in the head of the embryo, it could propagate in a posterior direction, triggering NCC emigration in a wave from head to tail.

Clearly some controlling event(s) must terminate NCC emigration at each location along the neural tube. Such an event has already been identified. At the time of NCC emigration, the ventral and lateral surfaces of the neural tube are covered by an intact basal lamina, which stabilizes the epithelium and separates it from the fibronectin layer around the tube [154]. During the few hours of emigration at any one site, as the NCC are leaving from the dorsal-most portion of the neural tube, basal lamina deposition progresses quickly up the sides of the tube and terminates local emigration when it becomes complete over the crest of the tube [152,154]. After they have emigrated from the neural tube, the NCC find themselves in an extracellular space filled with intact fibronectin and other ECM molecules that stimulate the focal adhesions of cell-ECM interactions of type I, thereby providing the substrate for migration. Upon arrival at their final destination, further interactions of type II stimulate differentiation into a wide range of phenotypes [51].

RELEVANCE FOR TISSUE ENGINEERING

Designing tissue and organ replacements that closely simulate normal physiology is a challenging endeavor. One avenue to achieve this goal is to study how tissues and organs arise during embryogenesis and during normal processes of repair, and how those functions are maintained. When developing tissue replacements, one needs to consider the following (Fig. 9.3).

(1) Avoiding an immune response that can cause inflammation and/or rejection. One possibility would involve using autologous cells; however, it is time-consuming and labor-intensive to collect and expand autologous cells for use in engineered tissues, decreasing the practicality of this approach. Alternatively, engineered tissues could incorporate progenitor cells that may suppress host immune responses directly or indirectly through decreased expression of MHC; these cells could be induced at a later time to differentiate into various cell types [155]. One example of a progenitor cell that appears to decrease immune responses and also maintains a broad differentiation capacity is the mesenchymal stem cell, which is capable of differentiating into multiple cell types, and may thus prove to be an invaluable asset in tissue engineering [155].

(2) Creating the proper substrate for cell survival and differentiation. One of the strategies to fulfill this goal is the use of biocompatible implants composed of extracellular matrix molecules seeded with autologous cells or with heterologous cells in conjunction with immunosuppressant drugs. Addition of growth and differentiation factors to these matrices as well as agonists or antagonists that favor cell-ECM interactions can potentially increase the rate of successful tissue replacement. However, growth factor activity in the engineered tissue is dependent upon its stability and its ability to diffuse both within

the engineered tissue and in peripheral host tissue. In normal tissue, VEGF has a short half-life (30 minutes), so it would be useful to increase the half-life, and thus activity, in an engineered tissue [156]. There are several methods that have been used to increase the duration and activity of growth factors: by adding components of the normal ECM that stabilize these factors and/or promote their activity, by covalently coupling the growth factors to a matrix molecule or biomaterial, and by removing proteolytic cleavage sites from growth factors to inhibit their degradation [59]. In the case of VEGF, covalent linkage to a fibrin matrix or addition of heparin and/or fibronectin fragments to the matrix promotes angiogenesis to a greater extent than addition of VEGF to a fibrin matrix alone [59]. In addition to restricting VEGF diffusion, heparin and fibronectin fragments likely promote VEGF signaling [92,106]. Preventing proteolytic cleavage of VEGF also increases its half-life and activity. Chronic wounds frequently exhibit excessive protease levels that would cleave wild type VEGF and limit its activity; in a mouse model of impaired healing, VEGF lacking proteolytic sites promoted VEGF duration and activity, leading to improved angiogenesis and healing [59]. It is possible that combinations of matrix molecules/fragments and inhibition of proteolytic activity could be even more effective in stimulating angiogenesis and wound healing in impaired wounds.

While the foregoing examples show that ECM molecules can be used successfully in tissue engineering, the use of natural ECM molecules in engineered tissue has several disadvantages, including the possibility of generating an immune response, possible contamination, and ease of degradation. Likewise, artificial biocompatible materials have drawbacks in that, unlike ECM, they are generally incapable of transmitting growth and differentiation cues to cells. Much attention is now devoted to the design of 'semi-synthetic biomaterials' in which functional regions of ECM molecules, including those that interact with receptors or growth factors or those that are cleaved by proteases, are incorporated into artificial biomaterials to impart additional functionality [59]. The inclusion of ECM-like cell-binding sites that promote cell adhesion, growth, and/or differentiation into such biomaterials may be critical in developing and maintaining functional engineered tissues by providing the appropriate cellular microenvironment. For example, because many integrins interact with the RGD motif on matrix molecules, it can be used as an agonist to make synthetic implants more biocompatible and to allow the development of tissue structure, or as an antagonist to prevent or moderate unwanted cell-ECM interactions. Similarly, collagen, fibronectin, and gelatin have been used to coat synthetic biomaterials to increase their biocompatibility and promote successful biological interactions [157]. However, the use of these biomaterials in engineered tissues requires additional knowledge regarding the types of cell-ECM interactions that result in the desired cellular effects.

(3) Providing the appropriate environmental conditions for tissue maintenance. To maintain tissue homeostasis, it is crucial to create a balanced environment with the appropriate cues for preservation of specific cell function(s). It is important to realize that such stasis on the level of a tissue is achieved via tissue remodeling — the dynamic equilibrium between cells and their environment. However, relatively little is known about the crosstalk between cells and ECM under such 'normal' conditions. As indicated above, the same ECM molecule may have multiple cellular effects; the ultimate cellular outcome likely depends upon the combination of variables, such as the domain of the molecule involved in the cellular interactions, the receptor used for these interactions, and the cellular microenvironment. These variables can, in turn, be influenced by matrix remodeling, as enzymatic degradation of the ECM can release functional fragments of ECM that then alter cell-ECM interactions by removing certain binding sites while exposing others. Another important consideration is the role of mechanical tension on matrix signaling, and, depending upon the desired effect, it may be necessary to control the stiffness and pore size of the biomaterial. One biomaterial that is 'tunable' in terms of stiffness and pore size is starPEG, which can use heparin as a functional crosslinker.

Altering the concentration or molecular weight of the starPEG changes its stiffness and pore size, and can thus change the mechanical tension exerted on cells, while the use of heparin as a crosslinker yields additional functionality due to its ability to bind growth factors and enhance their signaling [158]. Combinations of this hybrid biomaterial with RGD and growth factors increased endothelial cell survival and tube-like organization, suggesting the potential of this approach in designing engineered tissues [158].

Because organ transplantation is one of the least cost-effective therapies and is not always available, tissue engineering offers hope for more consistent and rapid treatment of those in need of body part replacement, and therefore has greater potential to improve patient quality of life. The selected examples presented above illustrate that further advances in tissue engineering require additional knowledge of the basic mechanisms of cell function and of the ways they interact with the environment. The recent surge in research on ECM molecules themselves and their interactions with particular cells and cell-surface receptors has led to realization that these interactions are many and complex, allow the modulation of fundamental events during development and wound repair, and are crucial for the maintenance of the differentiated phenotype and tissue homeostasis. As such, the manipulation of specific cell-ECM interactions has the potential to modulate particular cellular functions and processes in order to maximize the effectiveness of engineered tissues.

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CHAPTER 10

Matrix Molecules and Their Ligands

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INTRODUCTION

Cellular growth and differentiation, in two-dimensional cell culture as well as in the three-dimensional space of the developing organism, requires the presence of a structured environment with which the cells can interact. This extracellular matrix (ECM) is composed of polymeric networks of several types of macromolecules in which smaller molecules, ions and water are bound. The major types of macromolecules are polymer-forming proteins, such as collagens, elastin, fibrillins, fibronectin and laminins, and hydrophilic heteropolysaccharides, such as glycosaminoglycan chains in hyaluronan and proteoglycans. It is the combination of protein polymers and hydrated proteoglycans that give extracellular matrices their resistance to tensile and compressive mechanical forces.

The macromolecular components of the polymeric assemblies of the ECM are in many cases secreted by cells as precursor molecules that are significantly modified (proteolytically processed, oxidized and cross-linked) before they assemble with other components into functional polymers (Fig. 10.1). The formation of matrix assemblies *in vivo* is therefore in most instances a unidirectional, irreversible process and the disassembly of the matrix is not a simple reversal of assembly, but involves multiple, highly regulated processes. One consequence of this is that polymers reconstituted in the laboratory with components extracted from extracellular matrices do not have all the properties they have when assembled by cells *in vivo*. The ECM *in vivo* is also modified by cells as they proliferate, differentiate, and migrate, and cells in turn continuously interact with the matrix and communicate with each other through it [1].

The ECM is therefore not an inert product of secretory activities, but influences cellular shape, fate, and metabolism in ways that are as important to tissue and organ structure and function as the effects of many cytoplasmic processes. This realization has led to a reassessment of the need for a detailed molecular understanding of ECM. In the past, the ECM was primarily appreciated for its challenge to biochemists interested in protein and complex carbohydrate structure; a detailed characterization of ECM constituents is now considered essential for understanding cell behavior in the context of tissue and organ development and function. Some of these constituents are obviously most important for their structural properties (collagens and elastin), while others (fibronectin, fibrillin, laminin, thrombospondin, tenascin, perlecan and other proteoglycans) are multidomain molecules that are both structural constituents as well as regulators of cell behavior (Fig. 10.1). In a third category are matrix-bound signaling molecules (such as matrix-bound fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs)).

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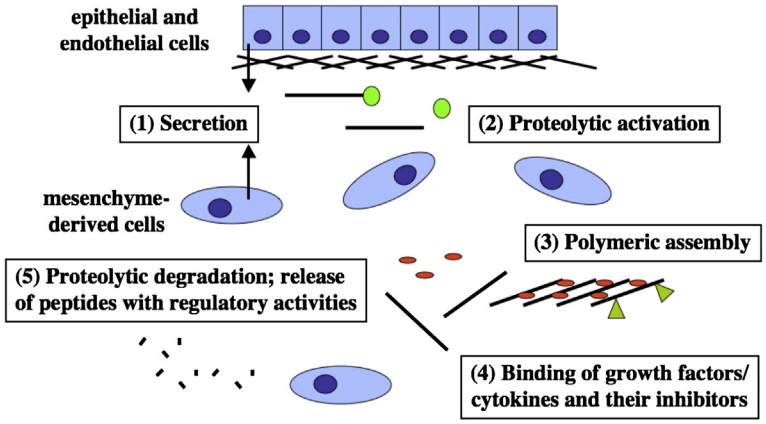

FIGURE 10.1

The life cycle of extracellular matrix molecules. Soluble matrix molecules are secreted by cells, modified by proteolysis, and assembled into polymeric complexes. These complexes serve as scaffolds for cells and as binding sites for small molecules such as growth and differentiation factors. Depending on the growth factor and cellular context, this may either inhibit or stimulate growth factor activity. Degradation of the scaffolds, during normal tissue turnover or during wound healing, may release bound growth factors and/or release peptide fragments from the larger scaffold proteins; such fragments may bind to cellular receptors and regulate cellular behavior.

COLLAGENS — MAJOR CONSTITUENTS OF ECM

Fibrillar collagens are major tissue scaffold proteins

Collagens constitute a large family of proteins that represent the major proteins (about 25%) in mammalian tissues [2]. A subfamily of these proteins, the fibrillar collagens, contains rigid, rod-like molecules with three subunits, α-chains, folded into a right-handed collagen triple helix. Within a fibrillar collagen triple-helical domain, each α-chain consists of about 1,000 amino acid residues and is coiled into an extended, left-handed polyproline II helix; three α-chains are in turn twisted into a right-handed superhelix (Fig. 10.2). The extended conformation of each α-chain does not allow the formation of intrachain hydrogen bonds; the stability of the triple helix is instead due to interchain hydrogen bonds. Such interchain bonds can only form if every third residue of each α-chain does not have a side chain and is packed close to the triple-helical axis. Only glycine residues can therefore be accommodated in this position. This explains why the amino acid sequence of each α-chain in fibrillar collagens consists of about 300 Gly-X-Y tripeptide repeats, where X and Y can be any residue, but Y is frequently proline or hydroxyproline. It also provides an explanation for why mutations in collagens that lead to a replacement of triple-helical glycine residues with more bulky residues can cause severe abnormalities.

Fibrillar collagen molecules are the major components of collagen fibrils. Their α-chains are synthesized as precursors, pro-α-chains, with large propeptide regions flanking the central triple-helical domain. The carboxyl propeptide (C-propeptide) is important for the assembly of trimeric molecules in the rough endoplasmic reticulum (RER). Formation of C-propeptide trimers, stabilized by intra- and interchain disulfide bonds, is the first step in the intracellular assembly and folding of trimeric procollagen molecules [3–5]. The folding of the triple-helical domain at body temperature requires post-translational hydroxylation of about 50% of the prolyl residues by prolyl hydroxylases, and this proceeds in a zipper-like fashion from the carboxyl towards the amino end of procollagen molecules. Mutations in fibrillar procollagens that affect the structure and folding of the C-propeptide domain are therefore likely to affect the participation of the mutated chains in triple-helical assemblies. In contrast, mutations upstream of the C-propeptide, such as in-frame deletions or glycine substitutions

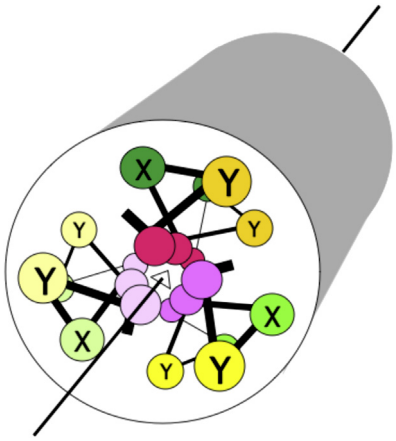

FIGURE 10.2
Diagram showing a segment of a triple-helical collagen molecule. The triple helix is composed of three left-handed helices (α-chains) that are twisted into a right-handed superhelix. The sequence of each α-chain is a repeat of the tripeptide Gly-X-Y. The Gly residues are packed close to the triple-helical axis (indicated by a line through a triangle). Only glycine (without a side chain) can be accommodated in this position. Although any residue can fit into the X- and Y-positions, Pro is frequently found in the Y-position.

in the triple-helical domains, exert a dominant negative effect, in that the mutated chains will participate in trimer assembly, but will interfere with subsequent folding of the triple-helical domain.

Fibrillar procollagen chains are the products of 11 genes. The similarities between these genes suggest that they arose by multiple duplications from a single ancestral gene. Despite their similarities and the high degree of sequence identity between their protein products, they exhibit specificity in the interactions of their C-propeptides during intracellular trimeric assembly in the RER. Thus, a relatively small number of chain combinations are found among triple-helical procollagens; these combinations represent fibrillar collagen types.

Collagens V/XI — regulators of fibril assembly, spatial organization and cell differentiation

Some collagen types are heterotrimers (types I, V, and XI), while others are homotrimers (types II, III, XXIV, and XXVII). Some chains participate in more than one type: For example, the α1(II) chain (encoded by the COL2A1 gene) forms the homotrimeric collagen II, but is also one of three different chains in collagen XI molecules. Between collagens V and XI there is extensive sharing of polypeptide subunits, and fibrillar collagen molecules previously described as belonging to either collagen V and XI are now referred to as belonging to the V/XI type. Thus, fibrillar procollagen molecules secreted by cells are members of a group of homologous proteins. They all contain a C-propeptide that is completely removed by an endoproteinase (BMP-1/tolloid) after secretion, and their triple-helical rod-like domains polymerize in a staggered fashion into fibrillar arrays (Fig. 10.3). They differ, however, in the structure of their amino propeptide (N-propeptide) domains and in the extent to which this domain is proteolytically removed. For some collagen types such as collagens I and II, the N-propeptide processing (by ADAMTS2, ADAMTS3 and ADAMTS14 proteinases) is complete in molecules within mature fibrils. For other types, such as collagens V/XI this is not the case, in that a large portion of the N-propeptides in these molecules remain attached to the triple-helical domain (Fig. 10.3). The incomplete processing of type V/XI molecules allows them to serve as regulators of fibril assembly. Collagen fibrils are heterotypic, i.e., they contain more than one collagen type, such that collagen I fibrils in skin, tendon, ligaments and bone contain 5—10% collagen V and collagen II fibrils in cartilage contain 5—10% collagen XI. The presence

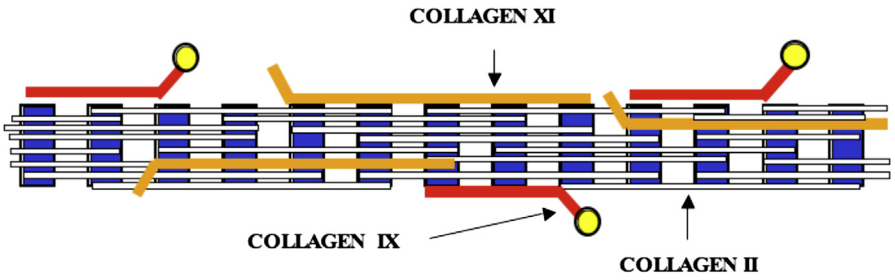

FIGURE 10.3
Diagram of a cartilage collagen fibril. Collagen II molecules are the major components. Molecules of collagen XI and IX are located on the surface. Collagen XI molecules, heterotrimers of three different α-chains, have amino-terminal domains that are thought to sterically block the addition of collagen II molecules at the fibril surface.

of N-propeptide domains on V/XI molecules represents a steric hindrance to addition of molecules at fibril surfaces. This heterotypic/steric hindrance model predicts that collagen fibril diameters in a tissue are determined by the ratio of the 'minor' component (V/XI) to the 'major' component (I or II). A high ratio results in thin fibrils; a low ratio results in thick fibrils. Direct support for this comes from studies of mutant and transgenic mice. For example, mice that are homozygous for a functional null mutation in α1(XI) collagen and transgenic mice overexpressing collagen II have cartilage collagen fibrils that are abnormally thick [6,7].

A characteristic feature of collagen fibrillar scaffolds is their precise three-dimensional patterns. These patterns follow mechanical stress lines and ensure a maximum of tensile strength with a minimum of material. Examples are the criss-crossing lamellae of collagen fibers in lamellar bone or in cornea, the arcades of collagen fibrils under the surface of articular cartilage and the parallel fiber bundles in tendons and ligaments. Ultimately, cells are responsible for establishing these patterns, but the cellular mechanisms involved are only beginning to be understood. A study by Canty et al. [8] suggests that the orientation of collagen fibrils in the extracellular space is linked to the cytoskeletal organization induced by cellular responses to mechanical stress. In tendon fibroblasts, Golgi-to-plasma transport carriers of collagen are formed on the exit side of the trans-Golgi network and move along cytoskeletal 'tracks' into long cytoplasmic extensions. Collagen fibrils, forming inside the carriers, are oriented along the longitudinal axis of the carriers. When the membrane at the distal tip of the carrier fuses with the cell membrane covering the tip of the extension, the space within the carrier becomes continuous with the extracellular space and the fibrils are, in effect, moved from an intracellular to an extracellular compartment. Thus, the parallel orientation of collagen fibrils in a tendon is a consequence of the polarized structure, intracellular movement, and polarized exocytosis of fibril-containing Golgi-derived transport carriers. This cellular mechanism for orientation of collagen fibrils is consistent with data showing that the same kind of heterotypic fibril can be part of scaffolds with very different spatial organization. Transgenic mice with an alteration in the N-propeptide region of collagen V molecules show a disruption of the lamellar arrangement of fibrils in the cornea of the eye, suggesting a role for fibril surface domains in generating and/or stabilizing the spatial pattern [2,9]. Finally, members of a unique subfamily of collagens, FACIT collagens (see below), are good candidates for molecules that modulate the surface properties of fibrils and allow tissue-specific fibril patterns to be generated and stabilized by cells.

The phenotypic consequences of mutations in fibrillar collagen genes indicate that a major function of these proteins is to provide elements of high tensile strength at the tissue level. Thus, mutations in COL1A1 or COL1A2, the human genes encoding the α1 and α2 subunits of fibrillar collagen I (in bone, ligaments, tendons and skin), cause osteogenesis imperfecta (brittle bone disease) or clinical forms of Ehlers-Danlos syndrome, characterized by skin hyperextensibility and fragility and joint hypermobility, with or without bone abnormalities [10,11]. Mutations in COL2A1, the gene encoding the α-chains of collagen II (in cartilage) cause a spectrum of human disorders, ranging from lethal deficiency in cartilage formation to relatively mild deficiencies in cartilage mechanical properties and function. Fibrillar collagens also have regulatory functions. For example, mutations in collagen V/XI genes suggest that

fibrillar collagen scaffolds are essential for normal cellular growth and differentiation. A functional null mutation in α1(XI) collagen, resulting in complete lack of collagen XI in cartilage, causes a severe disproportionate dwarfism in mice and perinatal death of homozygotes [7]. Histology of mutant long bone growth plates reveals a disorganized spatial distribution of cells and a defect in chondrocyte differentiation to hypertrophy. The explanation for this is likely related to the fact that proliferation and differentiation of chondrocytes in growth plates is regulated by locally produced growth factors and cytokines. Cells that produce these factors are localized close to cells that express the appropriate receptors. Lack of collagen XI may disrupt this relationship since it results in a dramatic decrease in cohesive properties of the matrix and a loss of cellular organization. Transgenic mice with a mutation in α2(V) collagen have a large number of hair follicles of unusual localization in the hypodermis; this may be related to a defect in the mechanical properties of the fibrillar collagen scaffold but could also be mediated by an effect on extracellular signaling molecules [9].

FACIT collagens — modulators of collagen fibril surface properties

Molecules that are associated with collagen fibrils, contain two or more triple-helical domains, and share characteristic protein domains (modules) with each other as well as with other non-collagenous matrix molecules (thrombospondin-1, fibronectin, von Willebrand factor) are classified as FACIT collagens [2,12]. Of the eight known members in the group (collagens IX, XII, XIV, XVI, XIX, XX, XXI, and XXII), collagen IX is the best characterized both structurally and functionally. Collagen IX molecules are heterotrimers of three different gene products [13]. Each of the three α-chains (α1(IX), α2(IX) and α3(IX)) contains three triple-helical domains separated and flanked by non-triple-helical sequence regions (Fig. 10.4). Between the

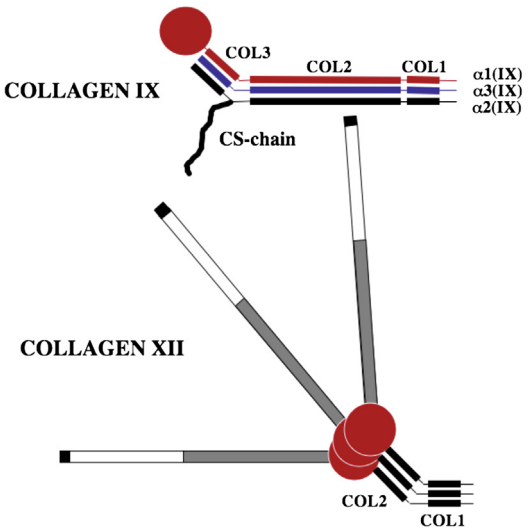

FIGURE 10.4

Diagrams of collagen IX and XII (long form) molecules. Collagen IX molecules contain the three chains α1(IX), α2(IX, and α3(IX). Each chain contains three triple-helical domains (COL1, COL2, COL3), interrupted and flanked by non-triple-helical sequences. In cartilage, the α1(IX) chain contains a large globular amino-terminal domain. The α2(IX) chain serves as a proteoglycan core protein in that it contains a chondroitin sulfate (CS-) side chain attached to the non-triple-helical region between the COL2 and COL3 domains. Collagen XII molecules are homotrimers of α1(XII) chains. The three chains form two short triple-helical domains separated by a flexible hinge region. A central globule is composed of three globular domains that are homologous to the amino-terminal globular domain of α1(IX) collagen chains. The amino-terminal region of the three α1(XII) chains contain multiple fibronectin type 3 repeats and von Willebrand factor A-like domains. These regions form three 'fingers' that extend from the central globule. Through alternative splicing a portion of the 'fingers' (white region in diagram) is spliced out in the short form of collagen XII. Hybrid molecules with both long and short 'fingers' can be extracted from tissues.

amino-terminal and central triple-helical domains a flexible hinge gives the molecule a kinked structure with two arms. The hinge region in the α2(IX) chain contains a sequence for attachment of a chondroitin sulfate glycosaminoglycan side chain, making type IX collagen both a collagen and a proteoglycan (see below) matrix component [14]. The use of two alternative promoters in the α1(IX) collagen gene gives rise to two alternative forms of the protein, one containing a thrombospondin-like module in the amino-terminal region and one without this module [15].

Type IX molecules are located on the surface of type II/XI-containing fibrils with the long arm parallel to the fibril surface and the short arm projecting into the perifibrillar space [16] (Fig. 10.3). Collagen IX functions as a bridging molecule between fibrils, between fibrils and other matrix constituents and between fibrils and cells. Transgenic mice with a dominant-negative mutation in the α1(IX) chain [17], as well as mice that are homozygous for null alleles of the gene (Col9a1) coding for α1(IX) [18], exhibit osteoarthritis in knee joints and mild chondrodysplasia. In humans, mutations in the α1(IX), α2(IX) or α3(IX) collagen chains cause a form of multiple epiphyseal dysplasia, an autosomal dominant disorder characterized by early onset osteoarthritis in large joints associated with short stature and stubby fingers [19].

Molecules of collagens XII and XIV are homotrimers of chains that are made up of several kinds of modules. Some modules are homologous to triple-helical and thrombospondin modules found in collagen IX, while others show homology to von Willebrand factor A domains and fibronectin type 3 repeats. Both types of molecules contain a central globule with three finger-like extensions and a thin triple-helical tail attached (Fig. 10.4). For collagen XII, two forms that differ greatly in the lengths of the finger-like extensions are generated by alternative splicing of RNA transcripts. Variations in the carboxyl regions also occur [20]. Both collagens XII and XIV are found in connective tissues containing type I collagen fibrils, except mineralized bone matrix, and immunolabeling studies show a fibril-associated distribution. Type XIV collagen can bind to heparan sulfate and the small fibril-associated proteoglycan decorin [21,22]. This would suggest an indirect fibril-association. A direct association is also possible, since collagen XII molecules form copolymers with collagen I even in the absence of proteoglycans. A functional interaction between fibrils and collagens XII and XIV is implied by studies showing that addition of the two collagens to type I collagen gels promote gel contraction mediated by fibroblasts [23]. The effect is dose-dependent and can be prevented by denaturation or addition of specific antisera. The association of collagens XII and XIV with fibrils may therefore modulate the frictional properties of fibril surfaces. The synthesis of different isoforms could be important in this context, since they could bind to fibrils with different affinities. Also, since the long form of collagen XII is a proteoglycan with heparan sulfate side chains, whereas the short form is not, variations in the relative proportion of the two splice variants may serve to modulate the hydrophilic properties of interfibrillar matrix compartments. Finally, the discovery that the collagen I N-propeptide processing enzyme (see below) binds to collagen XIV and can be purified as part of a complex with antibodies against collagen XIV, suggests that the FACIT collagens provide binding sites for fibril-modifying extracellular matrix enzymes [24].

Thus, FACIT collagens have multiple functions is a variety of tissues. For example, collagen XVI [25] connects and organizes fibrillar networks in cartilage and skin, but may also serve as substrate for adhesion and migration of cells, including tumor cells [26]. Collagen XIX [27] is expressed in skin and muscle cells [28] and it contributes to synapse-formation in the hippocampus [29]. Collagen XX [30], smaller but similar in structure to collagens XII and XIV, is present in several connective tissues, including cartilage, tendon and cornea, and collagen XXI [31] is expressed in heart, stomach, kidney, skeletal muscle and placenta. Collagen XXII is located in basement membrane zones of myotendinous junctions in skeletal and heart muscle, at hair follicles and at articular cartilage/synovial membrane junctions [32].

Basement membrane collagens and associated collagen molecules

At epithelial- and endothelial-stromal boundaries, basement membranes serve as specialized areas of ECM for cell attachment. Collagen IV molecules form a network-like scaffold in basement membranes by end-to-end and lateral interactions [33]. The products of the six different collagen IV genes (in mammals) interact to form at least three different types of heterotrimeric collagen IV molecules. These different isoforms show characteristic tissue-specific expression patterns. The physiological importance of collagen IV isoforms is highlighted by Alport syndrome [34]. This disease, characterized by progressive hereditary nephritis associated with sensorineural hearing loss and ocular lesions, can be caused by mutations within α3(IV) and α4(IV) collagen genes (autosomal Alport syndrome) or mutations in α5(IV) collagen (X-linked Alport syndrome). In cases of large deletions including both the α5(IV) and the neighboring α6(IV) collagen gene, renal disease is associated with inherited smooth muscle tumors.

Within basement membranes, the collagen IV networks are associated with a large number of non-collagenous molecules, such as various isoforms of laminin, nidogen, and the heparin sulfate proteoglycan perlecan (Fig. 10.5 [33]). Additional collagens are also associated with basement membranes. These include the transmembrane collagen XVII in hemidesmosomes and collagen VII in anchoring fibrils [2]. Collagens XVII and VII are important in regions of significant mechanical stress, such as skin, in that they anchor epithelial cells to the basement membrane (collagen XVII) and strap the basement membrane to the underlying stroma (collagen VII) (Fig. 10.6). In bullous pemphigoid, autoantibodies against collagen XVII cause blisters that separate epidermis from the basement membrane; dominant and recessive forms of epidermolysis bullosa can be caused by mutations in collagens VII and XVII [35,36].

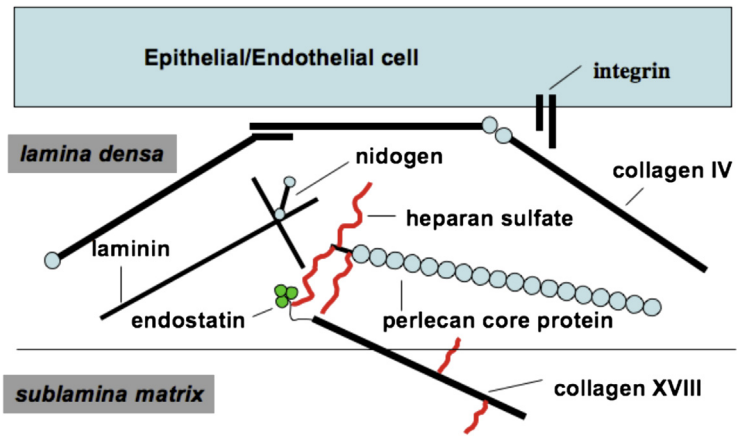

FIGURE 10.5
Components of basement membranes. Basement membranes contain interconnected networks of collagen IV and laminin polymers, together with nidogen, perlecan and collagen XVIII. Collagen XVIII molecules are located at the boundary between the lamina densa and the sublamina matrix, with their carboxyl endostatin domain within the lamina densa and the amino end projecting into the underlying matrix.

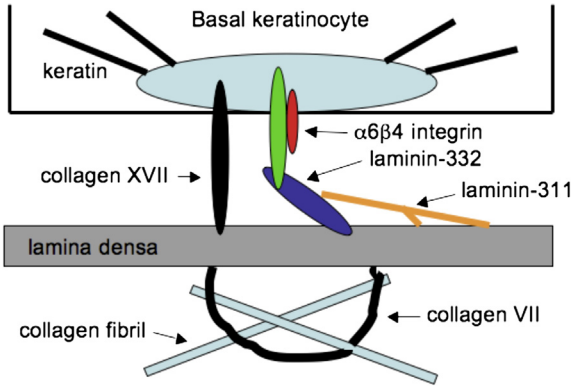

FIGURE 10.6
Epidermal basement membrane and associated collagens and laminins. Basal portion of a keratinocyte with hemidesmosome, anchoring filaments of collagen XVII and anchoring fibril of collagen VII. A complex of laminin-332, laminin-311 and integrin α6β4 provides further strength to the cell-basement membrane junction.

Two additional basement membrane-associated collagens, collagen VIII and collagen XVIII, are of interest because of their function in vascular physiology and pathology. Collagen VIII [2] is a short-chain, non-fibrillar collagen with significant homology to collagen X [37], a product of hypertrophic chondrocytes in long bone growth plates and cartilage growth regions (synchondroses) at the skull base. Mutations in collagen X cause metaphyseal dysplasia in mice and humans [38,39], likely as a result of endoplasmic reticulum stress [40].

Collagen VIII expression is upregulated during heart development [41], in human atherosclerotic lesions [42] and following experimental damage to the endothelium in large arteries [43]. Collagen VIII facilitates migration of smooth muscle cells from the medial layer into the intima during neointimal thickening following endothelial cell injury. Collagen VIII molecules are also major building blocks of Descemet's membrane, the thick basement membrane that bridges the corneal stroma with the corneal endothelium on the inside of the cornea [44]. Mutations in collagen VIII can cause clouding of the cornea and blurred vision (corneal dystrophy) in humans [45,46].

Collagen XVIII, together with collagen XV, belongs to a distinct subfamily of collagens called *multiplexins* because of their multiple triple helix domains and interruptions [47,48]. Because of the alternative utilization of two promoters and alternative splicing, the COL18A1 gene gives rise to three different transcripts that are translated into three protein variants. These are localized in various basement membranes (Fig. 10.5), including those that separate vascular endothelial cells from the underlying intima in blood vessels. Collagen XVIII α-chains contain several consensus sequences for attachment of heparan sulfate side chains and studies have, in fact, confirmed that collagen XVIII forms the core protein of a basement membrane proteoglycan [49]. Proteolytic processing of the carboxyl non-triple-helical domain of collagen XVIII in tissues leads to the release of a heparin-binding fragment with anti-angiogenic activity.

This fragment, named *endostatin*, represents the carboxyl-terminal 20 kDa portion of collagen XVIII chains (Fig. 10.5) [50]. Endostatin has been shown to inhibit the proliferation and migration of vascular endothelial cells, inhibit the growth of tumors in mice and rats, and cause regression of tumors in mice. The anti-tumor effects are mediated by inhibition of tumor-induced angiogenesis. The X-ray crystallographic structure of mouse and human endostatin proteins [51,52] shows a compact structure, consisting of two α-helices and a number of β-strands, stabilized by two intramolecular disulfide bonds. A coordinated zinc atom is part of the structure and on the surface a patch of basic residues forms a binding site for heparin. Studies of mutant endostatins have shown that specific arginines within this patch are required for heparin-binding [53].

The physiological function of collagen XVIII is highlighted by the consequences of loss-of-function mutations in this basement membrane component [54]. In humans, collagen XVIII mutations cause Knobloch syndrome, a recessive eye disorder in which affected individuals lose their eyesight at an early age because of degeneration of the retina and the vitreous [55]. Mice with inactivated collagen XVIII genes exhibit age-dependent changes in the retina and the pigment epithelial layer behind the retina. These changes are similar to what is seen in age-dependent macular degeneration in humans and lead (as in humans) to gradual loss of eyesight [54].

Of considerable interest is the finding that proteolytic fragments of basement membrane components other than collagen XVIII also have anti-angiogenic properties [56]. Molecules that give rise to such fragments include collagen IV and perlecan, the major heparan sulfate proteoglycan in basement membranes. The fragments involved show no sequence homology with endostatin, so it is likely that the molecular mechanisms underlying the anti-angiogenic effects are different. For example, endostatin is a heparin-binding molecule and its ability to inhibit angiogenesis is in many contexts heparan sulfate-dependent. In contrast, the fragment from perlecan, called *endorepellin*, does not bind to heparin and its inhibitory effects on

vascular endothelial cells are heparan sulfate-independent. In any case, release of such fragments as vascular basement membranes are degraded at sites of sprouting angiogenesis are likely to provide a local mechanism of negative control to balance the effects of factors that stimulate angiogenesis.

Bioactive fragments released by proteolytic cleavage of extracellular matrix proteins are not restricted to collagens. The recognition that such fragments may play an important role in both normal and pathological processes has led to the classification of such fragments as *matricryptins* [57]. A number of matricryptins have been identified as products of several collagens and proteoglycans [2].

ELASTIC FIBERS AND MICROFIBRILS

Collagen molecules and fibers have evolved as structures of high tensile strength, equivalent to that of steel when compared on a cross-sectional area basis, but three times lighter on a per-unit weight basis. In contrast, elastic fibers, composed of molecules of elastin and associated proteins, provide tissues with elasticity so that they can recoil after transient stretch [58,59]. In organs such as the large arteries, skin and lungs, elasticity is obviously crucial for normal functioning [60]. Elastic fibers derive their impressive elastic properties, an extensibility that is about five times that of a rubber band with the same cross-sectional area, from the structure of elastin molecules. Each molecule is composed of alternating segments of hydrophobic and α-helical Ala- and Lys-rich sequences. Oxidation of the lysine side chains by the enzyme lysyl oxidase leads to formation of reactive aldehydes and extensive covalent crosslinks between neighboring molecules in the fiber. It is thought that the elasticity of the fiber is due to the tendency of the hydrophobic segments to adopt a random-coil configuration following stretch.

On the surface of elastic fibers one finds a cover of microfibrils; beaded filaments with molecules of fibrillin as their major components [61,62]. The fibrillins, products of genes on chromosomes 5 (*FIB5*), 15 (*FIB15*) and 19 (*FIB19*) in humans, also form microfibrils that are found in almost all extracellular matrices in the absence of elastin. Fibrillin molecules are composed of multiple repeat domains, the most prominent being calcium-binding EGF-like repeats; similar repeats in latent TGF-β binding proteins indicate that the fibrillins belong to a superfamily of proteins. The physiological importance of fibrillin is highlighted by mutations causing the Marfan syndrome and congenital contractural arachnodactyly in humans [63]. The Marfan syndrome, caused by mutations in FIB15, is characterized by dislocation of the eye lens due to weakening of the suspensory ligaments of the zonule, congestive heart failure, aortic aneurysms, and skeletal growth abnormalities resulting in a tall frame, scoliosis, chest deformities, arachnodactyly and hypermobile joints [64]. In patients with congenital contractural arachnodactyly, mutations in FIB5 lead to similar skeletal abnormalities and severe contractures, but no ophthalmic and cardiovascular manifestations [65]. The tall stature and arachnodactyly seen in patients with the Marfan syndrome suggest that FIB15 is a negative regulator of longitudinal bone growth. Since fibrillin microfibrils are found in growth plate cartilage, it is conceivable that they affect chondrocyte proliferation and/or maturation.

A regulatory role for fibrillin in growth plates would be consistent with the function of fibrillin in other tissues [66]. In lung tissue and blood vessel walls, fibrillin functions as a regulator of TGF-β activity. Fibrillin mutations in mouse models of Marfan syndrome are associated with increased TGF-β activity in lungs and the aorta, causing impaired alveolar septation in lungs and widening and weakening (aneurysms) of the aorta. Inhibition of TGF-β largely prevents these defects [67,68]. Some of the major clinical abnormalities in patients with Marfan syndrome are therefore likely a consequence of altered fibrillin-mediated control of TGF-β activity and not loss of fibrillin as a structural molecule. The current data suggest that drugs to inhibit TGF-β activity may be useful in preventing early death caused by aortic aneurysms in Marfan syndrome patients. This represents a good example of how a genetic disease may be

effectively treated by pharmacological modulation of pathogenetic consequences of the mutation, without correcting the mutation.

A number of other proteins are associated with microfibrils or elastin [59]. These include MAGPs (microfibril-associated glycoproteins), fibulins, EMILIN as well as members of the lysyl oxidase (LOX) family, which catalyze the formation of covalent crosslinks between the proteins within elastic fiber networks. The importance of these different proteins for normal tissue formation and function is illustrated by the consequences of mutations in humans and experimental animals. For example, mice lacking the elastin gene die within a few days after birth due to obstruction of blood vessels as a result of increased proliferation of smooth muscle cells in vessel walls. Mice lacking fibrillin-1 (FIB-15) die within 2 weeks after birth because of aortic rupture and lung abnormalities. Mice lacking fibulin-1 die shortly after birth with kidney, lung and capillary defects; fibulin-4- or -5-deficient mice die either at birth (fibulin-4) with severe defects in lungs and blood vessels or exhibit loose skin and elastic fibers in abnormal aorta. Finally, several disease states have been linked to alterations in LOX activity (see reference [59]). Mutations in fibulin-4 and -5 have been associated with cutis laxa (loose skin, emphysema and aortic aneurysm) in humans (see reference [59]).

OTHER MULTIFUNCTIONAL PROTEINS IN ECM

Several proteins in the extracellular matrix contain binding sites for structural macromolecules and for cells, thus contributing both to the structural organization of ECM and its interaction with cells. The prototype of these adhesive proteins is fibronectin.

Fibronectin is a multidomain, multifunctional adhesive glycoprotein

Fibronectin is a disulfide-bonded dimer of 220–250 kDa subunits [69,70]. Each subunit is folded into rod-like domains separated by flexible 'joints'. The domains are composed of three types of multiple repeats or modules, Fn1, Fn2, and Fn3. Fn1 modules are found in the fibrin-binding amino- and carboxyl-terminal regions of fibronectin and in a collagen (gelatin)-binding region (Fig. 10.7). Single copies of Fn1 modules are also found in other proteins, such as tissue-type plasminogen activator (t-PA) and coagulation factor XII [71].

NMR and crystallographic studies of Fn1 modules demonstrate the presence of two layers of anti-parallel β-sheets (two strands in one layer and three strands in the other) held together by hydrophobic interactions [72,73]. The structure is further stabilized by disulfide- and salt-bridges. Fn2 modules are found together with Fn1 modules in the collagen-binding region of fibronectin and in many other proteins. Their structure, two double-stranded anti-parallel β-sheets connected by loops, suggests that a ligand such as collagen may bind to this module through interactions of hydrophobic amino acid side chains with its hydrophobic surface [74].

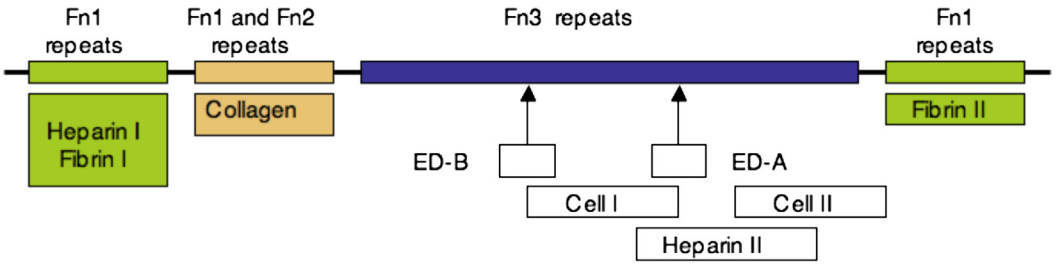

FIGURE 10.7

Diagram of a fibronectin polypeptide chan. The polypeptide chain is composed of several repeats (Fn1, Fn2, and Fn3) and contains binding sites for several matrix molecules and cells. Two regions can bind heparin and fibrin and two regions are involved in cell binding as well. By alternative splicing, isoforms are generated that may or may not contain certain Fn3 domains (labeled ED-A and ED-B in the diagram). Additional splice variations in the second cell-binding domain (Cell II) generate other isoforms.

Fn3 modules are the major structural units in fibronectin and are found in a large number of other proteins as well. Some of these proteins (for example, the long splice variant of collagen XII) contain more Fn3 modules than fibronectin itself. The structure of Fn3 is that of a sandwich of anti-parallel β-sheets (three strands in one layer and four strands in the other) with a hydrophobic core [75]. The binding of fibronectin to some integrins involves the tripeptide sequence Arg-Gly-Asp in the tenth Fn3 module; these residues lie in an exposed loop between two of the strands in one of the β-sheets [71].

Fibronectin can assemble into a fibrous network in the ECM through interactions involving cell surface receptors and the amino-terminal region of fibronectin. A fibrin binding site is also contained in this region; a second site is in the carboxyl domain. The ability to bind to collagen ensures association between the fibronectin network and the scaffold of collagen fibrils. Binding sites for heparin and chondroitin sulfate further make fibronectin an important bridging molecule between collagens and other matrix molecules (Fig. 10.7).

Transcripts of the fibronectin gene are alternatively spliced in a cell- and developmental stage-dependent manner. As a result there are many different isoforms of fibronectin [70]. The main form produced by the liver and circulating in plasma lacks two of the Fn3 repeats found in cell- and matrix-associated fibronectin. One alternatively spliced domain is adjacent to the heparin binding site, and this region binds to integrins α4β1 and α4β7. Thus, there is a mechanism for fine tuning of fibronectin structure and interaction properties. Not surprisingly, mice that are homozygous for fibronectin null alleles die early in embryogenesis with multiple defects [76].

The most important activity of fibronectin in biological terms is its interaction with cells. The ability of fibronectin to serve as a substrate for cell adhesion, spreading, and migration is based on the activities of several modules. The Arg-Gly-Asp sequence in the tenth Fn3 module plays a key role in the interaction with the integrin receptor α5β1, but synergistic interactions with other Fn3 modules are essential for high affinity binding of cells to fibronectin.

Laminins are major components of basement membranes

Laminins are trimeric basement membrane molecules of α, β, and γ chains [33]. With a large number of genetically distinct chains, more than 15 different trimeric isoforms are known from mice and humans. Based on a systematic approach to naming the different trimers, they are now named according to their chain composition (i.e., α1β1γ1) or by numbers only without the Greek letters (i.e., 111 instead of α1β1γ1) [77]. Several forms have a cross-shaped structure as visualized by rotary shadowing electron microscopy; some forms contain T-shaped molecules (Fig. 10.8).

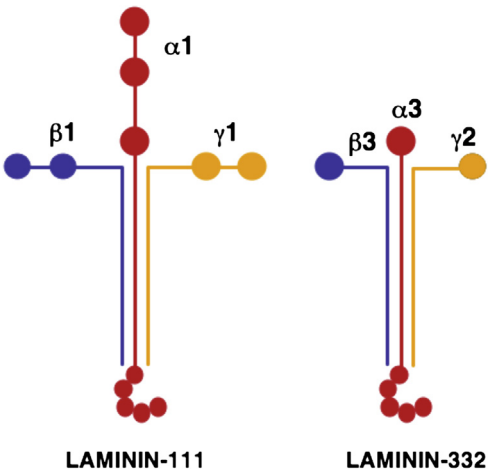

FIGURE 10.8
Diagrams of two types of laminins. Laminin-111 has a cross-shaped structure, while laminin 332 is T-shaped, due to a shorter α-chain.

In basement membranes, laminins provide interaction sites for many other constituents, including cell surface receptors. The functional and structural mapping of these sites and the complete sequencing of laminin chains has provided detailed insights into the organization of laminin molecules. Within the cross-shaped laminin-111 molecule, three similar short arms are formed by the N-terminal regions of the α1, β1, and γ1 chains, whereas a long arm is composed of the carboxyl regions of all three chains (Fig. 10.8). The three chains are connected at the center of the cross by interchain disulfide bridges. The short arms contain multiple EGF-like repeats of about 60 amino acid residues, terminated and interrupted by globular domains. The long arm consists of heptad repeats covering about 600 residues in all three chains folded into a coiled-coil structure. The α1 chain is about 1,000 amino acid residues longer than the β1 and γ1 chains and forms five homologous globular repeats at the 'base' of the cross; these globular repeats are similar to repeats found in the proteoglycan molecule perlecan, also a component of basement membranes (Fig. 10.5) [33].

Calcium-dependent polymerization of laminin is based on interactions between the globular domains at the N-termini and is thought to be important for the assembly and organization of basement membranes. Of significance for the assembly of basement membranes is also the high affinity interaction with nidogens (nidogen-1 and -2) [33]. The binding site in laminin for nidogen is on the γ1 chain, close to the center of the cross (Fig. 10.5). On nidogen, a rod-like molecule with three globular domains, the binding site for laminin is in the carboxyl globular domain, while another globular domain binds to collagen IV. Thus, nidogen is a bridging molecule that connects the laminin and collagen IV networks, and is critical for the assembly of normal basement membranes. Mice that are deficient in both nidogens or have the nidogen binding site in laminin γ1 deleted, have multiple defects and die before or at birth.

Laminin does not bind directly to collagen IV, but has binding sites for several other molecules besides nidogen. These are heparan sulfate, perlecan, and fibulin-1, which bind to the end of the long arm of the laminin cross. However, the biologically most significant interactions of laminin involve a variety of both integrin and non-integrin cell surface receptors. Several integrins show distinct preferences for different laminins and recognize binding sites on either the short or long arms of laminin molecules.

The different laminin genes likely arose through duplication of a single ancestral gene [78]. The laminins that are most closely related to this ancestral gene, laminin-111 and laminin-511, are crucial for early steps in development, including gastrulation, placentation and neural tube closure. In contrast, laminins that have evolved more recently are adapted to more specialized functions in the development and function of specific organs. For example, laminin-211 is a major laminin in the basement membranes surrounding skeletal muscle fibers where it provides binding sites for the dystroglycan-dystrophin complex, linking the muscle cell cytoskeleton to the basement membrane. In skin, a disulfide-linked complex of laminins-332 and -311 is crucial for the firm attachment of keratinocytes to the basement membrane by its interaction with α6β4 integrins in hemidesmosomes (Fig. 10.6). Mutations in any one of the genes encoding the subunits of laminin-332 cause autosomal recessive junctional epidermolysis bullosa, a lethal skin blistering disorder in which the epidermal cell layers are separated from the underlying epidermal basement membrane. Loss-of-function mutations in the laminin α2 gene cause congenital muscular dystrophy both in mice and humans.

Mice with targeted disruption of the laminin α3 gene develop a blistering skin disease similar to the disorder in human patients. In addition, the kidneys of the mutant animals show arrested development of glomeruli with a failure to develop glomerular capillaries with fenestrated endothelial cells, and lack of migration of mesangial cells into the glomeruli [79]. In humans, mutations resulting in laminin β2-deficiency cause a syndrome of loss of albumin and other plasma proteins through the glomerular basement membrane (congenital nephrotic syndrome), combined with sclerosis of the glomerular mesangium and severe impairment of vision and neurodevelopment [80].

Other modulators of cell-matrix interactions

Whereas proteins such as fibronectin and laminin are important for adhesion of cells to extracellular matrices, other ECM molecules function as both positive and negative modulators of such adhesive interactions. Examples of such modulators are thrombospondin [81] and tenascin [82]. Thrombospondins (TSPs) are a group of homologous trimeric (TSP-1 and TSP-2) and pentameric (TSP-3, TSP-4 and TSP-5/COMP) matrix proteins composed of several Ca^{++}-binding (type 3) domains, EGF-like repeats, (type 2) as well as other modules (Fig. 10.9). Different members of the group show differences in cellular expression and functional properties. The most highly conserved regions of the different thrombospondins are the carboxyl halves of the molecules, all consisting of a variable number of EGF-like domains, 7 Ca^{++}-binding TSP type 3 repeats and a C-terminal globular domain. In contrast, the N-terminal regions are quite variable, but all members have a short coiled-coil domain of heptad repeats in this region that is crucial for oligomerization into trimers in TSP-1 and TSP-2 or pentamers in TSP-3, TSP-4 and TSP-5/COMP. These oligomerization domains are stabilized by interchain disulfide bonds, but are quite stable even with the disulfides reduced. Since the subunits are held together at the coiled-coil domains, the assembled molecules have a flower-like appearance with three or five 'petals' extending out from the center, available for binding to cell surface receptors and other ECM molecules. The crystal structure of the five-stranded coiled-coil domain of TSP-5 shows that it forms a hydrophobic channel with some similarity to ion channels and can bind vitamin D and all-trans-retinoic acid. One function of pentameric thrombospondins may therefore be to store small hydrophobic signaling molecules in the ECM.

Interestingly, TSP type 1 repeats are found in many other proteins, including the large family of matrix metalloproteases called ADAMTS enzymes; in some members of this family, there are more copies of TSP type 1 domains than in TSP-1 or TSP-2 themselves. Members of the ADAMTS family have important biological functions [83]. For example, ADAMTS2, ADAMTS3 and ADAMTS14 are procollagen propeptidases, responsible for processing the amino propeptide in fibrillar procollagens (see above), and ADAMTS4 and ADAMTS5 are aggrecanases, able to degrade the major proteoglycan component of cartilage.

The carboxyl regions of thrombospondins can bind to a variety of ECM molecules, extracellular proteases and cell surface components such as integrins. Their oligomeric structure enable thrombospondins to be involved in multiple interactions and modulate both cellular behavior and ECM assembly. All thrombospondins support attachment of cells in a Ca^{++}-dependent manner and stimulate cell migration, proliferation, chemotaxis and phagocytosis. Trimeric thrombospondins (TSP-1 and TSP-2) have additional activities associated with the type 1 domains within their N-terminal regions. These activities include inhibition of angiogenesis through mechanisms by which thrombospondin induces endothelial cell apoptosis and inhibits mobilization of vascular endothelial growth factor (VEGF). The two

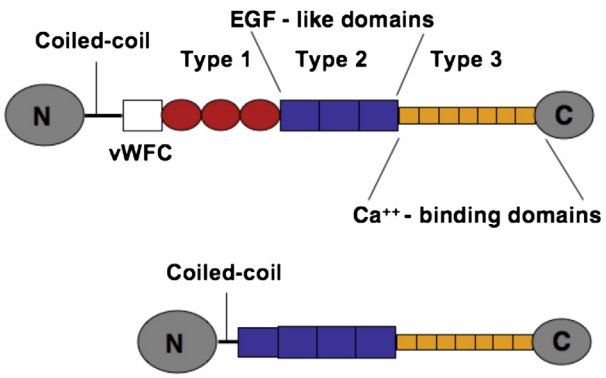

FIGURE 10.9
Diagram of thrombospondins. Diagram of trimeric thrombospondins (TSP-1 and TSP-2) on top showing the multidomain structure and the location of the coiled-coil domain important for trimerization. Diagram of pentameric thrombospondins (TSP-3, TSP-4 and TSP-5/COMP) at bottom, showing the lack of von Willebrand factor C-like domain (vWC) and type 1 repeats in this group of thrombospondins.

trimeric thrombospondins have also been shown to promote the formation of synapses in the central nervous system [84].

Cartilage oligomeric matrix protein (TSP-5/COMP) is thought to have evolved from the TSP-3 and TSP-4 branch of the thrombospondins. COMP is a secretory product of chondrocytes and is localized in their territorial matrix in cartilage. Beyond the fact that COMP interacts with collagens II and IX, little is known about its normal function in cartilage. Mice lacking COMP develop a normal skeleton and have no significant abnormalities. However, mutations in COMP cause pseudoachondroplasia and multiple epiphyseal dysplasia in humans [85,86]. At birth, affected individuals have normal weight and length but show reduced growth of long limb bones and striking defects in growth plate regions. These defects are caused by retention of mutant protein in the RER of chondrocytes, causing premature cell death. Mutations in COMP appear therefore to generate a mutant phenotype by a mechanism involving RER stress in chondrocytes.

The members of the vertebrate tenascin family (C, R, W and X) are large, multimeric proteins with subunits composed of multiple protein modules [82]. The modules include heptad repeats, fibronectin type 3 repeats, EGF-like domains, and a carboxyl domain with homology to the carboxyl-terminal domains of β-and γ-fibrinogen chains. These modules form rod-like structures that interact with their amino-terminal domains to form oligomers. Alternative splicing of tenascin-C generates multiple isoforms. The tenascins are differentially expressed in different tissues and at different times during development and growth [82]. For example, tenascin-R is only expressed in the central nervous system, in contrast to tenascin-C, which is found both in the central nervous system as well as in peripheral nerves. Tenascin-C expression is high during development and inflammation and around tumors, but is otherwise relatively low in postnatal tissues, with some interesting exceptions. In tissue regions of high mechanical stress, the levels of tenascin expression are high, suggesting a role for tenascin-C in the mechanisms used by cells to cope with mechanical stress. In fact, tenascin-C was first identified as a 'myotendinous antigen' because of the high level expression at tendon-muscle junctions [87]. It is also expressed by other cells that are exposed to mechanical stress, including osteoblasts, perichondrial cells around cartilage, smooth muscle cells and fibroblasts in healing wounds. This association between mechanical stress and expression is also seen for other tenascins. Thus, tenascin-W is expressed by both osteoblasts and smooth muscle cells and tenascin-X is expressed at high level in the connective tissue 'wraps' in skeletal muscle. Consistent with a 'mechanical' role in connective tissues is also the finding that a form of Ehlers-Danlos syndrome, with hypermobile joints and hyperelastic skin, is caused by a deficiency in tenascin-X [88].

The finding that collagen XII (see above) interacts with tenascin-X, combined with data showing that tenascin-X can bind to collagen I fibrils, possibly via interaction with the small proteoglycans decorin, suggests that a complex of collagen XII and tenascin-X are important interfibrillar bridges in skin [89]. This complex may also mediate attachment of collagen fibrils to cells, since tenascin can bind to integrin receptors. That a similar complex between collagen XII and tenascin-C may be present at myotendinous junctions is suggested by the high level expression of both collagen XII and tenascin-C at such junctions [90].

The interactions between tenascins and cells are relatively weak compared to other proteins such as fibronectin and thrombospondin. In certain experimental conditions tenascin-C can be an adhesive molecule for cells; it can also, however, have anti-adhesive effects [82]. The adhesive activity can be mediated by either cell surface proteoglycans or integrins, depending on cell type. Tenascin-C can bind heparin and this may be responsible for interactions with cell surface proteoglycans such as glypican. Tenascin-C can also block adhesion by covering up adhesive sites in other matrix molecules such as fibronectin and sterically block their interactions with cells. Tenascin-C has therefore been characterized as a cell adhesion-modulating

protein. Likewise, Tenascin-R can both promote neuronal cell adhesion and act as a repellant for neurites.

PROTEOGLYCANS — MULTIFUNCTIONAL MOLECULES IN THE EXTRACELLULAR MATRIX AND ON CELL SURFACES

A variety of proteoglycans play important roles in cellular growth and differentiation and in matrix structure. They range from the large hydrophilic space filling complexes of aggrecan and versican with hyaluronan, to the cell surface syndecan receptors. In basement membranes the major heparan sulfate proteoglycan is perlecan [91]. With three heparan sulfate side chains attached to the amino-terminal region, its core protein is multimodular in structure, having borrowed structural motifs from a variety of other genes. These include an LDL receptor-like module, regions with extensive homology to laminin chains, a long stretch of N-CAM-like IgG repeats, and a carboxyl-terminal region with three globular and four EGF-like repeats similar to a region of laminin. Alternative splicing can generate molecules of different lengths. Perlecan is present in a number of basement membranes, but is also found in the pericellular matrix of fibroblasts and in cartilage ECM. In fact, fibroblasts, rather than epithelial cells, appear to be major producers of perlecan (for example, in skin). In liver, perlecan is expressed by sinusoidal endothelial cells, and is localized in the perisinusoidal space. Mutations in the *unc-52* gene in *C. elegans*, encoding a short version of perlecan, cause disruptions of skeletal muscle [92]. This indicates that the molecule, as a component of skeletal muscle basement membranes, is important for assembly of myofilaments and their attachment to cell membranes. Binding of growth factors and cytokines to the heparan sulfate side chains also enables perlecan to serve as a storage vehicle for biologically active molecules such as bFGF. The critical role of perlecan is further highlighted by the dramatic effects of knocking out the perlecan gene in mice [93]. Most of the mutant embryos die halfway through pregnancy and the few embryos that survive to birth have severe defects in the brain and the skeleton. The skeletal defects include severe shortening of axial and limb bones and disruption of normal growth plate structure.

Several small leucine-rich repeat proteins and proteoglycans with homologous core proteins are found in a variety of tissues where they interact with other matrix macromolecules and regulate their functions [94]. They include decorin, biglycan, lumican, and fibromodulin. Decorin binds along collagen fibrils and plays a role in regulating fibril assembly and mechanical properties [95,96]. It also modulates the binding of cells to matrix constituents such as collagen, fibronectin and tenascin [97]. Through binding of TGF-β isoforms the small proteoglycans help sequester growth factors within the ECM and thus regulate their activities [98].

A variety of proteoglycans also have important functions at cell surfaces. These include members of the syndecan family, transmembrane molecules with highly conserved cytoplasmic domains, and glypican-related molecules that are linked to the cell surface via glycosyl phosphatidylinositol. Through their heparan sulfate side chains these molecules can bind growth factors, protease inhibitors, enzymes, and matrix macromolecules. They are therefore important modulators of cell signaling pathways and cell-matrix contacts [99,100].

Hyaluronan is an important component of most extracellular matrices [101]. It serves as a ligand for several proteins, including cartilage link protein and aggrecan and versican core proteins. In cartilage, based on such interactions, it is the backbone for the large chondroitin sulfate-containing aggrecan proteoglycan complexes responsible for the compressive properties of cartilage [102] (Fig. 10.10). It also is a ligand for cell surface receptors and regulates cell proliferation and migration [103,104]. One receptor for hyaluronan is the transmembrane molecule CD44. By alternative splicing and variations in post-translational modifications, a family of CD44 proteins is generated [105]. These show cell and

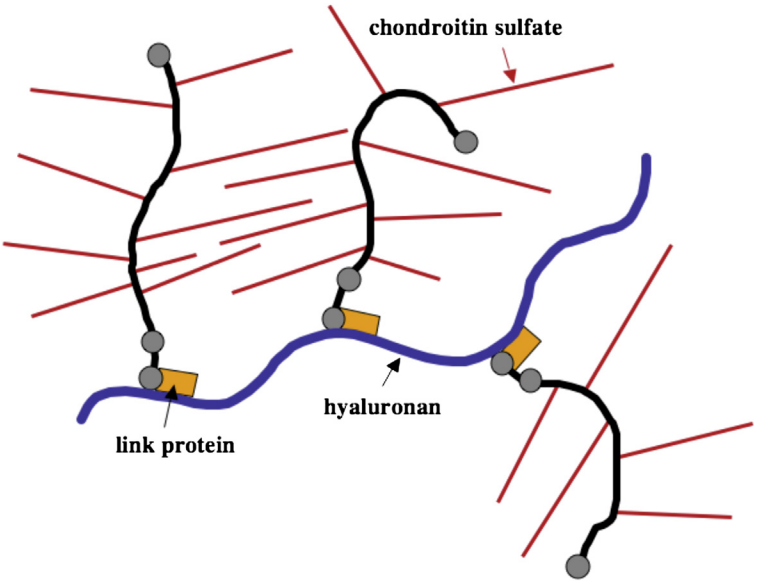

FIGURE 10.10
Diagram of a portion of a large proteoglycan complex from cartilage. Monomers of aggrecan, composed of core proteins with glycosaminoglycan side chains (mostly chondroitin sulfate) are bound to hyaluronan. The binding is stabilized by link proteins. For clarity, only some of the glycosaminoglycan side chains are shown in the monomers.

tissue-specific expression patterns and are thought to have distinct functional roles. Hyaluronan-mediated cell motility is based on the interaction of hyaluronan with a cell surface associated protein called RHAMM (receptor for hyaluronate-mediated motility) [106]. As a space filling molecule and through its interaction with cell surface receptors hyaluronan is important for several morphogenetic processes during development. It creates cell free spaces through which cells (for example neural crest cells) can migrate, and its degradation by hyaluronidase is important for processes of cellular condensation. Since hyaluronan is not immunogenic, is readily available and can be easily manipulated and chemically modified, it is receiving considerable attention as a tissue-engineering biopolymer [107].

CONCLUSION

Research efforts during the past 40 years have led to significant insights into the composition of extracellular matrices and the structure and function of the major components. We now realize that the evolution of vertebrates and mammals was associated with an expansion of several families of matrix molecules, providing cells with an increasing repertoire of isoforms and homologs to build different tissues. It is also evident that the increasing number of different families of genes encoding matrix molecules during evolution of more complex organisms involved shuffling and recombination of genes encoding a relatively small number of structural and functional modules. Finally, the data suggest that cells are building matrices by adding layer upon layer of components that can interact with various affinities (but mostly on the low side) and in multiple ways with their neighbors. The result is an extracellular matrix that readily can be fine-tuned to meet the demands of the moment but one that is relatively resistant to the effects of mutations that may cause dysfunction of specific components. As we learn to use these insights to identify the most critical matrix properties from a cellular point of view, exciting and rapid advances in tissue engineering should follow.

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CHAPTER 11

Morphogenesis and Tissue Engineering

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INTRODUCTION

Morphogenesis is the developmental cascade of pattern formation, the establishment of the body plan and architecture of mirror-image bilateral symmetry of musculoskeletal structures, culminating in the adult form. Tissue engineering is the emerging discipline of fabricating spare parts for the human body, including the skeleton, for the functional restoration and aging of lost parts due to cancer, disease or trauma. It is based on rational principles of molecular developmental biology and morphogenesis and is further governed by bioengineering. The three key ingredients for both morphogenesis and tissue engineering are inductive morphogenetic signals, responding stem cells, and extracellular matrix scaffolding [1] (Fig. 11.1). Recent advances in the molecular cell biology of morphogenesis will aid in the design principles and architecture for tissue engineering and regeneration.

The long-term goal of tissue engineering is to produce functional tissues *in vitro* for implantation *in vivo* to repair, enhance, and replace damaged tissue, and to preserve physiological function. Tissue engineering is based on the principles of developmental biology, evolution and self-assembly of supramolecular assemblies and higher hierarchal tissues and even whole embryos and organisms (Figs. 11.2 and 11.3). Regeneration recapitulates embryonic development and morphogenesis. Among the many tissues in the human body, bone has considerable powers for regeneration and therefore is a prototype model for tissue engineering. On the other hand, articular cartilage, a tissue adjacent to bone, is recalcitrant to repair and regeneration. Implantation of demineralized bone matrix into subcutaneous sites results in local bone induction. The sequential cascade of bone morphogenesis mimics sequential skeletal morphogenesis in limbs and permits the isolation of bone morphogens. Although it is traditional to study morphogenetic signals in embryos, bone morphogenetic proteins (BMPs), the primordial inductive signals for bone were isolated from demineralized bone matrix from adults. BMPs initiate, promote, and maintain chondrogenesis and osteogenesis and have actions beyond bone. The recently identified cartilage-derived morphogenetic proteins (CDMPs) are critical for cartilage and joint morphogenesis.

The symbiosis of bone's inductive and conductive strategies is critical for tissue engineering and is in turn governed by the context and biomechanics. The context is the microenvironment, consisting of extracellular matrix scaffolding, which can be duplicated by biomimetic biomaterials, such as collagens, hydroxyapatite, proteoglycans, and cell adhesion proteins, including fibronectins and laminins. The rules of architecture for tissue engineering are

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KEY INGREDIENTS FOR
TISSUE ENGINEERING

- Morphogenetic Signals
- Responding Stem Cells
- Extracellular Matrix Scaffolding

FIGURE 11.1
The tissue-engineering triad consists of signals, stem cells, and scaffolding.

an imitation and adoption of the laws and signals of developmental biology and morphogenesis, and thus they may be universal for all tissues, including bones and joints and associated musculoskeletal tissues in the limbs.

The traditional approach for identification and isolation of morphogens is first to identify genes in fly and frog embryos by means of genetic approaches, differential displays, substractive hybridization, and expression cloning. This information is subsequently extended to mice and men. An alternative approach is to isolate morphogens from bone, the premier tissue with the highest regenerative potential. Morphogenesis is the developmental cascade of pattern formation, the establishment of the body plan and architecture of mirror-image bilateral symmetry of musculoskeletal structures in the appendicular skeleton, culminating in the adult form. Our expanding knowledge of bone and cartilage morphogenesis is a prototypical paradigm for all of tissue engineering. The principles gleaned from bone

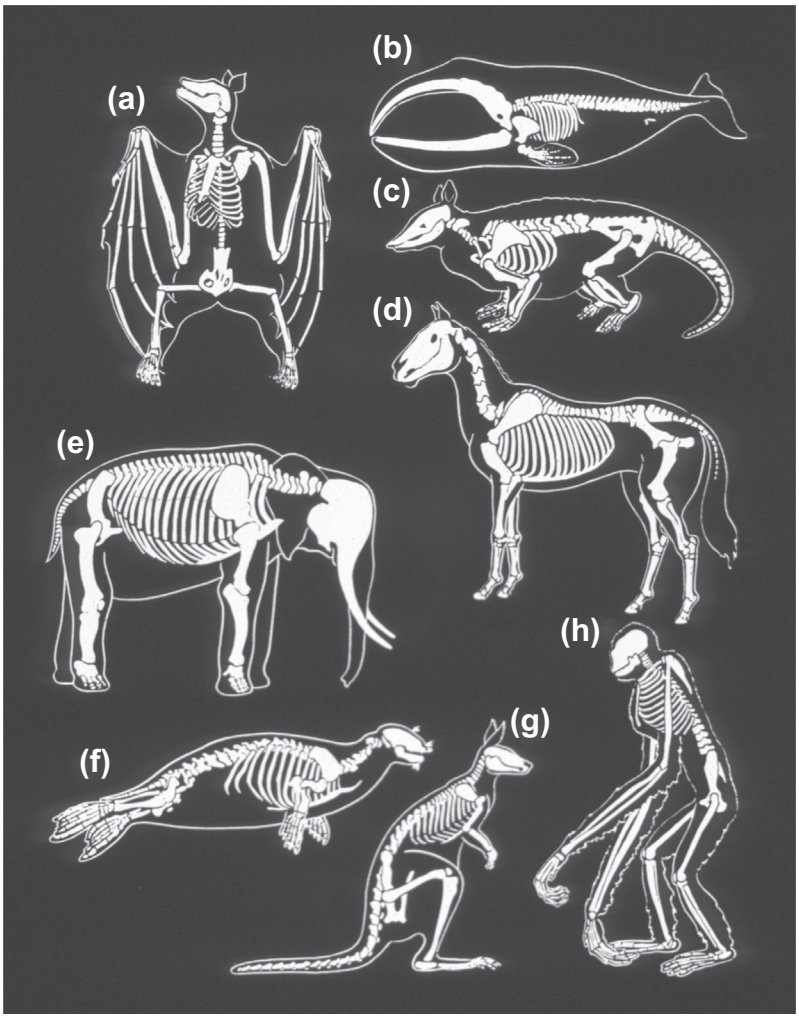

FIGURE 11.2
Evolution of skeletal structures in a variety of mammals adapted for flight (bat, a) and aquatic life (whale, b) and the use of hands in humans (h).

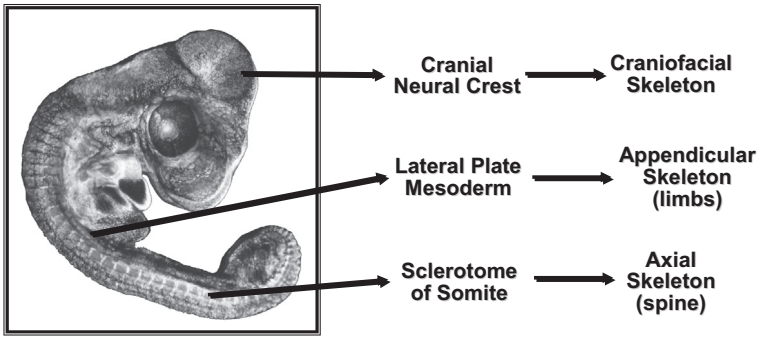

FIGURE 11.3
Developmental origins of skeleton in the chick embryo. The cranial neural crest gives rise to craniofacial skeleton. The lateral plate mesoderm gives rise to the limbs of the appendicular skeleton. The sclerotome of the somite gives rise to spine and the axial skeleton.

morphogenesis and BMPs can be extended to tissue engineering of bone and cartilage and other tissues.

BONE MORPHOGENETIC PROTEINS (BMPS)

Bone grafts have been used by orthopedic surgeons for nearly a century to aid and abet recalcitrant bone repair. Decalcified bone implants have been used to treat patients with osteomyelitis [2]. It was hypothesized that bone might contain a substance, osteogenin, that initiates bone growth [3]. Urist [77] made the key discovery that demineralized, lyophilized segments of rabbit bone, when implanted intramuscularly, induced new bone formation. The diaphysis (shafts) of long bones of rats were cleaned of marrow, pulverized and sieved. The demineralization of matrix was accomplished by 0.5 M HCl (Fig. 11.4). Bone induction, a sequential multistep cascade, is depicted in Fig. 11.5 [4–6]. The key steps in this cascade are chemotaxis, mitosis, and differentiation. Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the insoluble demineralized bone matrix.

The demineralized bone matrix is composed predominantly of type I insoluble collagen, and it binds plasma fibronectin [7]. Fibronectin has domains for binding to collagen, fibrin, and heparin. The responding mesenchymal cells attached to the collagenous matrix and proliferated as indicated by [3H]thymidine autoradiography and incorporation into acid-precipitable DNA on day 3 [8]. Chondroblast differentiation was evident on day 5, chondrocytes on days 7–8, and cartilage hypertrophy on day 9 (Fig. 11.5). Vascular invasion was concomitant on day 9 with osteoblast differentiation. On days 10–12 alkaline phosphatase was maximal. Osteocalcin, bone γ-carboxyglutamic acid containing gla protein (BGP), increased on day 28. Hematopoietic marrow differentiated in the ossicle and was maximal by day 21. This entire sequential bone development cascade is reminiscent of bone and cartilage morphogenesis in

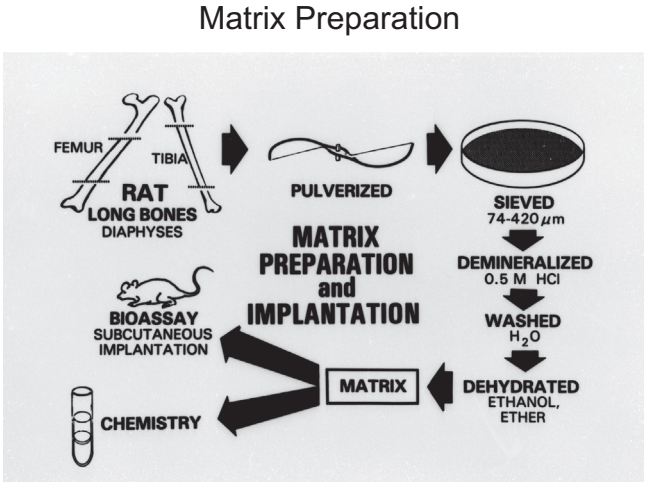

FIGURE 11.4
Preparation of the demineralized bone matrix (DBM). The diaphysis (shafts) of femur and tibia are cleansed of marrow and dried prior to pulverization. The pulverized bone matrix is sieved to a particle size of 74–420 μm and demineralized by 0.5 M HCl, dehydrated in ethanol and diethyl ether. The resulting matrix is a potent inducer of cartilage and bone, and we isolate, by means of chemical techniques, the active osteoinductive agent BMP.

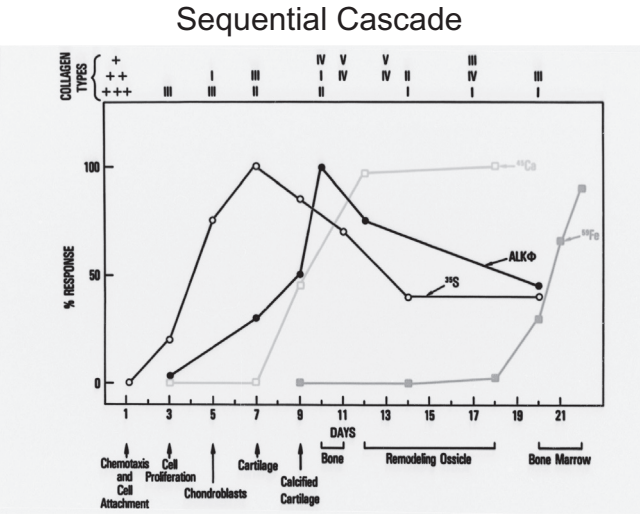

FIGURE 11.5

Developmental sequence of extracellular matrix-induced cartilage, bone, and marrow formation. Changes in 35SO4 incorporation into proteoglycans and 45Ca incorporation into the mineral phase indicate peaks of cartilage and bone formation, respectively. The 59Fe incorporation into heme is an index of erythropoiesis, as plotted from the data of Reddi and Anderson (1976) [5]. The values for alkaline phosphatase indicate early stages of bone formation [4]. The transitions in collagen types I to IV, summarized on top of the figure, are based on immunofluorescent localization = polymorphonuclear leukocytes. (Source: Ref. [6], with permission.)

the limb bud [6,9]. Hence, it has immense implications for isolation of inductive signals initiating cartilage and bone morphogenesis. In fact, a systematic investigation of the chemical components responsible for bone induction was undertaken.

The foregoing account of the demineralized bone matrix-induced bone morphogenesis in extra-skeletal sites demonstrated the potential role of morphogens tightly associated with the extracellular matrix. Next, we embarked on a systematic study of the isolation of putative morphogenetic proteins. A prerequisite for any quest for novel morphogens is the establishment of a battery of bioassays for new bone formation. A panel of *in vitro* assays was established for chemotaxis, mitogenesis, and chondrogenesis, and an *in vivo* bioassay was established for bone formation. Although the *in vitro* assays are expedient, we monitored routinely a labor-intensive *in vivo* bioassay, for it was the only bona fide bone induction assay.

A major stumbling block in the approach was that the demineralized bone matrix is insoluble and exists in the solid state. In view of this, dissociative extractants such as 4 M guanidine HCl or 8 M urea as 1% sodium dodecyl sulfate (SDS) at pH 7.4 were used [10] to solubilize the proteins. Approximately 3% of the proteins were solubilized from demineralized bone matrix, and the remaining residue was mainly insoluble type I bone collagen. The extract alone or the residue alone was incapable of new bone induction. However, addition of the extract to the residue (insoluble collagen) and then implantation in a subcutaneous site resulted in bone induction (Fig. 11.6). Thus, it would appear that in optimal osteogenic activity collaboration takes place between the soluble signal in the extract and insoluble substratum or scaffolding [10]. Thus, an operational concept of tissue engineering was established, in which soluble signals bound to extracellular matrix scaffold act on responding stem/progenitor cells to induce tissue digestion. This bioassay was a useful advance in the final purification of bone morphogenetic proteins and led to the determination of limited tryptic peptide sequences leading to the eventual cloning of BMPs [11–13].

In order to scale up the procedure, a switch was made to bovine bone. Demineralized bovine bone was not osteoinductive in rats, and the results were variable. However, when the guanidine extracts of demineralized bovine bone were fractionated on an S-200 molecular

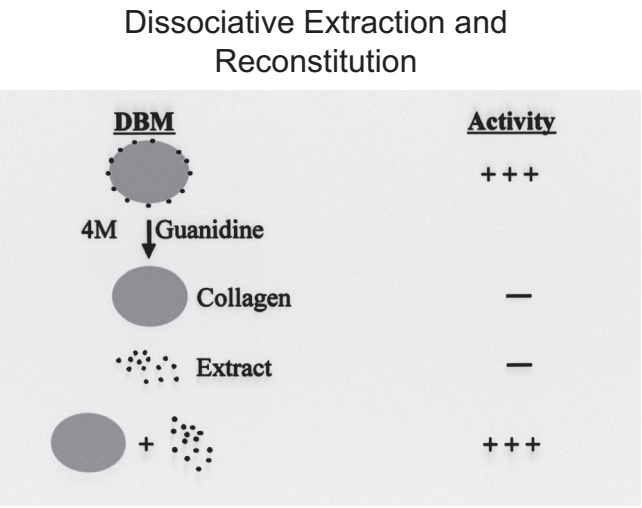

FIGURE 11.6
Dissociative extraction by chaotropic reagents such as 4M guanidine and reconstitution of osteoinductive activity with insoluble collagenous matrix. The results demonstrate a collaboration between a soluble signal and insoluble extracellular matrix. This experiment further established the basic tenets of tissue engineering in 1981 as signals, scaffolds, and responding stem cells.

sieve column, fractions less than 50 kD were consistently osteogenic when bioassayed after reconstitution with allogeneic insoluble [14,78]. Thus, protein fractions inducing bone were not species specific, and appeared to be homologous in several mammals. It is likely that larger molecular mass fractions and/or the insoluble xenogeneic (bovine and human) collagens were inhibitory or immunogenic. Initial estimates revealed 1 μg of active osteogenic fraction in a kilogram of bone. Hence, over a ton of bovine bone was processed to yield optimal amounts for amino acid sequence determination. The amino acid sequences revealed homology to TGF-β1 [14]. The important work of Wozney and colleagues [11] involved cloning BMP-2, BMP-2B (now called BMP-4), and BMP-3 (also called osteogenin). Osteogenic protein-1 and -2 (OP-1 and OP-2) were cloned by Ozkaynak and colleagues [13]. There are nearly 10 members of the BMP family (Table 11.1). The other members of the extended TGF-β/BMP superfamily include inhibins and activins (implicated in follicle-stimulating hormone release from pituitary), Müllerian duct inhibitory substance (MIS), growth/differentiation factors (GDFs), nodal, and lefty, a gene implicated in establishing right/left asymmetry [15,16]. BMPs are also involved in embryonic induction [15,17–19].

BMPs are dimeric molecules, and their conformation is critical for biological action. Reduction of the single interchain disulfide bond resulted in the loss of biological activity. The mature monomer molecule consists of about 120 amino acids, with seven canonical cysteine residues. There are three intrachain disulfides per monomer and one interchain disulfide bond in

TABLE 11.1 Bone morphogenetic proteins

BMP	Other names	Function
BMP-2	BMP-2A	Bone and cartilage morphogenesis
BMP-3	Osteogenin	Bone morphogenesis
BMP-3B	GDF-10	Intramembranous bone formation
BMP-4	BMP-2B	Bone morphogenesis
BMP-5		Bone morphogenesis
BMP-6		Cartilage hypertrophy
BMP-7	Osteogenic protein-1	Bone formation
BMP-8	Osteogenic protein-2	Bone formation
BMP-8B		Spermatogenesis
BMP-9		Liver differentiation
BMP-10		? Tooth differentiation
BMP-11	GDF-11	Odontoblast regulation
BMP-15		?

the dimer. In the critical core of the BMP monomer is the cysteine knot. The crystal structure of BMP-7 has been determined [20]. It is highly possible that in the near future the crystal structure of BMP receptor and receptor contact domains will be determined [20].

CARTILAGE-DERIVED MORPHOGENETIC PROTEINS (CDMPS)

Morphogenesis of the cartilage is the key rate-limiting step in the dynamics of bone development. Cartilage is the initial model for the architecture of bones. Bone can form either directly from mesenchyme, as in intramembranous bone formation, or with an intervening cartilage stage, as in endochondral bone development [6]. All BMPs first induce the cascade of chondrogenesis, and therefore in this sense they are cartilage morphogenetic proteins. The hypertrophic chondrocytes in the epiphyseal growth plate mineralize and serve as a template for appositional bone morphogenesis. Cartilage morphogenesis is critical for both bone and joint morphogenesis. The two lineages of cartilage are clear-cut. The first, at the ends of bone, forms articulating articular cartilage. The second is the growth plate chondrocytes, which, through hypertrophy, synthesize cartilage matrix destined to calcify prior to replacement by bone, and are the 'organizer' centers of longitudinal and circumferental growth of cartilage, setting into motion the orderly program of endochondral bone formation. The phenotypic stability of the articular (permanent) cartilage is at the crux of the osteoarthritis problem. The maintenance factors for articular chondrocytes include TGF-β isoforms and the BMP isoforms [21].

An *in vivo* chondrogenic bioassay with soluble purified proteins and insoluble collagen scored for chondrogenesis. A concurrent reverse transcription–polymerase chain reaction (RT-PCR) approach was taken with degenerate oligonucleotide primers. Two novel genes for CDMPs 1 and 2 were identified and cloned [22]. CDMPs 1 and 2 are also called GDF-5 and GDF-6 [23]. CDMPs are related to BMPs (Table 11.2). CDMPs are critical for cartilage and joint morphogenesis [24]. CDMPs stimulate proteoglycan synthesis in cartilage. CDMP 3 (also known as GDF-7) initiates tendon and ligament morphogenesis.

PLEIOTROPY AND THRESHOLDS

Morphogenesis is a sequential multistep cascade. BMPs regulate each of the key steps: chemotaxis, mitosis, and differentiation of cartilage and bone (Fig. 11.7). BMPs initiate chondrogenesis in the limb [25,26]. The apical ectodermal ridge is the source of BMPs in the developing limb bud. The intricate dynamic, reciprocal interactions between the ectodermally derived epithelium and mesodermally derived mesenchyme sets into motion the train of events culminating in the pattern of phalanges, radius, ulna, and the humerus.

The chemotaxis of human monocytes is optimal at femtomolar concentrations [27]. The apparent affinity was 100–200 pM. The mitogenic response was optimal at the 100 pM range. The initiation of differentiation was in the nanomolar range in solution. However, caution should be exercised, because BMPs may be sequestered by extracellular matrix components, and the local concentration may be higher when BMPs are bound on the extracellular matrix. A single recombinant BMP human 4 can govern chemotaxis and mitosis differentiation of cartilage and bone, maintain phenotype, stimulate extracellular matrix, and promote

TABLE 11.2 Cartilage-derived morphogenetic proteins

CDMP	Other names	Function
CDMP 1	GDF-5	Cartilage condensation
CDMP 2	GDF-6	Cartilage formation, hypertrophy
CDMP 3	GDF-7	Tendon/ligament morphogenesis

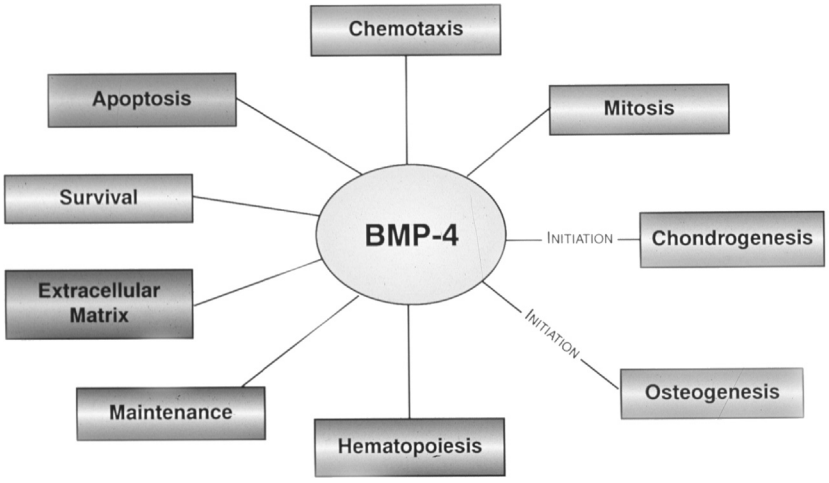

FIGURE 11.7
BMPs are pleiotropic molecules. Pleiotropy is the property of a single gene or protein to act on a multiplicity of cellular phenomena and targets.

survival of some cells but cause the death of others (Fig. 11.7). Thus BMPs are pleiotropic regulators that act in concentration-dependent thresholds.

BMPS BIND TO EXTRACELLULAR MATRIX

It is well known that extracellular matrix components play a critical role in morphogenesis. These structural macromolecules and their supramolecular assembly in the matrix do not explain their role in epithelial–mesenchymal interaction and morphogenesis. This riddle can now be explained by the binding of BMPs to heparan sulfate heparin and type IV collagen [28–30] of the basement membranes. In fact, this binding might explain in part the necessity for angiogenesis prior to osteogenesis during development. In addition, the actions of activin in development of the frog, in terms of dorsal mesoderm induction, are modified to neuralization by follistatin [31]. Similarly, Chordin and Noggin from the Spemann organizer induce neuralization via binding and inactivation of BMP-4 (Fig. 11.2). Thus neural induction is likely to be a default pathway when BMP-4 is non-functional [32,33]. Thus, an emerging principle in development and morphogenesis is that binding proteins can terminate a dominant morphogen's action and initiate a default pathway. Finally, the binding of a soluble morphogen to extracellular matrix converts it into an insoluble matrix-bound morphogen to act locally in the solid state [28].

Although BMPs were isolated and cloned from bone, work with gene knockouts has revealed a plethora of actions beyond bone. Targeted disruption of BMP-2 in mice caused embryonic lethality. The development of the heart is abnormal, indicating a need for BMP-2 in heart development [34]. BMP-4 knockouts exhibit no mesoderm induction, and gastrulation is impaired [35]. Transgenic overexpression of BMPs under the control of keratin 10 promoter leads to psoriasis. The targeted deletion of BMP-7 revealed the critical role of this molecule in kidney and eye development [36,37]. Thus the BMPs really are true morphogens for such disparate tissues as skin, heart, kidney and eye. In view of this, BMPs may also be called body morphogenetic proteins [38].

BMP RECEPTORS

Recombinant human BMP-4 and BMP-7 bind to BMP receptor IA (BMPR-IA) and BMP receptor IB (BMPR-IB) [39]. CDMP-1 also binds to both type I BMP receptors. There is collaboration between type I and type II BMP receptors [75]. The type I receptor serine/threonine kinase phosphorylates a signal-transducing protein substrate called Smad 1 or 5^{25}. Smad is a term derived from the fusion of the *Drosophila* Mad gene and the *Caenorhabtitis elegans* (nematode) Sma gene. Smads 1 and 5 signal in partnership with a common co-Smad, Smad 4 (Fig. 11.8). The

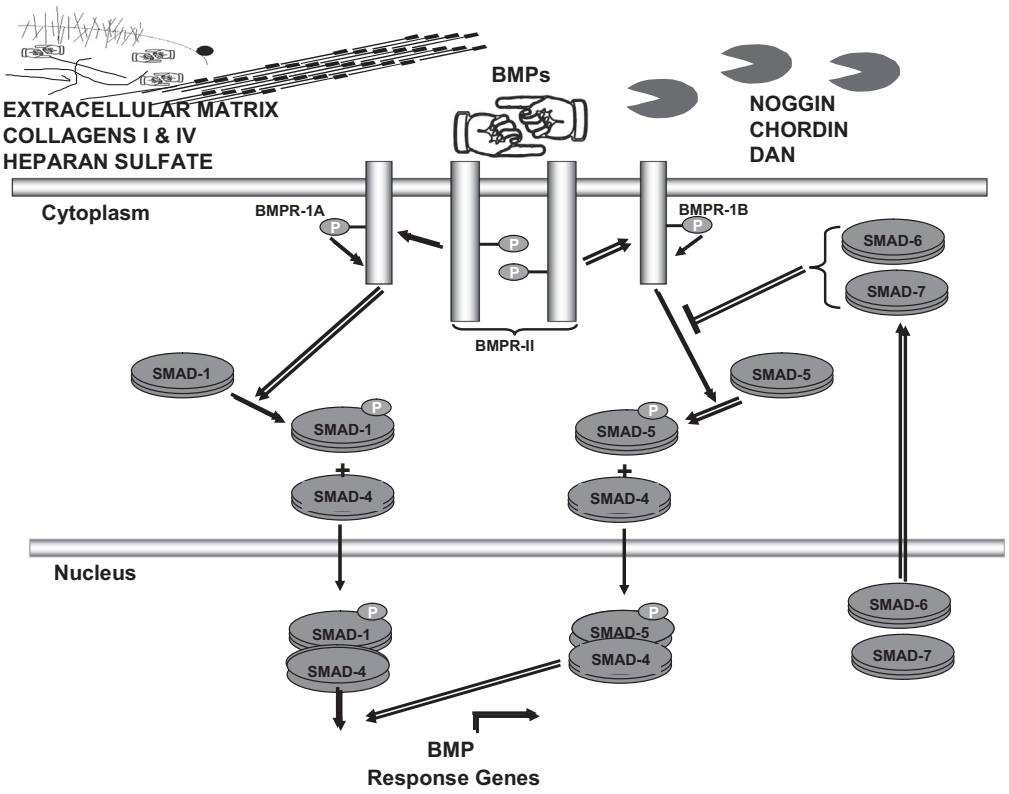

FIGURE 11.8

BMP receptors and signaling cascades. BMPs are dimeric ligands with cysteine knot in each monomer fold. Each monomer has two β-sheets, represented as two pointed fingers. In the functional dimer, the fingers are oriented in opposite directions. BMPs interact with both type I and II BMP receptors. The exact stoichiometry of the receptor complex is currently being elucidated. BMPR-II phosphorylates the GS domain of BMPR-I. The collaboration between type I and II receptors forms the signal-transducing complex. BMP type I receptor kinase complex phosphorylates the trimeric signaling substrates Smad 1 and Smad 5. This phosphorylation is inhibited and modulated by inhibitory interacts with Smad 4 (functional partner) and enters the nucleus to activate the transcriptional machinery for early BMP-response genes. A novel Smad-interacting protein (SIP) may interact and modulate the binding of heteromeric Smad 1/Smad 4 complexes to the DNA.

transcription of BMP-response genes are initiated by Smad 1/Smad 4 heterodimers. Smads are trimeric molecules, as discovered by X-ray crystallography. The phosphorylation of Smads 1 and 5 by type I BMP receptor kinase is inhibited by inhibitory Smads 6 and 7 [40]. Smad-interacting protein (SIP) may interact with Smad 1 and modulate BMP-response gene expression [15,41,42]. The downstream targets of BMP signaling are likely to be homeobox genes, the cardinal genes for morphogenesis and transcription. BMPs in turn may be regulated by members of the hedgehog family of genes, such as Sonic and Indian hedgehog [43], including receptors patched and smoothened and transcription factors such as Gli 1, 2, and 3. The actions of BMPs can be terminated by specific binding proteins, such as noggin [33].

RESPONDING STEM CELLS

It is well known that the embryonic mesoderm-derived mesenchymal cells are progenitors for bone, cartilage, tendons, ligaments, and muscle. However, certain stem cells in adult bone marrow, muscle and fascia can form bone and cartilage. The identification of stem cells readily sourced from bone marrow may lead to banks of stem cells for cell therapy and perhaps gene therapy with appropriate 'homing' characteristics to bone marrow and hence to the skeleton. The pioneering work of Friedenstein et al. [44] and Owen and Friedenstein [45] identified bone marrow stromal stem cells. These stromal cells are distinct from the hematopoietic stem cell lineage. The bone marrow stromal stem cells consist of inducible and

determined osteoprogenitors committed to osteogenesis. Determined osteogenic precursor cells have the propensity to form bone cells, without any external cues or signals. On the other hand, inducible osteogenic precursors require an inductive signal, such as BMP or demineralized bone matrix. It is noteworthy that operational distinctions between stromal stem cells and hematopoietic stem cells are getting more and more blurry! The stromal stem cells of Friedenstein and Owen are also called mesenchymal stem cells [46], with potential to form bone, cartilage, adipocytes, and myoblasts in response to cues from the environment and/or intrinsic factors. There is considerable hope and anticipation that these bone marrow stromal cells may be excellent vehicles for cell and gene therapy [47].

From a practical standpoint, these stromal stem cells can be obtained by bone marrow biopsies and expanded rapidly for use in cell therapy after pretreatment with BMPs. The potential uses in both cell and gene therapy is very promising. There are continuous improvements in the viral vectors and efficiency of gene therapy [48,79]. For example, it is possible to use BMP genes transfected in stromal stem cells to target the bone marrow.

MORPHOGENS AND GENE THERAPY

The recent advances in morphogens are ripe for techniques of regional gene therapy for orthopedic tissue engineering. The availability of cloned genes for BMPs and CDMPs and the requisite platform technology of gene therapy may have immediate applications. Whereas protein therapy provides an immediate bolus of morphogen, gene therapy achieves a sustained, prolonged secretion of gene products. Furthermore, recent improvements in regulated gene expression allow the turning on and off of gene expression. The progress in vectors for delivering genes also bodes well. The use of adenoviruses, adeno-associated viruses, and tetroviruses is poised for applications in bone and joint repair [48–51]. Although gene therapy has some advantages for orthopedic tissue engineering, an optimal delivery system for protein and gene therapy is needed, especially in the replacement of large segmented defects and in fibrous non-unions and mal-unions.

BIOMIMETIC BIOMATERIALS

Our earlier discussions of inductive signals (BMPs) and responding stem cells (stromal cells) lead us to the scaffolding (the microenvironment/extracellular matrix) for optimal tissue engineering. The natural biomaterials in the composite tissue of bones and joints are collagens, proteoglycans, and glycoproteins of cell adhesion, such as fibronectin and the mineral phase. The mineral phase in bone is predominantly hydroxyapatite. In its native state, the associated citrate, fluoride, carbonate, and trace elements constitute the physiological hydroxyapatite. Its high protein-binding capacity makes hydroxyapatite a natural delivery system. Comparison of insoluble collagen, hydroxyapatite, tricalcium phosphate, glass beads, and polymethylmethacrylate as carriers revealed collagen to be an optimal delivery system for BMPs [52]. It is well known that collagen is an ideal delivery system for growth factors in soft and hard tissue wound repair [53]. Hydrogels may be of great utility in cartilage tissue engineering [74].

During the course of systematic work on hydroxyapatite of two pore sizes (200 or 500 μm) in two geometrical forms (beads or disks), an unexpected observation was made. The geometry of the delivery system is critical for optimal bone induction. The disks were consistently osteoinductive with BMPs in rats, but the beads were inactive [54]. The chemical compositions of the two hydroxyapatite configurations were identical. In certain species, the hydroxyapatite alone appears to be 'osteoinductive' [55]. In subhuman primates, hydroxyapatite induces bone, albeit at a much slower rate. One interpretation is that osteoinductive endogenous BMPs in circulation progressively bind to an implanted disk of hydroxyapatite. When an optimal threshold concentration of native BMPs is achieved, the hydroxyapatite becomes

osteoinductive. Strictly speaking, most hydroxyapatite substrata are ideal osteoconductive materials. This example in certain species also serves to illustrate how an osteoconductive biomimetic biomaterial can progressively function as an osteoinductive substance by binding to endogenous BMPs. Thus, there is a physiological-physicochemical continuum between the hydroxyapatite alone and progressive composites with endogenous BMPs. Recognition of this experimental nuance will save unnecessary arguments among biomaterials scientists about the osteoinductive action of a conductive substratum such as hydroxyapatite.

Complete regeneration of baboon craniotomy defect was accomplished via recombinant human osteogenic protein (rhOP-1; human BMP-7) [76]. Recombinant BMP-2 was delivered by a poly(-hydroxy acid) carrier for calvarial regeneration [56].

Copolymers of polylactic acid and polyglycolic acid with recombinant BMP-2 were used in a non-union model in rabbit ulna, and complete unions were achieved in the bone [57].

An important problem in the clinical application of biomimetic biomaterials with BMPs and/or other morphogens is its sterilization. Although gas (ethylene oxide) can be used, one should always be concerned about reactive free radicals. Using allogeneic demineralized bone matrix with endogenous native BMPs, as long as a low temperature (4°C or less) is maintained, the samples tolerated up to 5—7 M rads of irradiation [58,59]. The standard dose acceptable to the Food and Drug Administration is 2.5 M rads. This information would be useful to the biotechnology companies preparing to market recombinant BMP-based osteogenic devices. Perhaps, the tissue-banking industry, with its interest in bone grafts [60], could also use this critical information. The various freeze-dried and demineralized allogeneic bone may be used in the interim as satisfactory carriers for BMPs. The moral of this experiment is it is not the irradiation dose but the ambient sample temperature during irradiation that is absolutely critical.

TISSUE ENGINEERING OF BONES AND JOINTS

Unlike bone, with its considerable prowess for repair and even regeneration, cartilage is recalcitrant. But why? In part this may be due to the relative avascularity of hyaline cartilage and the high concentration of protease inhibitors and perhaps even of growth inhibitors. The wound debridement phase is not optimal for preparing the cartilage wound bed for the optimal *milieu interieur* for repair. Although cartilage has been successfully engineered to predetermined shapes [61], true repair of the tissue continues to be a real challenge, in part due to hierarchical organization and geometry [62]. However, considerable excitement in the field has been generated by a group of Swedish workers in Gothenburg, using autologous culture-expanded human chondrocytes [63]. A continuous challenge in chondrocyte cell therapy is progressive dedifferentiation and loss of characteristic cartilage phenotype. The redifferentiation and maintenance of the chondrocytes for cell therapy can be aided by BMPs, CDMPs, TGF-β isoforms, and IGFs. It is also possible to repair cartilage using muscle-derived mesenchymal stem cells [64]. The possibility of problems posed by cartilage proteoglycans in preventing cell immigration for repair was investigated by means of chondroitinase ABC and trypsin pretreatment in partial thickness defects [65], with and without TGF. Pretreatment with chondroitinase ABC followed by TGF revealed a contiguous layer of cells from the synovial membrane, hinting at the potential source of repair cells from synovium. Multiple avenues of cartilage morphogens, cell therapy with chondrocytes and stem cells from marrow and muscle, and a biomaterial scaffolding may lead to an optimal tissue-engineered articular cartilage.

Recombinant human and BMP-2 and BMP-7 were approved in 2002 by the Food and Drug Administration (FDA) for tibial non-unions and single-level spine fusion. BMPs have been used in healing segmental defects (Fig. 11.9). The proof of the principle of tissue engineering based on BMPs as signals, molding via a scaffold, and responding cells can be demonstrated (Fig. 11.10).

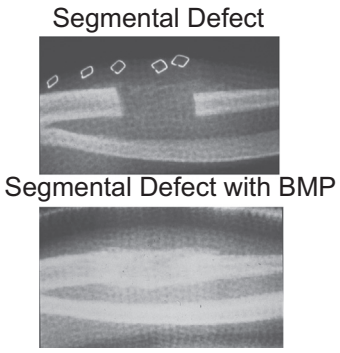

FIGURE 11.9
Repair of segmental defects in a primate bone by means of recombinant human BMP-7 and collagenous matrix scaffold. *(Photographs provided by Dr. T. K. Sampath.)*

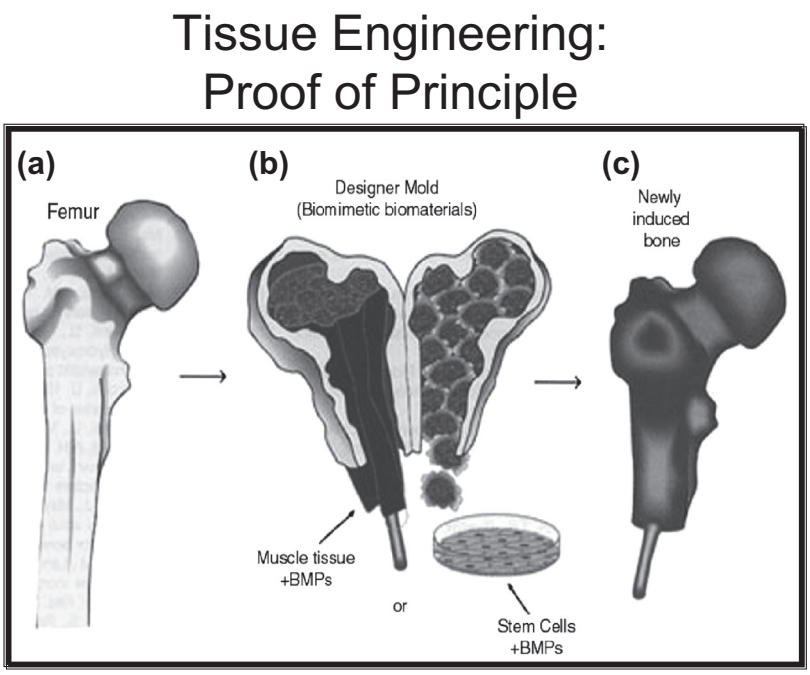

FIGURE 11.10
Proof of the principles of tissue engineering was established *in vivo* by Khouri et al. (1991) [73]. A mold was used to contain the vascularized muscle flap and treated with purified BMPs and collagen scaffold. The newly formed bone faithfully reproduced the shape of the mold. In the future, one can use stem cells directed by recombinant BMPs to induce bone.

FUTURE CHALLENGES

It is inevitable that as humans age that they will be confronted by impaired locomotion due to wear and tear in bones and joints. Therefore, the repair and possibly complete regeneration of the musculoskeletal system and other vital organs, such as skin, liver, and kidney, may potentially need optimal repair or a spare part for replacement. Can we create spare parts for the human body? There is much reason to be optimistic that tissue engineering can help patients. We are living in an extraordinary time with regard to biology, medicine, surgery, bioengineering, computer modeling of predictive tissue engineering and technology. The confluence of advances in molecular developmental biology and attendant advances in inductive signals for morphogenesis, stem cells, biomimetic biomaterials, and extracellular matrix biology augers well for imminent breakthroughs.

The symbiosis of biotechnology and biomaterials has set the stage for systematic advances in tissue engineering [14,66,67]. The recent advances in enabling platform technology include molecular imprinting [68]. In principle, specific recognition and catalytic sites are imprinted

using templates. The applications include biosensors, catalytic applications to antibodies and receptor recognition sites. For example, the cell-binding RGD site in fibronectin [69] or the YIGSR domain in laminin can be imprinted in complementary sites [70].

The rapidly advancing frontiers in morphogenesis with BMPs, hedgehogs, homeobox genes, and a veritable cornucopia of general and specific transcription factors, coactivators, and repressors will lead to co-crystallization of ligand–receptor complexes, protein–DNA complexes, and other macromolecular interactions. This will lead to peptidomimetic agonists for large proteins, as exemplified by erythroprotein [71]. To such advances one can add new developments in the self-assembly of millimeter scale structures floating at the interface of perfluorodecalin and water and interacting by means of capillary forces controlled by the pattern of wettablity [72]. The final self-assembly is due to minimization of free energy in the interface. These are truly incredible advances that will lead to man-made materials that mimic extracellular matrix in tissues. Superimpose on such chemical progress a biological platform in a bone and joint mold. Let us imagine a head of the femur and a mold fabricated via computer assisted design and manufacture. It faithfully reproduces the structural features and may be imprinted with morphogens, inductive signals, and cell adhesion sites. This assembly can be loaded with stem cells and BMPs and other inductive signals, with a nutrient medium optimized for the number of cell cycles, and then it predictably exits into the differentiation phase to reproduce a totally new bone femoral head. In fact, such a biological approach with vascularized muscle flap and BMPs yielded new bone with a defined shape and has demonstrated proof of the principle for further development and validation [73]. We indeed are entering a brave new world of prefabricated biological spare parts for the human body, based on sound architectural rules of inductive signals for morphogenesis, responding stem cells with lineage control, and with growth factors immobilized on a template of biomimetic biomaterial based on extracellular matrix. Like life itself, such technologies evolve with continuous refinements to benefit humankind by reducing the agony of human pain and suffering. In conclusion, based on principles of evolution, development, and self-assembly, the fields of tissue engineering and regenerative medicine are poised to make explosive advances with immense applications in the clinic.

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CHAPTER 12

Gene Expression, Cell Determination, and Differentiation

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INTRODUCTION

Studies of skeletal muscle development were the first to provide the principles for understanding the genetic and molecular bases of determination and differentiation. Molecular signals from adjacent embryonic structures activate specific genetic pathways within target cells. Important families of transcriptional regulators are expressed in response to these cues to initiate important developmental processes in skeletal muscle as well as in other tissues and organs. Both activators and repressors are essential to control the time and location at which development occurs, and self-regulating, positive feedback loops ensure that, once begun, development can proceed normally. An understanding of the mechanistic basis of the embryonic commitment to a unique developmental pathway, and the subsequent realization of the adult phenotype, are essential for understanding stem cell behavior and how this might be manipulated for therapeutic goals.

This chapter focuses on determination and differentiation, classical embryological concepts that emerged from descriptive embryology. It has been through the study of muscle development that the genetic basis of these processes was first revealed, laying out a mechanistic basis for understanding determination and differentiation. Following success in studies of skeletal muscle (reviewed in references [1] and [2]), genetic pathways involved in the determination of other systems, some of which are detailed in other chapters in this book, have also been uncovered, largely because of the underlying conservation of structure among the various effector molecules and mechanisms.

DETERMINATION AND DIFFERENTIATION

Determination describes the process whereby a cell becomes committed to a unique developmental pathway, which, under conditions of normal development, appears to be a stable state. In many cases, cells become committed early in development yet remain highly proliferative, expanding exponentially for long periods of time before differentiation occurs. Until recently, determination could only be defined post hoc. Prior to the discovery of transcriptional regulators there were few markers to indicate whether or not a cell was committed to a unique phenotype. Thus, determination was operationally defined as that state that existed immediately prior to differentiation, that is, before expression of a cell-type-specific

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phenotype. The identification of transcription factors that control the differential expression of large families of genes changed this concept.

Determination and differentiation are processes that are coupled during embryogenesis, where a small number of pluripotent cells (stem cells), expand and enter pathways through which they form the diverse cell types of the adult. The process of differentiation describes the acquisition of the phenotype of a cell, most often identified by the expression of specific proteins achieved as a result of differential gene expression. The differentiated state is easily determined by simple observation in most instances, because most differentiated cells display a unique phenotype as a result of the expression of specific structural proteins. Skeletal muscle cells are an extreme example of this, having a cytoplasmic matrix filled with highly ordered myosin, actin, and other contractile proteins within sarcomeres — the functional units of contraction — giving the fibers their cross-striated pattern. As development proceeds, there is a gradual narrowing of the possible final cell phenotypes that individual cells can adopt, with the final cell fate (set of genes expressed) determined by factors both extrinsic and intrinsic to the cell (Fig. 12.1).

Changes in gene expression responsible for directing cells to differentiate along particular developmental pathways result from the response to stimuli received from surrounding cells and the specific cellular phenotype of the cell itself at the time of interaction. For example, cells of recently formed somites have the potential to form either skeletal muscle or cartilage in response to adjacent tissues, and the fate adopted is a result of their location with respect to adjacent structures — the notochord, neural tube, and overlying ectoderm — that produce signaling molecules that determine phenotype [3]. In addition to the activation of muscle-specific structural and enzyme-encoding genes, the differentiated state is maintained by the continued expression of specific regulatory transcription factors, which can now be identified

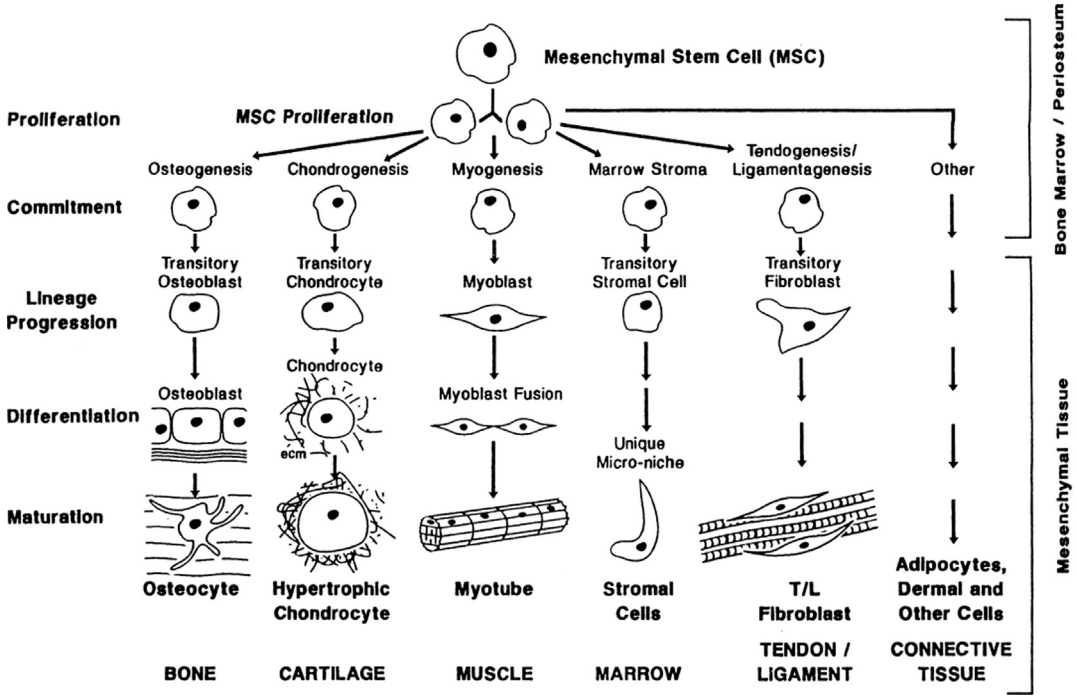

FIGURE 12.1

The process of commitment and differentiation. Cells arise during gastrulation in the vertebrate embryo that subsequently produce all the different cell type of the body. Cells that can be designated as mesenchymal stem cells (MSC) proliferate and, in response to cues from the cellular environment, enter lineages that undergo differentiation and subsequent maturation into the mature cell types.

using modern tools of cellular and molecular biology, including monoclonal antibodies, antisense nucleic acid probes, and gene chip analyses.

Commitment and differentiation to a skeletal muscle fate begins in the somites of the early vertebrate embryo. Within the embryonic somites, two distinct anatomical regions contain muscle progenitors. Specified by signals from the adjacent structures, the dorsal portion of each somite forms an epithelial structure, the dermomyotome, which contains the precursor cells of all skeletal muscles that will form in the vertebrate body (with the exception of those found in the head). The medial portion of dermomyotome contains cells that form the axial musculature surrounding the vertebral column, while cells of the ventral-lateral portion of the dermomyotome undergo a process of delamination and migrate into the forming appendages to produce the appendicular musculature of the limbs and body wall [4]. While the muscle fibers that form from the different regions of the somite are nearly indistinguishable, myogenesis in the axial and appendicular muscles is regulated by different effectors, demonstrating the complexity of determination and differentiation in the early embryo.

MyoD AND THE bHLH FAMILY OF DEVELOPMENTAL REGULATORY FACTORS

It was not until late in the twentieth century that experiments first demonstrated that cellular commitment to specific developmental fates could be determined by the expression of a single gene or a very small number of genes [5–9]. With the improvements in tissue culture methods and rapid advances in molecular biology that permitted the introduction of foreign genes into mammalian cells, the first factor capable of specifying a cell to a particular cellular phenotype, MyoD, was isolated and characterized in the laboratory of Dr. Harold Weintraub [10,11]. MyoD expression is specific to skeletal muscle, and introduction of MyoD cDNA into fibroblasts of the 10T1/2 cell line converts them at a high frequency into stable myoblasts, which in turn express skeletal muscle proteins. MyoD was only the first of a family of myogenic regulatory factors (MRFs) to be discovered; others include myf-5 [12], myogenin [13], and MRF4 [14]; reviewed by Berkes and Tapscott [1]. The importance of MyoD and myf-5 to the determination of skeletal muscle was demonstrated when double knockout of these two genes in transgenic mice resulted in a nearly complete absence of skeletal muscle [15].

MRF members share a common structure, a stretch of basic amino acids followed by a stretch of amino acids that form two amphipathic helices separated by an intervening loop (the helix-loop-helix [HLH] motif), and they are nuclear-located DNA-binding proteins that act as transcriptional regulators [1]. Experiments have demonstrated that the basic amino acids are required for DNA binding and are essential for the myogenic conversion of fibroblasts to muscle, while the HLH motif plays an essential role in the formation of heterodimers with other ubiquitously expressed HLH proteins (products of the E2a gene) as well as in DNA binding [16,17,18].

Nature being conservative, it is not surprising that the bHLH motif was found in transcriptional factors regulating the determination of cell types other than muscle. Based on homology with MyoD, the transcription factor NeuroD was isolated by the Weintraub laboratory and shown to act as a neuronal determination factor [19]. Expression of NeuroD in presumptive epidermal cells of Xenopus embryos converted many into fully differentiated neurons. Interestingly, NeuroD also plays an important role in the differentiation of pancreatic endocrine cells and the retina [20,21,22]. While NeuroD is involved primarily in neuronal differentiation and survival, neurogenin, whose expression precedes that of NeuroD in the embryo, functions more like a determination factor [23]. Overexpression of Xenopus neurogenin induces ectopic neurogenesis as well as ectopic expression of NeuroD. Additional bHLH family members, including HES, Math-5, and Mash-1, have been isolated and participate in the determination of neural cells as well.

Differences in the expression of various members of the neurogenic bHLH family help to explain the diversity of neuronal cell types. For example, genetic deletion of the Mash-1 gene eliminates sympathetic and parasympathetic neurons and enteric neurons of the foregut [54], while knockout of NeuroD leads to a loss of pancreatic endocrine cells as well as cells of the central and peripheral nervous system [20]. In addition to the various bHLH activators, other homeodomain-containing transcription factors are required for specification of neuronal subtypes.

Because cardiac muscle has so much in common with skeletal muscle, including a large number of contractile proteins, an exhaustive search was made for MyoD family members in the heart. Surprisingly, MyoD family members were not found in the developing heart, and thus they play no part in the differentiation of cardiac muscle cells. However, a different family of bHLH-containing factors, including dHAND and eHAND, were found in the developing heart, autonomic nervous system, neural crest, and deciduum. In the heart these factors are important for cardiac morphogenesis and the specification of cardiac chambers [25,26]. Unlike their MyoD family cousins, neither of the HAND proteins plays a role in the differentiation of cardiac muscle cells.

Acting as dominant negative regulators of the bHLH family of transcriptional regulators is a ubiquitously expressed family of proteins that contain the helix-loop-helix structure but lack the upstream run of basic amino acids essential for specific DNA binding by MyoD family members. Termed inhibitors of differentiation (Id), these proteins can associate specifically with MyoD or products of the E2A gene, and attenuate their ability to bind DNA by forming non-functional heterodimeric complexes [27]. In proliferating myoblasts, Id inhibit the terminal differentiation program by complexing with the E12/E47 protein until the cell receives an appropriate stimulus. Id levels decrease on terminal differentiation.

Neuronal development is also regulated in part by repressors of the neurogenic family of bHLH activators. The HES family of HLH-containing proteins is expressed in neural stem cells, where they maintain proliferation of neuronal precursors and prevent premature differentiation in cells expressing NeuroD and neurogenin. The interaction of unique sets of positively acting bHLH activators and negatively acting members of the HES family helps explain how different subsets of neurons undergo differentiation at different times during development, so that the complex structure of the brain can be produced [29,30].

MEFS AND microRNAs — COREGULATORS OF DEVELOPMENT

MEF2 proteins are transcriptional activators that bind to A+T-rich DNA sequences found in many muscle-specific genes, including those encoding contractile proteins, muscle fiber enzymes, and the muscle differentiation factor myogenin. While some members of this family of MADS-box regulatory factors show a nearly ubiquitous distribution among tissue types, a few show expression that is more restricted to striated muscle [31]. Because they do not act alone, MEF2 family members must physically interact with MyoD family members at their DNA-binding domains to positively regulate transcription of downstream muscle-specific differentiation genes [32]. Additionally, the transcriptional activation of muscle-specific genes requires that either the MyoD or MEF2 protein provide a transcriptional activation domain. Interestingly, although the wide tissue distribution of some MEF2 family members suggested that they may act in combination with bHLH family members found in other cell types (such as neurogenin in neural precursors) to activate downstream genes (Molkentin and Olson, 1996), no evidence has been found to that effect [33,34].

A common method of regulation in most differentiating cells which results in specification, maintenance, and regulation of all complex regulatory circuits, are non-coding RNAs which includes microRNAs [35]. Differentiation requires not only the acquisition of new functions through gene activation and transcription, but also the suppression of gene transcription at

the expression level [29]. MicroRNAs (miRNAs), which regulate gene expression at the transcriptional and post-transcriptional levels, play key roles in differentiation [35]. They can suppress gene expression by binding to mRNAs through complementarity in sequence between the two and altered expression by alternative splicing [36]. In particular, they have been shown to play key roles in the post-transcription regulation in skeletal muscle cell differentiation. Specific microRNAs interact with MyoD and other bHLH transcription factors, such as Twist, in cooperation with MEF2 by binding of MyoD and myogenin [37]. Some of the key functions of MEF2 that are central to muscle cell gene transcription are controlled by microRNAs. Particular miRNAs are absent from undifferentiated myoblasts and are strongly upregulated upon differentiation [36]. Experiments suggest that miRNAs also act as modulators of myogenic differentiation, affecting expression of genes not involving Mef2. An example is that an increase of miR1 expression in muscle cell culture accelerates myoblast differentiation by downregulating histone deacetylase 4 (HDAC4), a repressor of muscle differentiation [38]. The interplay between transcription factors and microRNAs provides the fine tuned modulation of protein expression characteristic of differentiating cells.

Pax IN DEVELOPMENT

Much of what has been learned about the role of MyoD and myf-5 in the determination of skeletal muscle has come from studies on transgenic mice in which various genetic loci have been deleted [15,39–43]. This work suggested that MyoD and myf-5 acted as redundant activators of myogenesis, albeit with some slight distinctions. Subsequently, Pax3, a DNA-binding protein with both a paired box and a paired-type homeodomain, was identified as a key regulator of myogenesis [44,45]. Double knockout of Pax3 and myf-5 leads to a complete absence of skeletal muscle and places Pax3 genetically upstream of MyoD [46]. Using knock-in experiments where the lacZ marker gene replaced Pax3, Buckingham's group demonstrated that Pax3 and myf-5 are activated independently of one another. The implication is that axial muscle (myf-5-dependent) and appendicular muscle (MyoD-dependent) are specified separately in the embryonic somites by two different pathways [47,48], and that a Pax gene (or more than one) is required for this specification (determination).

A second member of the paired box transcription factor family, Pax7, has also been implicated in myogenesis. Pax7 was isolated from satellite cells, a population of muscle-committed stem cells found in intimate association with mature muscle fibers and involved in muscle growth and repair in the adult. Pax7 is specifically expressed in proliferative myogenic precursors, both embryonic myoblasts as well as satellite cells, and is down regulated at differentiation [49]. Transgenic mice lacking the Pax7 gene have normal musculature, albeit with reduced muscle mass, but a complete absence or markedly reduced numbers of satellite cells [49,50]. These investigators found that in these Pax7 mutants, satellite cells, which are cells responsible for postnatal growth of skeletal muscle, are progressively lost by cell death. These results suggest that specification of skeletal muscle satellite cells requires Pax7 expression, or that Pax7 expression is responsible for survival of satellite cells. The interplay of the many factors that control the initiation and maintenance of myogenesis are shown in Fig. 12.2.

SATELLITE AND STEM CELLS IN SKELETAL MUSCLE DIFFERENTIATION AND REPAIR

The reason that a discussion of skeletal muscle differentiation is so central to a basic understanding of cell differentiation is that this tissue unites concepts of tissue formation and tissue repair. The unifying concept is the stem cell. Among the first to be considered as stem cells were the satellite cells of skeletal muscle (reviewed by Wang and Rudnicki [51]). These cells lie just beneath the basal lamina that surrounds each muscle fiber within an anatomic muscle. These cells meet the definition of stem cells [46] because they are self-renewing. They are cells set aside in early muscle development, during which they contribute to fiber lengthening as

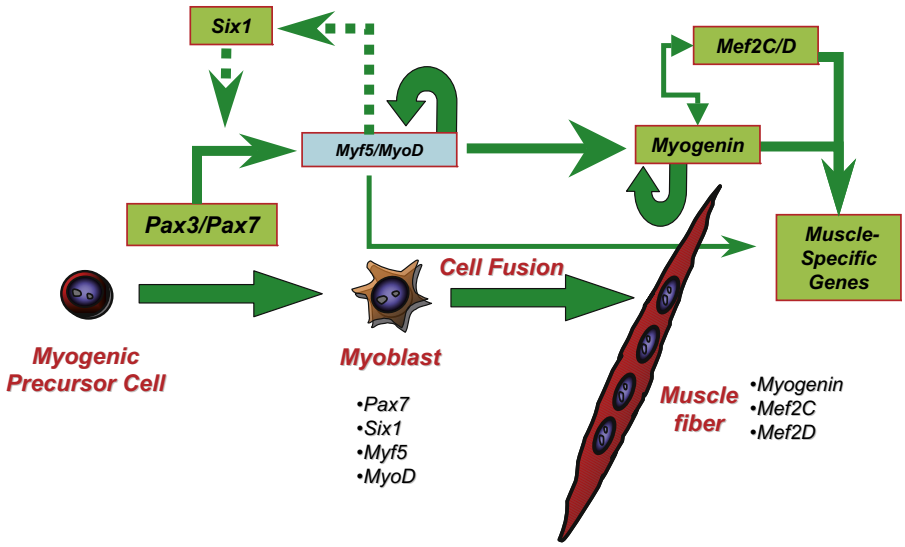

FIGURE 12.2
A regulatory network controls muscle cell differentiation. *(Provided by Dr. Michael Rudnicki and modified.)*

muscles grow, as well as to the formation of additional fibers as the neonate matures. On the other hand, muscle hypertrophy, as in weight lifters, is primarily produced by expansion is size of the fibers rather than fiber number. However, because of injury of small numbers of fibers, satellite cells can fuse with existing fibers during this process. They are located within special niches formed by the basal lamina. These niches keep satellite cells in a quiescent state until there is injury to the fibers of a muscle on which they lie. Injury results in the release of cytokines that initiate the proliferation and migration of the satellite cells on the surface of the injured fibers. The cell divisions of satellite cells are asymmetric – one daughter forms another satellite cell (stem cell) and is retained in the niche and the other goes forward as a myogenic cell, which most often fuses with an injured fiber to reconstitute its multinuclear state, or occasionally, depending on the nature of the injury, to form a new fiber [52]. Thus, while muscle regeneration in higher vertebrates is very limited, new fibers can form and old fibers can be repaired while still retaining a supply of stem cells for future tissue repair. The challenge to investigators of tissue engineering is to develop strategies to facilitate extensive muscle stem cell proliferation, cell survival, and efficient cell differentiation of transplanted cells. Equally challenging is gaining an understanding of the cells that form the connective tissue scaffolding of an anatomic muscle, if such satellite cells or stem cells are to successfully be used to treat neuromuscular disease or to reconstitute muscles following trauma [53].

Among the most promising new approaches to repair of skeletal muscles damaged by disease or trauma are embryonic stem cells (ES cells) and induced pluripotent stems (iPS cells). In recent times there has been a flurry of experimental activity in model systems for regenerating and primary muscular diseases, which exploit these two types of cells [54–56]. ES cells are derived from the inner cell mass of pre-implantation human and other vertebrate embryos. These cells can differentiate into a variety of cell types, including skeletal and cardiac muscle, under the influence of factors added to culture media in which they are grown. On the other hand iPS cells can be derived from many adult human cell types obviating, in the case of humans, the need for human embryos. iPS cells can be obtained through direct reprogramming of different human somatic cells to a pluripotent state by a limited number of genes, or by transfection with retroviruses, lentiviruses or plasmids [54]. There is also a variety of other cells types that have been specifically linked to skeletal muscle differentiation [57], including bone marrow-derived side-population cells [58], mesenchymal stem cells [59], pericytes [60], CD133+ progenitor cells [61] , and mesoangioblasts [62] because they show a high myogenic propensity *in vitro* and *in vivo*. But only ES and iPS cells overcome the disadvantage of the latter cell types because they can be produced in sufficient quantities for study and transplantation.

The use of ES cells from humans is complicated by ethical considerations, whereas iPS cells are not, because they do not require human embryos. iPS cells also have the advantage that each individual could have their own cells (autologous patient-specific stem cells) used to form iPS cells, thus obviating the immunological rejection that accompanies the use of ES cells. The hurdles to overcome in tissue engineering of muscle are the size of muscles in humans (human anatomic muscles are relatively large), which require large numbers of cells for adequate repair. This means that transplanted cells must proliferate and endure *in vivo*, and that they become co-opted into the satellite cell lineage for subsequent hypertrophy of the muscle or its repair. In any situation where cells for transplantation are encouraged to proliferate and differentiate *in vitro* following viral transfection, an important challenge is the formation of teratomas [63] or insertional mutagenesis.

The advantage of iPS cells for skeletal muscle transplantation has been exploited in murine model systems. Darabi and colleagues [64,65] and Mizuno and colleagues [66] have used iPS cells from the mouse transfected with a gene (Pax 7) fostering myogenic differentiation showing that these engineered cells are effective in engraftment, improving muscle function in disease models. The studies demonstrate in principle that the use of stem cell transplantation can be effective.

However, for human transplantation it will be important that human iPS cells be directed into the myogenic lineage without the use of transfection or other approaches that alter the structure of the genome [67]. The use of non-transfection strategies is desirable, as the integration of transfected genes can be problematic for the functioning of the cells. Recently, the hurdle of making human iPS cells initially obtained from retrovirally transformed cells that can undergo myogenesis *in vivo* may have been overcome when tested in an immunoincompetent model mouse systems [68]. Under specific culture conditions, myogenic iPS cells were obtained which formed human muscle when transplanted, supplying satellite cells for subsequent regeneration in response to injury, and reportedly do not form tumors. While these approaches with human iPS cells are exciting and hold great promise, additional work is needed before induced pluripotent stem cell transplantation can be applied in humans.

CONCLUSIONS

Determination and differentiation are in large part controlled by the expression of transcriptional regulators. The processes begin early in development and involve the formation of stem cells that become committed to specific pathways of regulated gene expression. The regulators responsible were first characterized in studies examining commitment to, and differentiation of, skeletal muscle, and muscle development serves as a model for the mechanisms involved. Some other developing organs, such as the central and peripheral nervous systems and the pancreas, employ very similar mechanisms and closely related members of the bHLH family of proteins to drive development. However, for reasons that are not clear, other organs, such as the heart, in which cardiac cells express many of the same contractile protein genes as their skeletal muscle cousins, use other mechanisms. As our understanding of the mechanisms and effectors of determination and differentiation during embryonic development increase, we will be better able to conceive and apply strategies to engineer stem cells, embryonic or adult-derived, to address medical problems through transplantation.

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PART 2

In Vitro Control of Tissue Development

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CHAPTER 13

Engineering Functional Tissues: *In Vitro* Culture Parameters

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INTRODUCTION

Clinical disorders typically associated with musculoskeletal and cardiovascular tissues (i.e., osteoarthritis and myocardial infarction, respectively) often result in the loss of native tissue structural organization and mechanical function [1,2]. Tissue engineering is a rapidly growing field that seeks to restore the structure and function of tissues damaged due to injury, aging or disease through the use of cells, biomaterials, and biologically active molecules (Fig. 13.1). Despite many early successes, there are few engineered tissue products available for clinical use, and significant challenges remain with regard to translating tissue-engineering technologies to the clinic for successful long-term repair of dysfunctional tissues. The precise reasons for graft failure are not fully understood, but they include a combination of factors that lead to the breakdown of repair tissues under conditions of physiologic loading.

Two critical aspects of tissue-function in many tissue and organ systems are the transmission or generation of mechanical forces and the maintenance of blood circulation. In particular, articular cartilage and myocardium have highly specialized tissue composition and structure to allow for the specific mechanical and transport properties of these tissues. Although different in many respects, cartilage and cardiac tissues both perform critical biomechanical functions *in vivo* and lack intrinsic capacity for self repair. Therefore, cartilage and cardiac tissues are ideal targets for functional tissue engineering.

At the time of implantation, tissue-engineered constructs rarely possess mechanical properties that can withstand the high magnitudes of mechanical stresses experienced *in vivo*. For many native tissues, however, the potential range of *in vivo* stresses and strains are not well characterized, thus making it difficult to incorporate a true 'safety factor' into the design criteria for engineered tissues. In addition, matching a single mechanical parameter, such as modulus or strength, is rarely sufficient, as most tissues possess complex viscoelastic, non-linear, and anisotropic mechanical properties that may vary with age, site, and other factors [3].

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00013-6

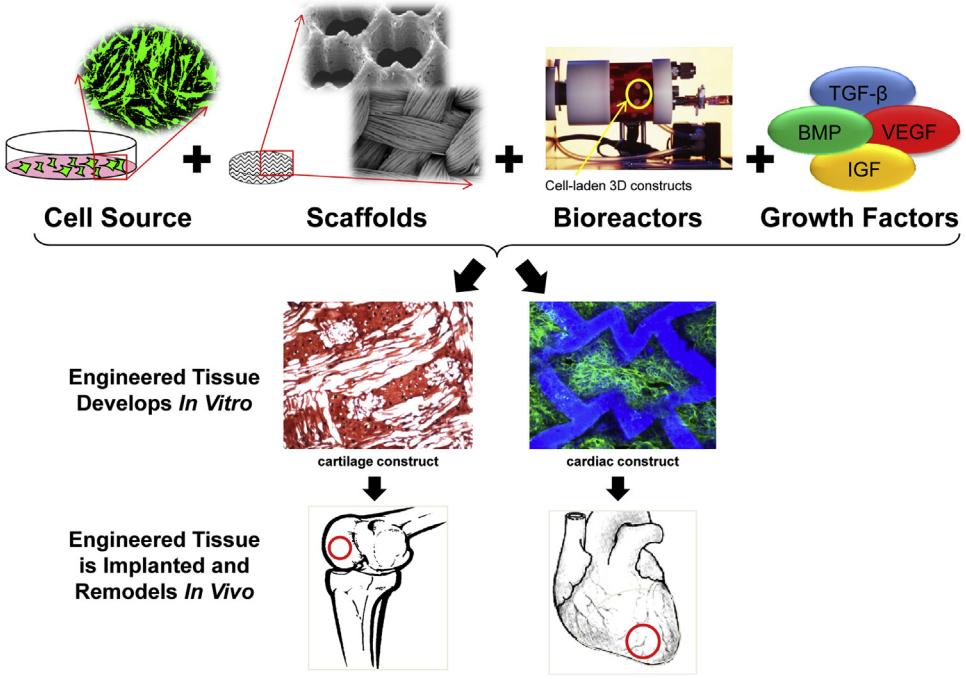

FIGURE 13.1

Strategy for engineering functional tissues *in vitro*. Cells, scaffolds, bioreactors, and growth factors are used as tools to create functional engineered tissues. This chapter focuses on the *in vitro* culture of engineered cartilage and cardiac constructs that can be utilized for basic research and, potentially, for repair of damaged articular cartilage and myocardium. *Top Panel: scaffold images adapted from Engelmayr, Jr., G.C. et al., Nature Materials 7:1003, 2008 [67] (top) and Moutos and Guilak, Tissue Eng Part A 16:1291, 2010 [42] (bottom). Middle Panel: engineered cartilage image adapted from Valonen, P.K., et al., Biomaterials, 31(8):2193, 2010 [93]; engineered cardiac image adapted from Engelmayr, Jr., G. C. et al., Nature Materials 7:1003, 2008 [67].*

A number of complex interactions must also be considered, as the graft and surrounding host tissues are expected to integrate and remodel in response to their changing environments post-implantation [4]. For example, convective transport of oxygen, nutrients, and waste products may become a limiting factor. Currently, most tissue-engineered constructs do not contain a functioning vasculature at the time of implantation, instead relying on either anastomosis of implanted capillary networks to the host vasculature or *de novo* angiogenesis to provide transport capability. Because oxygen has low solubility in aqueous media and diffuses over distances of only 100 to 200 μm, oxygen transport is often a limitation in cell survival within large, anatomically relevant grafts [5–7].

An evolving discipline referred to as 'functional tissue-engineering' has sought to address the aforementioned challenges by developing guidelines for rationally investigating the role of biological and mechanical factors in tissue engineering. A series of formal goals and principles for functional tissue engineering have been proposed in a generalized format [3,8,9]. In brief, these guidelines include development of:

1) Improved definitions of functional success for tissue-engineering applications;
2) Improved understanding of the *in vivo* mechanical requirements and intrinsic properties of native tissues;
3) Improved understanding of the biophysical environment of cells within engineered constructs;
4) Scaffold design criteria that aim to enhance cell survival, differentiation, and tissue mechanical function;
5) Bioreactor design criteria that aim to enhance cell survival and the regeneration of functional tissue-engineered constructs;

6) Construct design criteria that aim to meet the metabolic and mechanical demands of specific tissue-engineering applications; and
7) Improved understanding of biological and mechanical responses of an engineered tissue construct following implantation.

Since these guidelines were proposed, significant progress has been made toward developing *in vitro* culture systems and techniques to enhance graft mechanical properties and engineer functional tissues.

One of the key challenges in functional tissue engineering is optimizing the *in vitro* culture environment in order to produce three-dimensional (3D) implants that can meet the requirements of the *in vivo* milieu. In particular, the ability to precisely define and control *in vitro* culture conditions can be exploited to improve and ultimately control the structure, composition and mechanical properties of engineered tissues. This chapter focuses on how advanced *in vitro* culture strategies, including the use of scaffold systems, bioreactors, growth factors and mechanical conditioning, can influence the development and performance of engineered tissues (Fig. 13.1). Although the discussion addresses cartilage and cardiac tissues specifically, the concepts are also of relevance to other tissues and organs that serve some mechanical function (e.g., muscle, tendon, ligament, bone, blood vessels, heart valves, bladder) and are the targets of tissue-engineering research efforts. The following sections of this chapter will consider key concepts, the importance of *in vitro* studies, and the influence of selected *in vitro* culture parameters on the development and performance of engineered tissues. Illustrative examples and alternative approaches for engineering cartilage and cardiac tissues are provided.

KEY CONCEPTS FOR ENGINEERING FUNCTIONAL TISSUES

Fundamental parameters for engineering functional tissues

Many tissue-engineering approaches involve the *in vitro* culture of cells on biomaterial scaffolds to generate functional engineered constructs. The working hypothesis is that *in vitro* culture conditions have a significant influence on the structural and mechanical properties of engineered tissues, and therefore can be exploited to manipulate the growth and functionality of engineered tissues. ***In vitro culture conditions*** will refer to tissue-engineering scaffold systems, bioreactors, growth factors, and mechanical conditioning regimens that mediate cell behavior and functional tissue assembly [10,11]. ***Scaffolds*** will be defined as 3D material structures designed to perform some or all of the following functions:

1) Promote cell-biomaterial interactions, cell adhesion, and extracellular matrix (ECM) deposition;
2) Permit sufficient transport of gases, nutrients and regulatory factors to allow cell survival, proliferation and differentiation;
3) Biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest; and
4) Provoke a minimal degree of inflammation or toxicity *in vivo* [7,12].

Bioreactors will be defined as laboratory devices designed to perform some or all of the following functions:

1) Provide control over the initial cell distribution on 3D scaffolds;
2) Provide efficient mass transfer of gases, nutrients, and growth factors to tissue-engineered constructs during their *in vitro* cultivation; and
3) Expose the developing constructs to convective mixing, perfusion, and/or mechanical, electrical, or other biophysical factors in a controlled manner [5,6,13–17].

Mechanical conditioning will be defined as the *in vitro* application of dynamic mechanical loads (i.e., compression, tension, pressure, and/or shear) to cells, tissues, and/or three-dimensional

(3D) engineered tissue using custom designed systems. These fundamental *in vitro* culture parameters can be controlled independently or in combination to strategically meet the requirements of the specific tissue to be engineered.

Fundamental criteria for engineering functional tissues

The biological and mechanical requirements of an engineered tissue depend on the specific application. For example, engineered cartilage should provide a low-friction, articulating surface and be able to withstand and transmit load in compression, tension, and shear; whereas engineered cardiac tissue should propagate electrical signals, contract in a coordinated manner, and withstand dynamic changes in pressure, tension, and shear. In addition to tissue-specific requirements that serve as design principles for functional tissue engineering, there are fundamental criteria that all engineered tissues should meet:

1) At the time of implantation, an engineered tissue should possess sufficient size and mechanical integrity to allow for handling and permit survival under physiological conditions;
2) Immediately following implantation, an engineered tissue should provide some minimal level of biomechanical function that should improve progressively until normal tissue function has been restored; and
3) After implantation, an engineered tissue should mature and integrate with surrounding host tissues.

IMPORTANCE OF *IN VITRO* STUDIES FOR ENGINEERING FUNCTIONAL TISSUES

Cells cultured *in vitro* tend to retain their differentiated phenotype and function under conditions that resemble their natural *in vivo* environment; these conditions may be generated using a combination of scaffolds, bioreactors, growth factors, and mechanical conditioning [3,8–11]. *In vitro*-grown tissue-engineered constructs can potentially be transplanted *in vivo* for tissue engineering and regenerative medicine applications or utilized as platforms for *in vitro* testing of cell and tissue-level responses to molecular, mechanical, or genetic manipulations.

In vitro studies relevant to tissue engineering and regenerative medicine

In vivo models are essential for clinical translation of engineered tissues as they provide insight into the functional performance of engineered tissues. However, these models are complicated by high variability and biological and mechanical environments that differ from those existing in the human condition. To overcome these limitations, *in vitro* studies can be designed to understand the performance of the engineered tissue *in vivo* by: (i) addressing the challenges of *in vivo* complexity in more controllable *in vitro* systems, and (ii) exploring how an *in vitro*-grown construct may behave when implanted *in vivo*. For example, tissue-engineered cartilage constructs pose a significant challenge with regard to variable and incomplete integration upon implantation *in vivo* [18]. To address the challenge of tissue integration, researchers have taken the approach of evaluating the integration of engineered tissues with native tissues using not only *in vivo* models [19], but also *in vitro* models (Fig. 13.2). In one study, cells were combined with three types of scaffolds [fibrin, agarose, and poly(glycolic acid) (PGA)], incorporated with explants of native articular cartilage, and cultured as composites for 20 or 40 days [20]. The presence of native cartilage significantly altered cell proliferation and matrix accumulation in the composites. Additionally, although engineered constructs based on all three scaffold materials adhered to the native cartilage, there were significant differences in the adhesive strength between the groups, suggesting that the type of scaffold may influence construct integration *in vivo*.

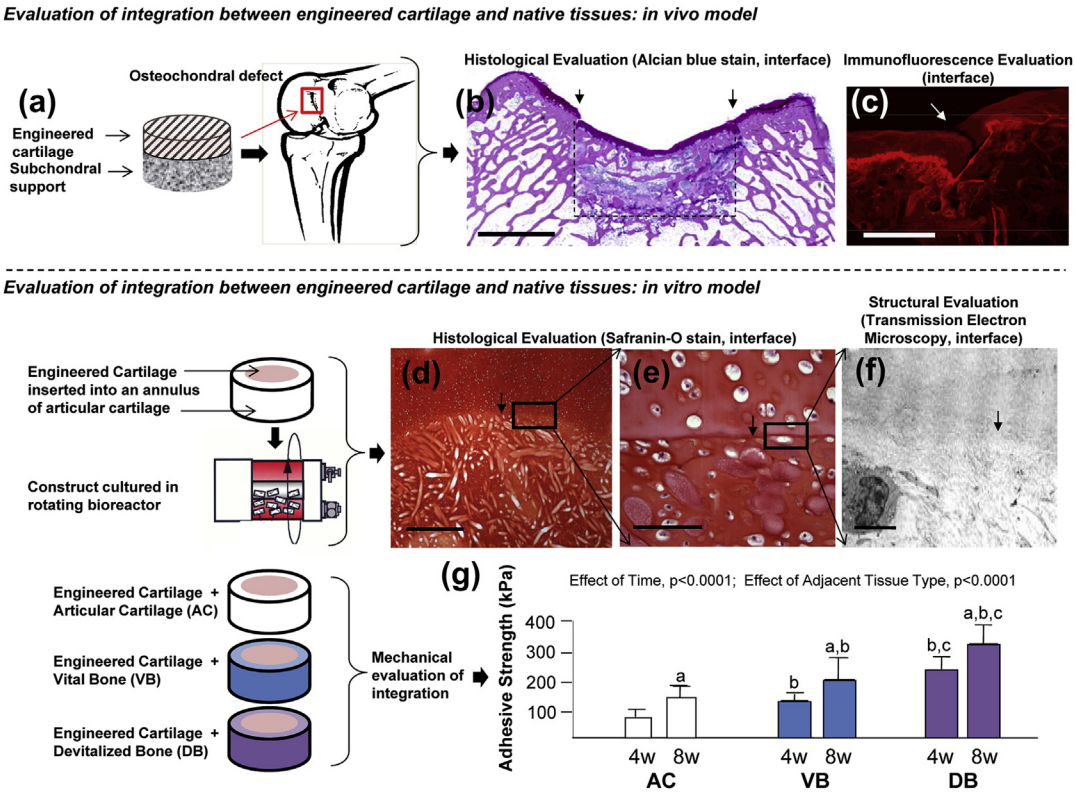

FIGURE 13.2

Studies of the clinically relevant problem of engineered cartilage integration. Top Panel: a) Osteochondral implant integration was studied using adult rabbits with surgically created osteochondral defects. b, c) Histological appearances of explants harvested after 6 months. b) Alcian blue stain, scale bar: 2.5 mm, dashed line shows borders of original defect; c) Immunofluorescence stain, scale bar: 400 μm. Arrows indicate areas of incomplete integration between engineered and host cartilages. Bottom Panel: Engineered cartilage integration was studied using rotating bioreactors to culture engineered cartilage construct disks within rings of articular cartilage (AC), vital bone (VB), and devitalized bone (DB). d, e) Histology of the construct-AC interface (Safranin-O stain, scale bars: 500 μm and 50 μm for D and E, respectively). f) Transmission electron micrograph of the construct-AC interface (scale bar: 5 μm). Arrows at interfaces point toward the construct and arrowheads indicate the scaffold. g) Adhesive strength for construct disks cultured in rings of AC (*white*), VB *(blue)* or DB *(purple)* for 4 or 8 weeks *(4w, 8w)*. Data represented are mean ± SD. [a]Significant difference due to time; [b]Significantly different from the corresponding AC composite; [c]Significantly different from the corresponding VB composite. *Top panel: adapted from Schaefer et al., Arthritis & Rheumatism, 46(9):2524, 2002 [19]. Middle and Bottom panels: adapted from Tognana et al., Osteoarthritis Cartilage 13(2):129, 2005 [21].*

In another *in vitro* study, cells were combined with hyaluronan benzyl ester scaffolds, incorporated with three types of explants (native articular cartilage, vital bone, or devitalized bone), and cultured in rotating bioreactors for 4 or 8 weeks (Fig. 13.2) [21]. Engineered cartilage constructs interfaced with the solid matrix of adjacent cartilage without any gaps or intervening capsules. Additionally, focal intermingling between construct collagen fibers and native cartilage collagen fibers provided evidence of structural integration (Figs. 13.2d–f). Interestingly, adhesive strength was higher for constructs cultured adjacent to bone than cartilage and highest for constructs cultured adjacent to devitalized bone (Fig. 13.2g). These findings could be explained by the differences in adjacent tissue architecture (histological features) and transport properties (diffusivity) [21]. Perfused bioreactors consistently yielded significantly higher amounts of glycosaminoglycans (GAG) and total collagen in engineered cartilage co-cultured adjacent to engineered bone than either engineered cartilage, native cartilage, or native bone [22]. These results suggest that the type of native tissue with which the engineered tissue is combined may influence construct integration *in vivo*. Collectively, information can be gleaned from *in vitro* studies with respect to the integration potential of a tissue-engineered construct prior to *in vivo* implantation.

In vitro platforms relevant for high throughput screening of drugs and other agents

In vitro models can also be utilized to generate physiologically responsive tissue for screening pharmaceutical and therapeutic drugs (reviewed in Vandenburgh et al. [23]). This type of model can be designed for high throughput screening, thereby reducing the need for human tissue and organ harvest. Moreover, if based on human rather than animal cells (e.g., Schaaf et al. [24]), *in vitro* models can provide a more relevant system than *in vivo* animal models, which may differ significantly from the human case in their physiologic responses.

For example, a tissue-engineered drug screening platform referred to as 'engineered heart tissue' was developed by combining neonatal rat heart cells with ECM components such as Matrigel™ and fibrin [25]. The cell-ECM-fibrin mixture was cast between two flexible posts such that maturation of the neonatal cells and condensation of the maturing tissue between the flexible posts yielded strips of cardiac-like tissue which contracted regularly. Pharmaceutical agents with known cardiac effects induced the expected changes within the engineered heart tissue in a repeatable fashion, suggesting that engineered tissues of this type could be used for relatively high throughput drug screening. Moreover, human embryonic stem cells (ESCs) were recently cultured to form engineered heart tissue that was successfully used to screen a panel of drugs with known pro-arrhythmic effects [24]. Engineered heart tissue may also be used as a surrogate cardiac tissue to explore *in vitro* the integration into native heart tissue of various cell populations, such as murine cardiomyocytes, cardiac fibroblasts, and murine ESC-derived cardiac cells [26,27]. These studies together suggest that engineered heart tissue can provide a valuable platform for screening cardiac pharmaceutical and cell therapeutics.

In vitro experiments, such as those described above, are designed to elucidate cell- and tissue-level responses to molecular and mechanical stimuli, and thus improve the understanding of complex *in vivo* phenomena and promote clinical translation of tissue-engineering technologies. In this context, the *in vitro* culture environment plays a key role by allowing for controlled and reproducible test conditions that limit the variability associated with the *in vivo* environment.

INFLUENCE OF SELECTED *IN VITRO* CULTURE PARAMETERS ON THE DEVELOPMENT AND PERFORMANCE OF ENGINEERED TISSUES

It is well-understood that traditional two-dimensional (2D) *in vitro* culture techniques cannot sufficiently recapitulate the microenvironment experienced by cells and tissues *in vivo*. Furthermore, physical forces are critical for the development of tissues and organs during embryogenesis and post-natal growth and remodeling. Therefore, functional tissue-engineering strategies aim to recreate a 3D microenvironment *in vitro* that mimics the *in vivo* milieu through the use of scaffold systems, bioreactors, growth factors, and mechanical conditioning [3,8,9,11,14,28].

Culture duration

CARTILAGE TISSUE ENGINEERING

With increasing *in vitro* culture duration, chondrocytes assemble a mechanically functional ECM (e.g., Buschmann et al. [29]), and cardiomyocytes develop contractile responsiveness to electrical impulses (e.g., Radisic et al. [30]). For example, over a 40 day period of *in vitro* culture, constructs based on bovine calf chondrocytes and PGA scaffolds contained increasing amounts of GAG and type II collagen, and decreasing amounts of the PGA scaffold (Fig. 13.3a) [31]. In this system, the relatively high rates of ECM synthesis and deposition by the calf chondrocytes approximately matched the relatively high degradation rate of the PGA scaffold, a finding that did not hold true when the same scaffold was studied with other cell types

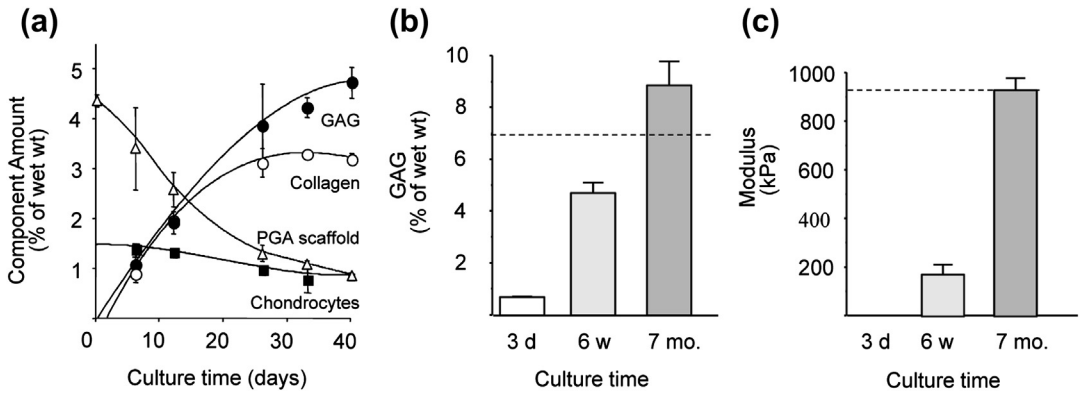

FIGURE 13.3

Effects of culture duration on the composition and function of engineered cartilage. a, b) Bovine calf chondrocytes were cultured on non-woven PGA scaffolds in rotating bioreactors for up to 7 months. a) Short-term changes in the construct amounts of GAG *(closed circles)*, total collagen *(open circles)*, cells *(squares)*, and PGA scaffold *(triangles)*. b, c) Long-term changes in construct amounts of GAG and Aggregate modulus (H_A), measured at 3 days, 6 weeks, and 7 months *(3d, 6w, and 7mo)*. Data are represented as mean ± SD. Dashed lines indicate average values obtained for native bovine calf cartilage. *a): adapted from Freed et al., Experimental Cell Research, 240:58, 1998 [31]; b) and c): adapted from Freed et al., Proc Natl Acad Sci USA, 94:13885, 1997 [32].*

(e.g., bone-marrow-derived mesenchymal stem cells [MSCs]) [11]. The structural and functional properties of engineered cartilage constructs can be improved to some degree by further extending the culture duration. For example, 7 month long cultures carried out in rotating bioreactors operated on Earth yielded engineered cartilage constructs with very high GAG fractions (~8% of wet weight) and compressive moduli (~0.9 MPa) that were comparable to normal articular cartilage (Fig. 13.3b, c), although the collagen fraction and dynamic stiffness of the 7 month constructs remained sub-normal [32].

Optimal maturation of tissue-engineered cartilage *in vitro* may improve cartilage repair with respect to ECM quality and integration after implantation *in vivo* [33]. A recent study correlated engineered cartilage maturity with *in vivo* repair [34]. Engineered cartilage was prepared by culturing chondrocytes on ECM scaffolds for 2 days, 2 weeks, or 4 weeks. Constructs were then implanted into full thickness cartilage defects in rabbit knee joints. In this study, the use of more mature engineered cartilage improved osteochondral defect repair; however, another study showed that prolonged *in vitro* culture of engineered constructs prior to *in vivo* implantation may lead to ECM degradation, resulting in sub-optimal performance *in vivo* [35]. Collectively, these findings suggest that while *in vitro* tissue maturation may enhance the *in vivo* performance of the engineered tissue, the optimal tissue maturation conditions are likely to be cell type-, scaffold-, culture condition-, and/or animal model-dependent.

CARDIAC TISSUE ENGINEERING

Engineered cardiac tissue slowly forms contractile units over time in culture, beginning with spontaneous beating of single cells or groups of cells. As neonatal rat heart cells mature, elongate, and form networks, coordinated, coherent contractions develop throughout the construct [36,37]. For example, engineered cardiac tissue cultured for up to 8 days exhibited a temporal increase in contractile amplitude [30]. In another study, engineered heart tissue exhibited coherent contraction at 7–9 days in culture, increased in force of contraction between 9 and 15 days in culture, and then stabilized [25]. Cells within these constructs showed an elongated phenotype consistent with more mature cardiomyocytes. For cardiac tissue engineering, however, increasing culture duration alone is not sufficient to produce functional tissue for implantation. At present the maximal contractile force generation reported for an engineered cardiac construct (~4 mN/mm^2) remains more than an order of magnitude below that of normal heart muscle [28].

Scaffold systems

CARTILAGE TISSUE-ENGINEERING SCAFFOLDS

Novel 3D scaffold designs have attempted to mimic aspects of native ECM using composite scaffold structures. As biomimetic physical and mechanical properties are difficult to achieve with a single, homogeneous material, several approaches for tissue engineering have employed composite scaffold systems, which are often designed with fiber reinforcement and layered structures [7].

Fiber-reinforced constructs for cartilage repair

This type of design typically utilizes a fibrous phase comprised of a non-woven, knitted, or 3D woven fabric within a cell-supporting phase comprised of a hydrogel or sponge-like material. The cell-supporting phase generally provides a favorable environment for proliferation, differentiation, and ECM synthesis, while the fiber phase functions as a mechanical reinforcement and stabilizer for the construct.

For example, hydrogels known to support chondrogenesis (e.g., agarose, alginate, and fibrin) have been combined with degradable non-woven or woven 3D scaffolds to engineer functional cartilage. To test the influence of a hydrogel cell-carrier system for chondrogenesis, non-woven poly(lactic-co-glycolic acid) (PLGA) meshes were seeded with either dissociated bovine chondrocytes or with chondrocytes suspended in alginate [38] and then implanted subcutaneously in nude mice. The alginate cell-carrier increased seeding efficiency by assisting in the retention and uniform distribution of cells throughout the pores of the non-woven mesh. The fiber-reinforced hydrogel also yielded a physically robust construct that maintained its initial geometry over time, without a negative effect on ECM synthesis. Similar studies showed success when non-woven PGA was either combined with a chondrocyte-laden fibrin gel [39] or with MSCs in a type I collagen and alginate gel [40].

Woven 3D fabrics embedded with hydrogels have also been utilized and replicate the complex biomechanical behavior of native articular cartilage [41]. A microscale 3D weaving technique was employed to fabricate multiple layers of continuous fibers in three orthogonal directions (Fig. 13.4a–d). Composite scaffolds comprised of 3D woven fiber bundles of PGA [41] or poly(ϵ-caprolactone) (PCL) [42] were used in combination with fibrin gel to mimic the physical properties of native articular cartilage, specifically, its inhomogeneous, anisotropic, non-linear, and viscoelastic mechanical properties. Construct compressive mechanical properties and equilibrium coefficient of friction were found to be similar to those of native articular cartilage throughout the defined culture period (Fig. 13.4e–g). Further, constructs seeded with human adipose-derived stem cells (ASCs) supported the elaboration of ECM which stained positive for the presence of chondroitin 4-sulfate and type II collagen (Fig. 13.4h, i). In other studies, the infiltration of this 3D woven PCL scaffold with a slurry of cartilage-derived ECM enhanced the chondrogenesis of ASCs, while providing a mechanically functional construct that resisted cell-mediated contraction [43]. A unique advantage of this composite structure is that scaffolds can be designed and fabricated with predetermined control of site-dependent variations in mechanical properties and porosity within a biocompatible matrix.

Fiber reinforcement can also improve the mechanical properties of sponge-like scaffolds that otherwise have insufficient mechanical properties to support mechanical loading. For example, a scaffold comprising a web-like collagen micro-sponge and knitted PLGA fabric was fabricated for engineering cartilage tissue [44]. The knitted fabric provided the mechanical integrity lacking in the collagen micro-sponge, and the collagen micro-sponge filled in the large pores of the fabric to facilitate uniform cell distribution and cartilage-like tissue formation. Similarly, reinforced PLGA foam scaffolds were produced by embedding short PGA fibers into the bulk polymer prior to foaming [45]. The mechanical properties of these scaffolds

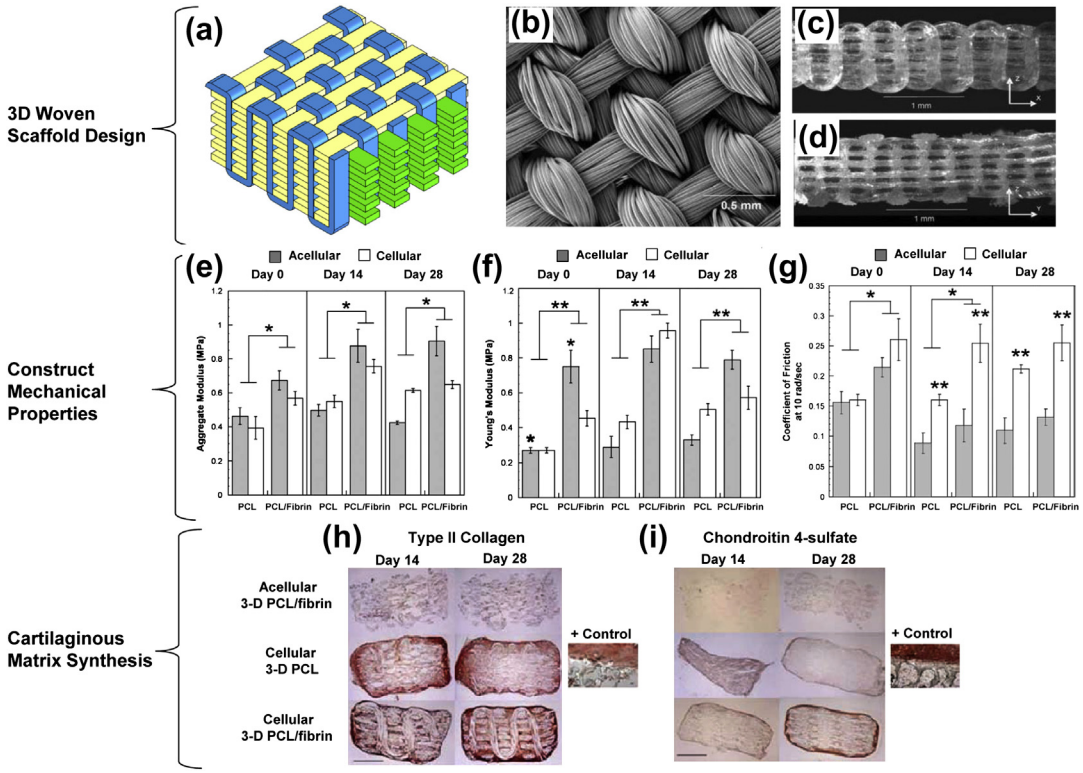

FIGURE 13.4

Structural and mechanical properties of a representative cartilage tissue-engineering scaffold. Top Panel: Fiber architecture of 3D orthogonally woven PCL scaffold. a) Schematic and b, c, d) scaffold weave and fiber morphology as shown with scanning electron microscopy (a — surface view, scale bar: 0.5 mm; b, c — Cross-sectional view through the X-Z plane b), and Y-Z plane c), scale bar: 1 mm). Middle Panel: Compressive biomechanical properties of scaffolds without and with cultured cells at days 0, 14, and 28. e) Aggregate modulus (H_A) and f) Young's modulus as determined by confined and unconfined compression, respectively. The addition of fibrin to 3D PCL scaffolds significantly increased H_A and E for both acellular and cellular groups (ANOVA, *$p<0.05$, **$p<0.0001$). g) Equilibrium coefficient of friction measured under steady frictional shear. Cellular constructs displayed significantly higher coefficients of friction against a rotating stainless steel platen than did acellular scaffolds (ANOVA, *$p<0.05$, **$p<0.001$). Data represented as mean ± SEM. Human adipose-derived stem cells used as cell source. Bottom Panel: Immunohistochemistry of acellular 3D PCL—fibrin composite scaffolds, cellular 3D PCL constructs, and cellular 3D PCL-fibrin composite constructs at days 14 and 28. h) Type II collagen and h) Chondroitin 4-sulfate (scale bar: 1 mm). Human adipose-derived stem cells used as cell source. *Adapted from Moutos and Guilak, Tissue Eng Part A 16: 1291, 2010 [42].*

could be tailored for potential use in articular cartilage repair by adjusting the material composition [46]. Collectively, this work demonstrates that fiber reinforcement is a controllable design variable that can be manipulated in order to engineer scaffolds to suit the load-bearing requirements of an engineered tissue.

Stratified constructs for cartilage repair

In an attempt to recapitulate the structural, compositional and mechanical inhomogeneity of articular cartilage, which varies with depth from the tissue surface, several groups have designed stratified constructs (reviewed in Klein et al. [47]). In one study, a two-layered stratified structure based on agarose was developed in an effort to mimic the depth-dependent mechanical properties of articular cartilage [48]. Constructs with a top layer composed of 2% agarose and a bottom layer composed of 3% agarose exhibited zonal differences in the compressive elastic modulus immediately after fabrication. Another study reported on the use of a photopolymerizing poly(ethylene glycol) diacrylate (PEGDA) hydrogel system that supported chondrocyte-mediated matrix synthesis which histologically resembled the depth-dependent composition of native articular cartilage [49]. Furthermore, in a recent study the layered organization of PEG-based hydrogels comprised of chondroitin sulfate (CS), matrix

metalloproteinase-sensitive peptides (MMP-pep), and/or hyaluronic acid (HA), promoted the differentiation of bone-marrow derived MSCs into chondrogenic phenotypes that resembled those found in the zones of articular cartilage [50,51]. The three-layered, PEG-based construct incorporated CS and MMP-pep into the top layer (superficial zone), CS into the middle layer (transitional zone) and HA into the bottom layer (deep zone), thus resulting in a construct with spatially-varying mechanical and biochemical properties. Overall, these stratified structures resulted in depth-dependent variations in construct properties, thereby mimicking some of the inhomogeneous aspects of native articular cartilage.

Osteochondral constructs

Since bone-to-bone interfaces are known to integrate more effectively than cartilage-to-bone interfaces, osteochondral constructs are considered a promising technique for repairing full thickness articular cartilage defects. Progress in the development of osteochondral constructs using multi-material strategies has been reviewed by several groups [52–54]. In one study, an anatomically-shaped osteochondral construct was developed by casting a layer of chondrocyte-seeded agarose gel on top of devitalized trabecular bone [55]. Other approaches have included: engineered cartilage grown on non-woven PGA sutured to a subchondral support made of Collagraft™ [19], a hyaluronan benzyl ester sponge for cartilage regeneration attached with fibrin glue to a calcium phosphate ceramic sponge for bone regeneration [56], differentially applied chondrogenic and osteogenic growth factors on MSCs seeded in stacked silk scaffolds [57], a 3D printed scaffold of mixed PLGA/PLA for the upper cartilage region with a PLGA/tricalcium phosphate mix for the lower bone region [58], a collagen gel for chondrogenesis cultured upon a bone-inducing hydroxyapatite base [59], a cartilaginous tissue layer overlying a porous biodegradable calcium polyphosphate substrate [60] and chondrocyte-laden agarose hydrogels layered on constructs composed of mineral-containing microspheres [61] or directly containing a mineral phase [62].

CARDIAC TISSUE-ENGINEERING SCAFFOLDS

Scaffolds designed for cardiac tissue engineering must meet very different requirements to those developed for cartilage tissue engineering. Cardiac tissue-engineering scaffolds should provide the necessary microvascular and mechanical properties to meet the demands of a continuously contracting tissue, considering the cellular, geometric, mass transport, and oxygen supply concerns of native cardiac tissue. Scaffold approaches for cardiac tissue engineering have been reviewed by several groups [7,17,28] and are discussed briefly here.

Many natural and synthetic materials have been examined for use in cardiac tissue engineering. As described previously, Matrigel™, combined with fibrin and/or thrombin [25,63,64] or with collagen sponges [30,65] has been used to generate contractile engineered cardiac tissue *in vitro*. However, when considering clinical translation of these scaffolds, material limitations become apparent. Specifically considering the mechanical requirements of cardiac tissue, collagen gels and sponges may lack the mechanical strength required to withstand suturing and/or repeated cycles of stretch and relaxation. On the other hand, synthetic polymers popular in other tissue-engineering applications (e.g., PCL, PLGA) may be too stiff to form engineered cardiac tissue that possesses appropriate contractile properties. Therefore, development of other scaffolds that more closely mimic the native structural, mechanical, and transport properties of cardiac tissue is an area of active research.

In pioneering work, Ott et al. developed a biomimetic scaffold for cardiac tissue engineering through de-cellularization of adult rat hearts [66]. These de-cellularized hearts were repopulated by intramural injection and coronary artery perfusion with neonatal heart cells and aortic endothelial cells and cultured with pulsatile flow and electrical stimulation to regenerate the nascent pump function of the heart. Although this system for cardiac tissue engineering has proven successful in many respects, the efficiency of re-cellularization remains

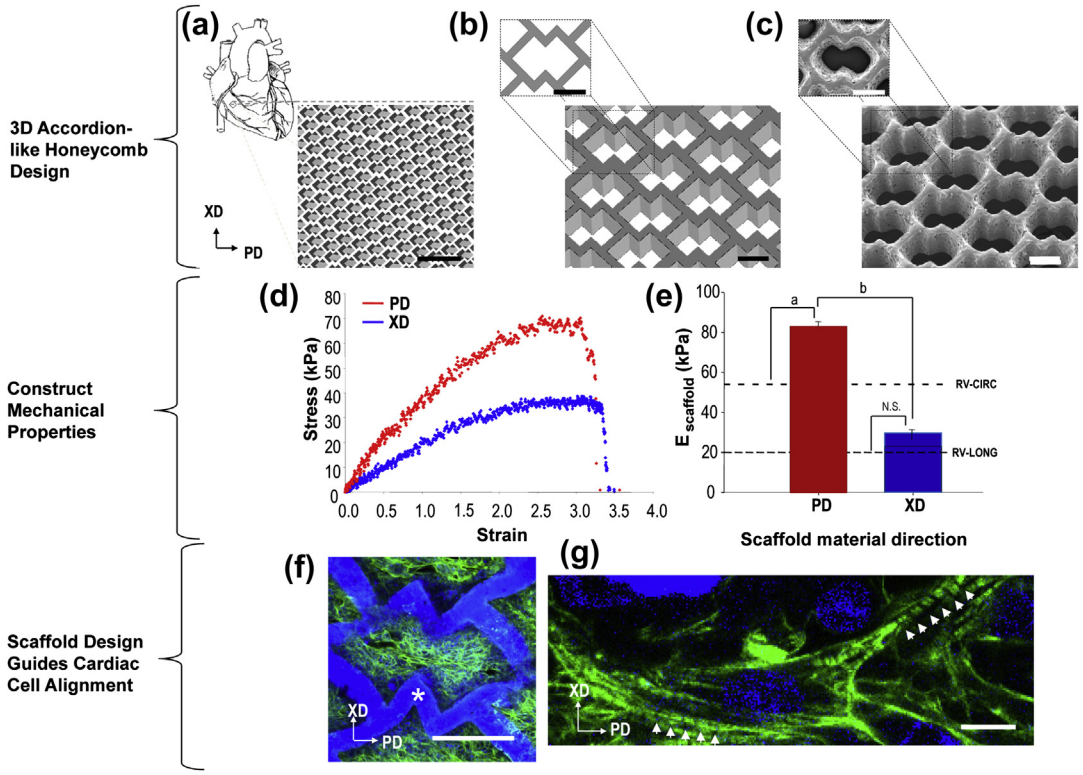

FIGURE 13.5

Scaffold design for engineering cardiac tissue. Top Panel: a, b) Schematics of accordion-like honeycomb scaffold design (a — scale bar: 1 mm, b — scale bar: 200 μm). c) Scanning electron micrograph of the scaffold architecture fabricated by laser microablation of a biodegradable elastomer, poly(glycerol sebacate) (PGS) (scale bar: 200 μm). Preferred direction (PD) and orthogonal cross-preferred direction (XD) material directions are indicated. Middle Panel: d) Representative uniaxial stress strain plot of PGS scaffold with cultured neonatal rat heart cells (scaffolds were fabricated from PGS membranes cured for 7.5h at 160°C; cells were cultured for 1 week). e) Scaffold anisotropic effective stiffnesses (E_{PD} and E_{XD}) are compared to specimens of native adult rat right ventricular (RV) myocardium harvested in two orthogonal directions (RV_{CIRC} and RV_{LONG}, respectively). Data are represented as mean ± SD. [a]Significant difference between scaffold and RV; [b]Significant difference due to scaffold test direction; N.S. indicates not significant. Bottom Panel: f, g) Confocal micrographs of neonatal rat heart cells cultured on accordion-like honeycomb scaffolds for 1 week. (Stain: filamentous F-actin (green), counterstain: DAPI (blue), scale bars: 200 μm and 10 μm, for f and g, respectively). Scaffold indicated by white asterisks; cross-striations indicated by white arrows. *Adapted from Engelmayr, Jr., G. C. et al., Nature Materials 7: 1003, 2008 [67].*

low and the mechanical stiffness of the de-cellularized and re-cellularized tissues remains considerably higher than that of native myocardium.

In order to design a scaffold to address some of the specific structural and mechanical requirements of cardiac tissue, an elastomeric scaffold with accordion-like honeycomb pores was microfabricated from poly(glycerol sebacate) (PGS) (Fig. 13.5) [67]. The accordion-like honeycomb pores were chosen as a first step toward mimicking the native structure of collagen within the myocardial ECM (Fig. 13.5a–c). These scaffolds, when combined with neonatal rat heart cells, yielded constructs with anisotropic mechanical properties that closely matched to the right ventricular myocardium, coordinated contractions in response to electrical stimulation, and allowed for some degree of elongation and alignment of the neonatal heart cells (Fig. 13.5d–g). In addition, PGS scaffolds designed based on finite element simulations provided a platform for cell delivery while simultaneously recapitulating the mechanical properties of the left ventricular myocardium [68].

Many other scaffold designs have also utilized PGS as an elastomer for cardiac tissue engineering. One study used salt leaching to produce a porous scaffold, combined with laser cutting to produce microvascular channels for transport and oxygen diffusion [69], and

another utilized semi-automated layer-by-layer assembly of planar polymer sheets with through-pores to create 3D structural patterns that directed the orientation of meso-scale cardiac muscle-like fibers [70]. Scaffold modifications that sought to provide greater oxygen, nutrient, and waste transport (i.e., microvascular channels), or to condition the construct with mechanical stresses (i.e., cyclic stretch), can potentially increase the viability of the cells within the scaffold over time.

Bioreactors and growth factors

Bioreactors, which are capable of initiating, maintaining, and directing cell growth and tissue development in a well-defined and tightly controlled culture environment, have proven to be crucial tools for 3D tissue culture. Tissue culture bioreactors represent a controllable model system for:

1) Studying the effects of biophysical stimuli and therapeutic agents on cells and developing tissues;
2) Simulating responses of an *in vitro*-grown construct to *in vivo* implantation and thereby helping to define its potential for survival and functional integration; and
3) Developing and testing physical therapy regimens for patients who have received engineered tissue implants.

As described in the studies highlighted below, bioreactors have been utilized to:

1) Improve cell infiltration and distribution by dynamically seed cells within 3D scaffolds;
2) Overcome the limitations associated with oxygen and nutrient transport that are often observed in tissues cultured in static environments; and
3) Enhance matrix synthesis and mechanical properties by biophysical stimulation of the developing constructs [13–15,17,71,72].

CELL SEEDING

Applying an appropriate cell type to a biomaterial scaffold is the first step in the tissue-engineering process, and the seeding technique may have a critical role in directing subsequent tissue formation [73,74]. Scaffold cell seeding has traditionally been done statically and performed manually using pipettes, but these static seeding techniques often lead to inefficient and spatially non-uniform cell distribution within the scaffold [75], which may result in disparate ECM deposition throughout the construct [73,76]. In order to overcome the challenges associated with static seeding, bioreactor-based dynamic cell seeding techniques have been developed. Numerous types of bioreactors have been explored, including spinner flasks [77], wavy-walled reactors [78], and perfused vessels [79].

For some combinations of cells and scaffolds, dynamic cell seeding resulted in scaffolds with high and spatially uniform initial cell densities, which increased ECM deposition and compressive modulus in the resulting engineered cartilage [31,77,80]. For other combinations, perfusion of a cell suspension directly through a scaffold enabled spatially uniform seeding and enhanced tissue regeneration [75,79,81]. Engineered cartilage seeded in perfused bioreactors with alternating medium flow reportedly exhibited higher cell viability and uniformity than controls seeded statically and in spinner flasks [75]. Likewise, engineered cardiac tissue seeded in perfused bioreactors with alternating medium flow exhibited higher cell viability and spatial uniformity than controls seeded in mixed petri dishes [79].

CONSTRUCT CULTIVATION

Cellular apoptosis and the formation of necrotic regions within 3D engineered constructs cultured under static conditions suggest that diffusion alone does not provide sufficient mass transport of oxygen, nutrients, and wastes for cell survival within a construct. Bioreactors help

to mitigate these mass transfer limitations and provide a controlled microenvironment for 3D construct development. Several groups have demonstrated that mass transport limitations could be minimized, cell viability, differentiation, and function enhanced, and matrix synthesis improved within engineered constructs with the use of bioreactors that induce convective mixing (spinner flasks [77,80] and rotating bioreactors [31,77,82]), and perfusion [14,15,65,79,83–86].

Convective mixing, flow, and mass transport are required to supply the oxygen, nutrients, and regulatory factors that are in turn required for the *in vitro* cultivation of large tissue constructs [5,6,87]. Oxygen is the factor that generally limits cell survival and tissue growth, due to its relatively low solubility, slow diffusion rate and high consumption rate [6,88]. Different tissue types have different oxygen requirements, depending on cell type(s), concentrations and metabolic activities. For example, articular cartilage, an avascular tissue, has a lower requirement for oxygen than myocardium, a highly vascularized tissue. Experimental and modeling studies have correlated oxygen gradients within engineered tissues with morphology and composition [6,89,90].

CARTILAGE TISSUE-ENGINEERING BIOREACTORS

A variety of bioreactors have been used to engineer cartilage constructs. Rotating bioreactors supported the growth of engineered cartilage constructs 5 to 8 mm thick based on bovine calf chondrocytes and PGS scaffolds [31,32,76,77]. More recently, human chondrocytes were expanded in 2D and then cultured on hyaluronan benzyl ester scaffolds in rotating bioreactors for up to 4 weeks [91]. While constructs cultured statically and in bioreactors contained similar amounts of GAG and collagen, the bioreactor-grown constructs exhibited a bi-zonal structure, consisting of a collagenous surface capsule deficient in GAG and an inner region that stained more positively for GAG. As compared to bovine calf chondrocytes, expanded human chondrocytes deposited relatively lower amounts of matrix. In another study, a wavy-walled bioreactor was used to culture bovine calf chondrocytes on PGA scaffolds, and increased construct growth, defined by weight, cell proliferation, and ECM deposition, was observed in bioreactors as compared to spinner flasks [92].

In an effort to explore the influences of bioreactors and exogenous growth factors, an oscillatory perfused bioreactor providing slow, bi-directional perfusion was used to study cartilage constructs made by culturing adult human MSCs on 3D woven PCL scaffolds for 3 weeks (Fig. 13.6) [93]. Constructs cultured in bioreactors had higher aggregate moduli, higher total collagen contents, and similar GAG contents compared to constructs cultured statically (Fig. 13.6a, b). Constructs cultured statically in medium containing chondrogenic growth factors but not serum exhibited better chondrogenesis and more homogeneously positive matrix staining for GAG and collagen type II than otherwise identical medium containing serum (Fig. 13.6c–e). Constructs cultured in medium without chondrogenic growth factors and with serum did not exhibit chondrogenic differentiation (Fig. 13.6f). Together, these studies show that bioreactors and growth factors can influence cell morphology, proliferation, and ECM deposition in engineered cartilage.

CARDIAC TISSUE-ENGINEERING BIOREACTORS

In the case of engineered cardiac tissue, convective mixing in rotating bioreactors and spinner flasks supported the growth of a tissue-like surface layer ~100 to 200 μm thick [36,37,88]. Moreover, perfusion of culture medium directly through an engineered cardiac construct can significantly improve construct thickness and spatial homogeneity [65,79,83,88]. Specific design of perfusion bioreactors for cardiac tissue engineering is described in [14], and bioreactors designed specifically for electrical stimulation of cardiac constructs are described in [16]. In one example, perfused bioreactors enhanced the survival of heart cells cultured on porous collagen sponges by increasing the transport of oxygen and insulin-like growth factor-I

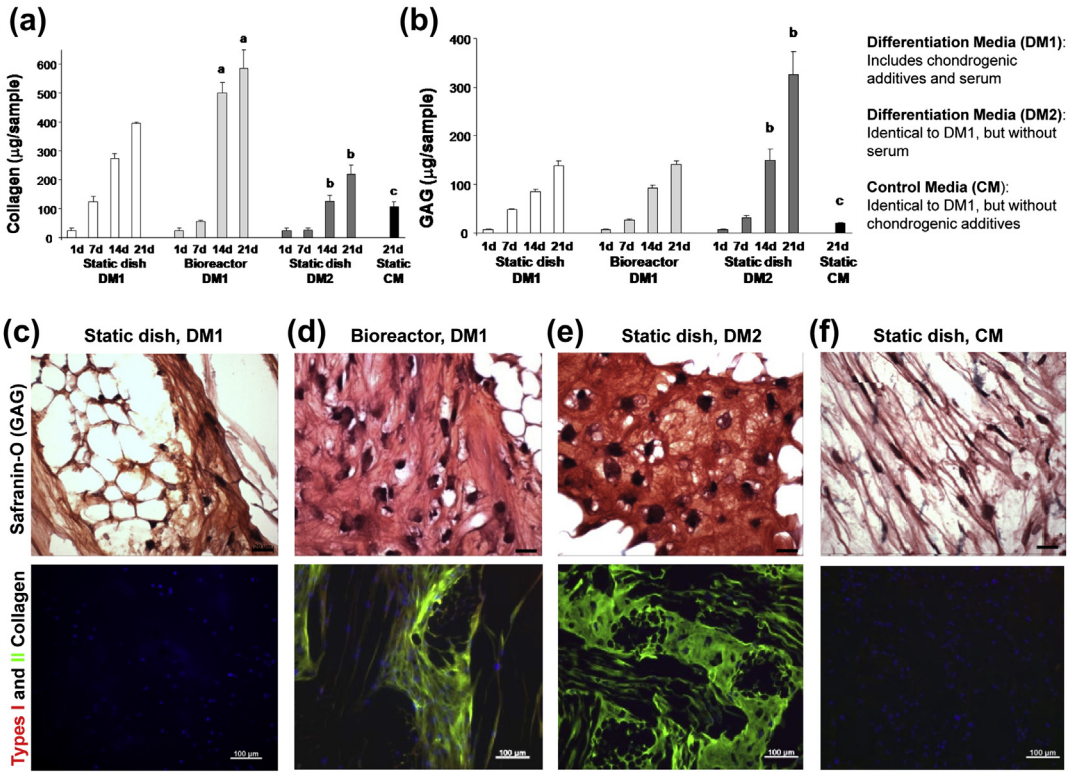

FIGURE 13.6

Bioreactors and growth factors influence the structure and composition of engineered cartilage. Human MSCs were cultured on 3D woven PCL scaffolds in static dishes or bioreactors and in three different culture media (differentiation medium 1 (DM1), differentiation medium 2 (DM2), and control medium (CM)) for up to 21 days. Top Panel: Time evolutions of construct amounts of a) total collagen and b) GAG. Data are represented as mean ± SEM. [a]Significant difference due to type of culture vessel, [b]Significant difference due to presence of serum, [c]Significant difference due to chondrogenic additives, which included TGF-β-3, insulin-transferrin-selenium (ITS), dexamethasone, and ascorbic acid. Bottom Panel: Histological sections of 21-day constructs cultured c) statically in DM1, d) in bioreactors in DM1, e) statically in DM2, and f) statically in CM and stained with safranin-O for GAG (top, scale bars: 20 μm) or immunostained for collagen type II (green) and type I (red, not seen) with DAPI (blue) counterstain (bottom, scale bars:100 μm). *Adapted from Valonen, P.K., et al., Biomaterials, 31(8): p. 2193, 2010 [93].*

(IGF-I) (Fig. 13.7) [86]. Neonatal rat heart cells were seeded on scaffolds at high density by hydrogel-entrapment, and then slow, bi-directional perfusion culture was carried out in an oscillatory perfused bioreactor for 8 days. Bioreactor-grown constructs grown exhibited improvements over static controls with respect to several benchmarks, including reduced apoptosis, increased contractile amplitude, and increased expression of the contractile protein cardiac troponin-I (Fig. 13.7a–c). In the static control group (Fig. 13.7d), heart cells remained rounded and did not exhibit cross-striations, whereas in the bioreactor group (Fig. 13.7e), some cells were elongated and striated, albeit to a lesser degree than native adult rat ventricular myocardium (Fig. 13.7f). Moreover, perfusion of medium supplemented with IGF-I yielded further improvements in construct properties, presumably due to enhanced transport of growth factor to the heart cells within the 3D construct.

In another study done in the oscillating perfused bioreactor, heart cells cultured on two-layered, 500 μm thick PGS scaffolds with fully interconnected accordion-like honeycomb pores exhibited increases in the gap junctional protein connexin-43 and MMP-2, an enzyme associated with tissue remodeling [94]. In other studies, sustained release and delivery of IGF [95] or sequential delivery of IGF-I and hepatocyte growth factor (HGF) [96] enhanced survival and maturation of heart cells in a 3D model and protected against oxidative stress. Together, these studies suggest that bioreactors and growth factors may work in tandem to enhance heart cell viability, contractility and differentiation in 3D myocardial grafts.

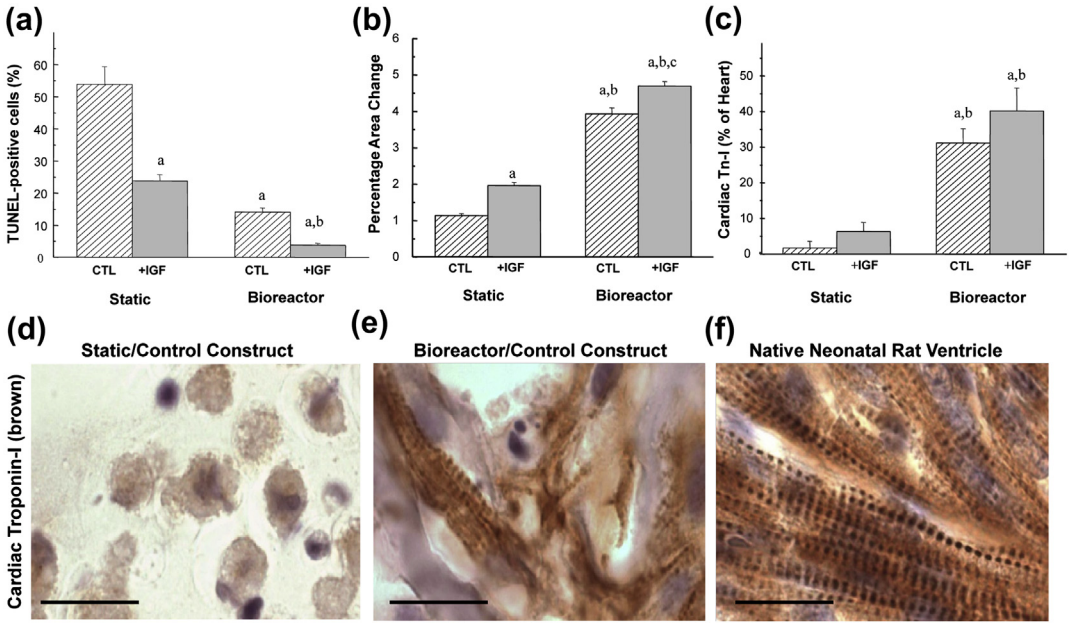

FIGURE 13.7

Bioreactors and growth factors influence the structure and composition of engineered cardiac tissue. Neonatal rat heart cells were seeded in hydrogel and cultured on collagen sponge scaffolds either statically or in bioreactors in two different media (control, CTL, or with supplemental growth factor, IGF-I). Top Panel: a) Apoptosis (TUNEL-positive cells, % of total cells), b) Contractile amplitude (% area change), c) Cardiac troponin-I (Western blot, % of native neonatal rat heart). Data are represented as mean ± SEM. [a]Significantly different from Static/CTL; [b]Significantly different from Static/IGF; [c]Significantly different from Bioreactor/CTL. Bottom Panel: Histological appearances of d) static control construct, e) bioreactor control construct, and f) native neonatal rat ventricular myocardium immunostained for cardiac troponin-I (brown). Scale bars: 20 μm. *Adapted from Cheng, M., et al., Tissue Eng Part A, 15(3): 645, 2009 [86].*

Bioreactors and mechanical forces

Bioreactors can be utilized to apply biomechanical signals (shear, compression, tension, pressure, or a combination thereof) to growing tissues [97,98]. Chondrocytes are quite responsive to mechanical signals and remodel the matrix according to the loads applied; thus the choice of loading regime can directly influence the development of the structure, composition, and mechanical properties of cartilaginous constructs (reviewed in Grad et al. [99]). Likewise, skeletal [100], smooth [101], and cardiac [28,63,64] muscle cells are quite responsive to mechanical signals.

EFFECTS OF HYDRODYNAMIC FORCES

Hydrodynamic forces associated with convective mixing can have a significant effect on the composition, structure, and properties of engineered tissues. For example, engineered cartilage cultured in rotating bioreactors had thinner surface capsules and higher fractional amounts of GAG than constructs grown in spinner flasks [77,82,102,103]. The flow field in the spinner flask was unsteady, turbulent (Reynolds number of 1758), and characterized by large spatial variations in the velocity field and maximum shear stresses [104]. In contrast, the flow field in the rotating bioreactor (slow turning lateral vessel, STLV) was predominately laminar with shear stresses of ~1 dyn/cm^{2}and a well-mixed interior due to secondary flow patterns induced by the freely-settling constructs [102]. A model of tissue growth in the rotating bioreactor that accounted for the intensity of convection over 6 weeks of *in vitro* culture was used to predict the morphological evolution of an engineered cartilage construct [105]. In particular, the model predicted that high shear and mass transfer at the lower corners of a settling, discoid construct would preferentially induce tissue growth in these regions, and that temporal changes in construct size and shape would further enhance local variations in the flow field in a manner that accentuated localized tissue growth. The computed velocity fields and shear

stress data corresponded well with the morphological evolution of engineered cartilage, as shown by superimposing a calculated flow field on a histological cross-section of an actual construct. The above examples suggest that combining experimental studies and computational modeling of hydrodynamic shear stresses and concentration gradients in bioreactors may help to explain underlying mechanisms that control the growth of engineered tissue constructs.

EFFECTS OF MECHANICAL TENSION, COMPRESSION, AND SHEAR LOADING

It is well known that mechanical forces are critical for determining the architecture of native tissues such as bone [106], and there is growing evidence that mechanical factors are important factors in determining stem cell fate [107]. In particular, the role of *in vitro* mechanical stress in maintaining and promoting the chondrogenic phenotype has been the topic of several investigations, but the specific influences of different physical stimuli and their interactions with the biochemical environment are not fully understood. For example, dynamic compression caused a ~2-fold increase in cartilage nodule density and a 2.5-fold increase in GAG synthesis in stage 23/24 chick limb-bud cells cultured in agarose gel [108]. Likewise, cyclic hydrostatic pressure significantly increased the amounts of proteoglycan and collagen in aggregates of human bone-marrow-derived MSCs [109]. In another study, compression enhanced chondrogenic differentiation in mouse embryonic E10 stage cells embedded in collagen type I compared to unloaded controls, as shown by upregulation of SOX-9 and downregulation of IL-1β expression [110].

A variety of devices have been custom designed and built to study the effects of mechanical conditioning (i.e., compression, tension, pressure or shear) on cells and tissues *in vitro* (reviewed in Brown et al. [111], Darling et al. [112], and Waldman et al. [113]). For engineering cartilage, devices typically apply dynamic compression (e.g., Buschman et al. [29], Mauck et al. [114]), hydrostatic pressure (e.g., Mizuno et al. [115], Toyoda et al. [116]) or mechanical shear (e.g., Waldman et al. [117]). For engineering skeletal, smooth, and cardiac muscle tissues, devices typically apply dynamic tensile strain [63,64,97,100,101,118–121] or pulsatile hydrostatic pressure [122–125].

MECHANICAL EFFECTS ON ENGINEERED CARTILAGE TISSUE

In the case of engineered cartilage, a number of studies have shown that mechanical conditioning can enhance chondrogenesis. Dynamic loading has been shown to increase GAG accumulation and ECM assembly, and therefore, the mechanical properties of constructs based on bovine calf articular chondrocytes and a variety of 3D scaffolds including agarose gel [29,114], PGA non-woven mesh [126], and self-assembling peptide gel [127]. Similar results were also observed for adult canine chondrocytes in an agrose gel under dynamic loading conditions [128]. Application of dynamic loading was also investigated in a layered agarose construct, with encapsulated bovine articular chondrocytes, with varying mechanical properties (2% agarose vs. 3% agarose) [129]. These results indicated preferential matrix formation in the 2% agarose layer and an increased elastic modulus in only the initially softer, more permeable layer (2% agarose) [129]. Although the aforementioned studies focused on cells encapsulated in hydrogels, similar results (i.e., increased ECM production and compressive modulus of construct) were found when dynamic loading was applied to calf chondrocytes cultured within a porous calcium polyphosphate scaffold. Overall, the response of chondrocytes to dynamic loading depended on the amount and composition of ECM in the developing construct [130], and in some studies loading increased both synthesis of new GAG and its loss into the culture media [127,131].

The influence of dynamic loading on engineering cartilage has also been investigated in MSC-laden hydrogel systems. In a hyaluronic acid-based hydrogel seeded with human MSCs, dynamic compressive loading enhanced cartilage-specific matrix synthesis and more uniform distribution, increased construct mechanical properties, and suppressed the expression of

hypertrophic markers [132]. In another study [133], mechanical loading of MSC-laden agarose constructs prior to chondrogenesis decreased functional maturation and increased chondrogenic gene expression. In contrast, loading initiated after chondrogenesis and matrix elaboration further improved the mechanical properties of engineered constructs, but only when TGF-β3 levels were maintained and under specific loading parameters. Overall, these results demonstrated that the combination of dynamic compressive loading initiated after chondrogenesis and sustained TGF-β exposure may enhance the mechanical properties and matrix distribution of engineered cartilage constructs.

The effects of mechanical stimuli on engineered cartilage may vary among different scaffold systems. In one study, fibrin hydrogels seeded with chondrocytes were cultured under unconfined compression (static and oscillatory) [134]. Compared to the free-swelling control condition, static loading had minimal influence on matrix synthesis or construct stiffness. When comparing the constructs exposed to static versus oscillatory loading, the constructs cultured under dynamic conditions were found to be softer with less matrix accumulation [134]. Although dynamic compressive loading often results in favorable outcomes in terms of engineering functional cartilage tissue, the scaffold material in which the cells are cultured may influence the effects of mechanical conditioning.

In the aforementioned studies, the cell-laden constructs were loaded in dynamic compression; however, the influence of shear, hydrostatic pressure, and tensile forces has also been explored for cartilage regeneration. For example, the application of shear stress yielded constructs with higher amounts of ECM and higher compressive moduli than those exposed to compressive stress [117]. Likewise, application of dynamic hydrostatic pressure affected chondrogenesis in 3D cultures of bovine [115] and human [116] chondrocytes. In another study, the influence of intermittent loading induced with hydrostatic pressure was investigated during cartilage formation [135]. The application of hydrostatic pressure increased the GAG content of the engineered cartilage construct. Finally, the influence of oscillatory tensile loading on chondrocytes derived from distinct tissue zones of articular cartilage (superficial, middle, and deep) was investigated [136]. Tensile loading stimulated proteoglycan synthesis only in superficial zone chondrocyte populations. The results of these studies collectively suggest that loading conditions other than compression may enhance the properties of engineered cartilage constructs, and a combination of loading regimens may be necessary to engineer the different zones of articular cartilage (reviewed in Klein et al. [47]).

MECHANICAL EFFECTS ON ENGINEERED MUSCLE TISSUE

In the case of engineered muscle, dynamic tensile and pulsatile loading can affect construct composition, contractility, and pharmacological responsiveness [23,63,64,97,100,101,118–120,122–124]. Cyclic stretch affects not only the structure (e.g., orientation of cells and collagen) [119] but also the mechanical function (i.e., contractility) [28,63,64,118] of engineered cardiac tissue. Recently, scaffolds have been specifically designed for mechanical stimulation in cardiac tissue engineering [121]. In this study, chitosan-collagen scaffolds with an array of parallel channels were seeded with rat neonatal heart cells and subjected to dynamic tensile stretch for 6 days using a custom designed bioreactor. Mechanical conditioning promoted cardiomyocyte alignment and elongation, and increased cell-to-cell connections as evidenced by increased connexin-43 expression, although these results were dependent on high local stress conditions and were not achieved in areas of the scaffold with lower stress.

Instead of utilizing cyclic stretch to provide mechanical stimulation, some studies utilize pulsatile hydrostatic pressure. One such example used a bioreactor designed to provide physiologically relevant shear stresses and flow rates via pulsatile perfusion [125]. Culture under these pulsatile perfusion conditions enhanced the contractility of the constructs by increasing the contractile amplitude and lowering the excitation threshold.

Together, these mechanical conditioning studies demonstrate that specific loading protocols may be exploited to stimulate specific responses in engineered tissue constructs, and emphasize the potential utility of bioreactors which provide mechanical conditioning in the form of stretch or pulsatile flow in studying and promoting *in vitro* construct formation.

CONCLUSIONS

As the field of functional tissue engineering progresses, the development of engineered tissue replacements may require additional exogenous influences to achieve many of the important requirements for long-term success. Scaffolds can provide structural, biochemical and mechanical cues, and in combination with bioreactors, growth factors, and mechanical conditioning, may enhance *in vitro* generation of functional tissue-engineered constructs. *In vitro* models can be used to assess construct physiologic and pharmacologic responses, including responses to environments mimicking those into which the constructs will eventually be implanted *in vivo*. Nonetheless, it is important to note that other rapidly evolving technologies also may have a significant impact on tissue engineering, and it is important to consider *in vitro* culture parameters in light of the role of novel molecular and gene therapies, and other changing technologies.

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CHAPTER 14

Principles of Bioreactor Design for Tissue Engineering

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INTRODUCTION

The fascinating development of human tissues and organs entails tightly regulated mechanisms, which synchronize biological cues such as cell-secreted cytokines with external stimuli such as physical signals and structural framework. By mimicking the native milieu of tissue development and regeneration, bioreactors can coax the cells to form viable and functional tissues *in vitro*.

In this chapter, we discuss the design of tissue engineering bioreactors with physiologic-like environments and their application in regenerative medicine as well as in the studies of development and disease. We first review representative bioreactor designs, then focus on three different areas of application for advanced bioreactors:

1) Regenerative medicine,
2) Biological research,
3) High-throughput drug screening.

PRINCIPLES OF BIOREACTOR DESIGN

Cell/tissue culture techniques arose from the historical concept that, in an appropriate environment, cells and tissues can survive and remain viable outside the body. To date, traditional cell culture provides a tool for controllable proliferation of the cells and induction of specific differentiated phenotypes. However, the conventional cell culture materials — well plates and culture flasks — lack the ability to generate native-like functional tissues.

In nature, cells are the key architects of the tissues and organs. Under suitable conditions, they can remodel their immediate microenvironment and form functional tissue units. The 'biomimetic' approach to tissue engineering combines these biological principles with engineering design, directing metabolically active cells into three-dimensional (3D) spatial arrangements that, in response to genetic and environmental signals, gradually form tissue structures. In both *in vivo* (development/regeneration) and *in vitro* (tissue engineering) settings, the environmental cues are the principal determinants of the cell phenotype. Therefore, advances in bioreactor design have focused on mimicking the native environments

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in vitro. In general, a bioreactor must support 3D tissue development, maintain cell viability and function within the tissue, and provide appropriate molecular and physical cues. This section describes how tissue engineers perceive the physiology of the human body, and also reviews the key engineering concepts and approaches to mimic the native environment.

An engineering perspective of physiological systems

The approaches to mimicking the native environment are based on the knowledge gleaned from developmental biology, cell biology and normal and pathological tissue function. Progress in cell and developmental biology has advanced our understanding of genetic expression and regulatory pathways that affect cellular function and tissue development. For example, a blastocyst develops into three distinguished germ layers via spatial and temporal regulation of specific signaling pathways: SMAD (portmanteau between small body size and mothers against decapentaplegic) and Mix paired-like homeobox (Mixl1) for endoderm; Fibroblast growth factor (FGF), Wingless-related integration site (WNT) and Bone morphogenetic protein (BMP) for mesoderm; and BMP and downregulation of WNT for ectoderm [1]. Tissue engineers can apply such knowledge to study biological systems, optimize biological responses and generate functional tissues. In order to mimic the native tissue environment, the engineering perspective is implemented both microscopically (at the molecular and cellular levels) and macroscopically (at the tissue and organ levels) (Fig. 14.1).

At the microscopic level, cellular mechanisms are regulated by spatial and temporal presentation of molecular factors. Molecular transport is dominated by diffusion (an exception being transport through motor protein such as kinesin and dynein), while cell behavior is influenced by cell-cell and cell-matrix interactions. Fundamental biochemistry has demonstrated that the activation and deactivation of cell signaling pathways relies on the presence of promoters and inhibitors and their affinities for the receptors.

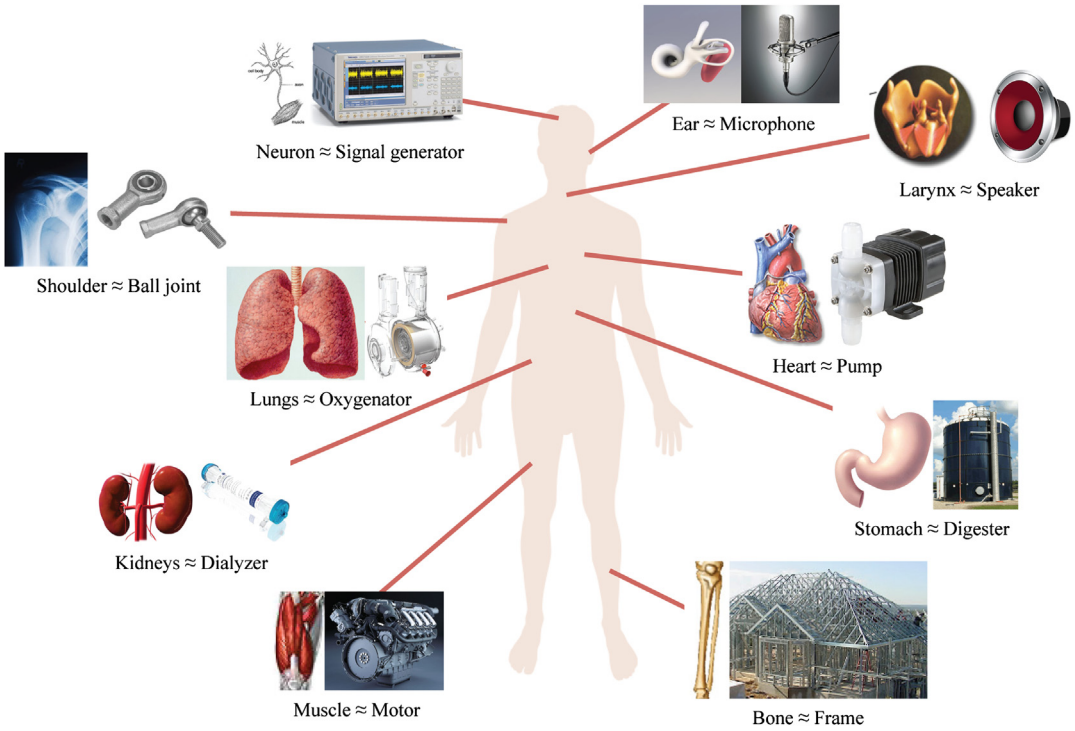

FIGURE 14.1

Engineering perception of human organs and tissues. The understanding of tissue environment and physiological function provides a basis for the design of various types of bioreactors.

For many individual pathways, the essential molecules and factors regulating the desired processes have been identified. However, tissue engineering requires control over a combination of multiple factors that alter cell behavior through multiple regulatory pathways. Such ability would result in the optimization of cellular responses and diverse solutions for enhancement of tissue formation that cannot be achieved either *in vivo* (due to the complexity of the systemic environment that limits control over regulatory factors) or using conventional tissue culture systems (due to the batch-wise operation that prevents control of cellular environment in space and time, and the lack of physical factors). Innovative bioreactor designs offer solutions for quantitative studies of cellular mechanisms in response to changes in molecular transport, mechanotransduction, and the presence of other cells or extracellular matrix.

At the macroscopic level, the cell populations and extracellular matrix (ECM) interact to provide 'engineering' functions (Fig. 14.1). For example, the heart chamber is comparable to a pump moving fluid (blood) through pipes (blood vessels), the lungs work like a gas exchanger and the kidney combines filtration, osmosis, passive and active mechanisms to transport large amounts of molecules and water. Capillary networks provide a supply of oxygen and nutrients to the tissue by combining the convective and diffusive schemes. Cartilage and meniscus absorb impact forces and lubricate joints. Signaling in nerve cells is analogous to electrical signals. Despite the 'engineering' components and functions of our tissues, the biological systems are much more complex, versatile and adaptive than anything we have ever built. For example, blood vessels contract or expand to modulate the flow resistance in response to blood pressure or to a change in blood flow rate, and non-critical bone fractures heal perfectly over time. Viewing physiological systems from an engineering perspective provides guidance for the design of bioreactors, and is critical for defining their operating regimes.

Key engineering concepts for mimicking native environment

Today, bioreactors are developed based on our increasing understanding of physiological systems and their function, in conjunction with multi-disciplinary approaches to enhance and optimize cellular responses. The key concepts and approaches to mimic physiological phenomena in the human body are summarized in Table 14.1.

MASS TRANSPORT

To promote cell viability and well-being, bioreactors must provide adequate mass transport of nutrients, biochemical factors, and most importantly oxygen to the cells [2,53]. In addition, the system must allow rapid removal of metabolic products from the cellular environment. In physiological settings, these functions are provided by the vasculature, which readily adapts to the changes in metabolic demands of the cells.

To generate and maintain viable and functional tissues, tissue engineering needs to effectively build a vascular supply, or at least replicate the convective-diffusive transport normally provided by the vasculature. Static cell culture techniques rely on diffusional transport mechanisms that are efficient only within the thin superficial layer in contact with culture medium (in most cases only $\sim$100–200 μm thick), and fail to support three-dimensional tissues [3]. As the cell density increases, so does the nutrient demand. As a result, nutrients are depleted over short distances, and the cells positioned further from the source of nutrients and oxygen (i.e., those in the tissue interior) cannot remain viable [2,3,53,54].

Several different bioreactor technologies have been developed to increase transport rates between the cells and culture medium. Wave-wall bioreactors create an undulating motion of the culture medium to limit the size of the stagnant cell layer present at the surfaces of tissue constructs [2]. Spinner flasks achieve mixing of the external environment through the use of stirring components. A drawback of both of these simple systems is that they generate hydrodynamic shear stresses that can cause cell dedifferentiation [3]. If too high, shear stress can result in the formation of a fibrous capsule surrounding the tissue construct.

TABLE 14.1 Bioreactor designs and important parameters to mimic physiological phenomena

Physiological phenomena	Physical/ biological cues	Bioreactor design	Important parameters	References
Blood supply through capillary network	Adequate supply of oxygen, growth factors, and nutrition through convective transport	–Spinner flask –Rotating wall vessel –Wave –Perfusion	–Movement speed –Flow rate	[2–4]
Weight bearing joints during locomotion	Mechanical compression resulting in tissue pressurization	–Compressing platen –Pressurized chamber	–Static/dynamic compression –Length –Frequency –Strain	[5–10]
Stretching muscles and tendons	Tensile force pulling tissue and cells	–uni-/bi-stretching motion –Radial stretching motion	–Strain –Tensile force –Tensile stress –Frequency	[11–17]
Fluid flow over cells	Hydro static/dynamic shear force	–Perfusion in thin wall –3D Perfusion	–Shear stress –Fluid flow rate –Complex flow based on simulation	[18–21]
Joint movement	Shear force from tissue sliding	–Sliding surfaces	–Translational speed –Friction	[22–24]
Development, wound healing, cell firing	Electrical current and potential difference	–Electrical stimulator –Electromagnetic Current	–Voltage –Current –Frequency (AC and DC pulsed)	[25–28]
Muscle contraction	Electrical signal depolarizes cells	–Electrical stimulator	–Voltage –Current –Frequency	[29–31]
Cell adhesion to tissue matrix	Cell-substrate attachment through antigens and protein binding	–Micropatterning substrate	–Substrate chemistry, spatial distribution, and stiffness	[32–35]
Cell-cell communication	Local cellular contact	–Microcontact printing –Micromolding contact area	–Cell types –Cell density –Cell surface area in contact	[36–39]
Cellular response to biological signals	Biochemical concentration	–Microfluidic multi-well system	–Biochemical concentration –Temporal Presentation	[40, 41]
Chemotaxis	Concentration gradient	–Microfluidic gradient chamber	–Chemicals and factors –Gradient strength –Gradient Profile	[42–47]
In vivo response by multiple cell types, factors, and properties	Tissue *in vivo* response Cell type and stimulating factors	–*In vivo* implantation	–Complex tissue development, formation and function (ex. Angiogenesis, or bone/ cartilage tissue)	[48–52]

Rotating wall bioreactors were created to provide mixing while limiting the shear stress applied to the cells. The bioreactor rotation creates a dynamic flow environment by balancing the rotational, gravitational, and drag forces, so that the tissue constructs are suspended in culture medium in a state of free fall [2,3]. These conditions were termed 'simulated microgravity',

since they effectively randomize the gravity vector without having effect on gravity force. The dynamic nature of the settling of the tissue constructs in the rotating flow, which is associated with fluctuations in the amplitudes and directions of flow velocity and shear vectors, prevented the formation of fibrous capsules and significantly improved the cultivation of some tissues, cartilage in particular. Still, the size and viability of the tissue constructs depended on the internal diffusion of nutrients.

In tissue engineering, the most common practice to increase transport is to augment diffusion with convection, by mechanisms governing blood-tissue exchange of nutrients, oxygen and metabolites, using perfusion bioreactors [2]. In a perfusion bioreactor, medium is pumped through the scaffold, providing transport of nutrients inside the porous material. Perfusion bioreactors have been shown to be superior over both static culture [20] and spinner flask bioreactors [55] in terms of the resulting homogeneity of cell distribution.

MECHANICAL CUES

Physical forces play important roles in regulating all phases of tissue formation. As the cells proliferate and produce extracellular matrix, the volume of the tissue changes and exerts forces to the adjacent cells. For example, during mandibular development, shear forces exerted along the longitudinal growth of the mandible induce bone formation, while compression at the tip of the mandibular body induces cartilage formation. Interestingly, the type of cartilage strongly depends on the acting mechanical forces. Hyaline cartilage in the knee joint is thick and can withstand high compressive forces, while fibrocartilage on the mandibular condyle is very thin, consistent with its role in lubrication rather than in absorbing compressive forces. As a result, various types of bioreactors were designed to utilize various physical stimulation regimes.

Bioreactors applying mechanical compression were first developed for cartilage tissue engineering. These bioreactors generally comprise of a platen, which compresses tissue constructs statically or dynamically at a defined displacement or load. Different compressive regimes have been investigated and demonstrated to enhance deposition and functionality of cartilage matrix [5–7]. Hydrostatic pressure, a simpler set up than platen compression, has also been employed to show similar effects [8]. This is not surprising, as cartilage is a dense viscoelastic tissue containing large amounts of water. When the tissue is compressed, the low hydraulic conductivity prevents water from flowing out, leading to an increase in hydrostatic pressure, which stimulates the chondrocytes.

Bioreactors that apply mechanical tension have been developed to study the formation of tissues such as ligament, muscle, or blood vessels. These tissues are stretched and exert tensile forces during their physiologic function. A tensile stress can be applied unilaterally, bilaterally or radially with either displacement or force control. Axial stress was shown to stretch the cells attached to the scaffold and induce a number of cellular responses, including cell elongation, alignment, differentiation and coupling [11–15]. Radial stress was shown to regulate smooth muscle cell organization and differentiation in vascular grafts [16].

Bioreactors applying shear force have utilized two different mechanisms: by the flow of fluid or by sliding a rigid material over the cell or tissue surface. The same mechanisms are associated with the physiological flow of fluid over osteocytes during bone compression and sliding of cartilage over meniscus during joint movement. By varying shear stress through changes in fluid viscosity and flow rate, primary osteoblast and mesenchymal stem cells showed increased osteogenic expression and produced more bone proteins and minerals [18,20,21]. Likewise, surface motion over cartilage enhanced chondrogenic responses [56].

ELECTRICAL SIGNALS

Electrical signals are inherent to most tissues. Depending on the specific state of the tissue (development, damage, or homeostasis) the electrical signals change with the specific tissue

activities [25]. Electrical currents have been measured in tissue development, wound healing and preconditioning of engineered tissues [25]. These studies have shown the importance of electrical currents in the differentiation, maturation and assembly of electrically excitable cells.

The presence of large electrical currents within the embryo shows that electrical signals are important for stem cell differentiation and early development. Studies of animal embryos show the presence of an electrical field during first cell divisions. Notably, the reversal of the direction of this electrical field caused developmental defects [25]. Equipping bioreactors with electrical fields has enabled the use of electrical signals to modulate stem cell differentiation. By applying an alternating current with a 1 Hz frequency, neural stem cells were induced to differentiate into astrocytes [26]. Similarly, a 15 Hz frequency of a pulsed electromagnetic field was able to increase osteogenic differentiation of human mesenchymal stem cells [27].

In wounds, an electrical field is generated across the damaged tissue. Its strength depends on the tissue type, but in general, a stronger field is recorded at the edges of a wound in comparison to its center [25]. The upregulation of sarcoma and inositol-phospholipid signaling pathways establish the directionality of the electrical field, which mediates cell migration into the wound [28]. Similar electrical fields also promoted directed cell migration [28].

The incorporation of electrical stimuli in the bioreactor design for electrically active tissues, such as muscle and nerves, allows for better tissue development and augmented functionality of engineered tissues. For example, the application of pulsed electrical signals 2 ms duration at a frequency of 1 Hz resulted in significant structural organization of the myocardial grafts [29]. Electrical stimulation resulted in improved cardiac cell alignment and significant improvements of the amplitude and synchronicity of myocyte contractions [29].

CELLULAR MICROENVIRONMENT

Ideally, biological studies should be performed under conditions that recapitulate specific aspects of the complex native environment. The capability to modulate regulatory factors and measure cellular responses can lead to the recreation of native-like environments and quantitative studies of cell behavior. Of key interest are cell responses to other cells, matrix, and biophysical factors.

Microscale technologies enable fast and accurate control of the cell microenvironment and exploration of the underlying mechanisms through which the local microenvironment regulates cell behavior. The structure and organization of cellular assemblies can be precisely controlled by patterning techniques [35], through which cells can be guided to adhere onto specific locations with specific physical properties. Commonly used techniques include:

1) Microcontact printing of alkanethiols and proteins on gold-coated glass or other substrates;
2) Replica molding for fabrication of micro and nanostructures in polyurethane or epoxy;
3) Solvent-assisted micromolding of nanostructures in poly-methyl-methacrylate.

Patterns of adhesive and non-fouling proteins determine the spatial distribution of cells and the formation of focal adhesions. Pioneering studies [32] have proved that cell shape, regardless of the type of ECM printed on the substrate to mediate cell adhesion, plays a major role in determining cell fate and function. Micro- and nano-scale geometric cues can induce different functional outputs on cultured cells (for example growth or apoptosis) as a result of exogenously controlled microenvironmental stimuli. Combination of specific mechanical and biochemical properties of the cell culture substrate enabled the study of a plethora of previously unexplored biological phenomena.

Lineage specification in stem cells could be directed by substrate stiffness. By mimicking *in vivo*-like matrix elasticity using variably compliant polyacrylamide gels, and directing cell attachment by coating the surfaces with collagen I or other adhesion proteins, evidence was presented that soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and more rigid matrices that mimic bone prove osteogenic [33,34].

Another important component of the *in vivo* microenvironment is the tightly controlled spatial and temporal pattern of soluble factors. Spatial gradients of morphogens and other chemical cues play a fundamental role in determining cellular response *in vivo*. Since the advent of microscale technologies, researchers have invested heavily into the development of highly controllable platforms for delivering complex physiological signals.

One of the earliest, still widely used and re-adapted, microfluidic devices was developed by Jeon's group to generate dynamic spatial-temporal gradients of morphogen concentrations [45]. Their simple device combined a modular mixer with a gradient-maker network, capable of controlling multiple gradient shapes. Different concentration profiles could be generated depending on the configuration and number of fluidic inputs, the design of the microchannels network and the flow rates of the incoming streams. Splitters and mergers induced the mixing (primarily by diffusion) of fluid streams with different compositions, thus allowing sequential generation of a variety of concentration gradient profiles such as linear, saw-tooth, and bimodal.

Microfluidic technologies have enabled the investigation of many previously unexplored biological questions. Among them, one study demonstrated the effect of the concentration gradient of growth factors on human neural stem cells [42]. After seven days in culture under continuous flow with concentration gradients, cells proliferated and differentiated into astrocytes in a graded fashion, in proportion to the growth factor concentration. Similarly, neural progenitors cells were studied under conditions mimicking the *in vivo*-like presentation of morphogens known to act via the formation of concentration gradients during embryo development [46]. Neural progenitor cells derived from human embryonic stem cells were cultured for up to eight days under concentration gradients of cytokine cocktails (sonic hedgehog, fibroblast growth factor 8, bone morphogenetic protein 4). Differentiation of progenitor cells was successfully guided in a manner directly proportional to the concentration gradient, with complex neural networks formed inside the culture channel.

Cellular migration

Chemotaxis and chemokinesis regulate cancer metastasis, embryogenesis, inflammation, wound healing and many other biological processes. Evidence has been found for the role of fast-switching spatial and temporal regulation patterns *in vivo*. However, effective study of these complex biological events has long been hindered by a lack of necessary technologies. Recent technological advances, such as microstructured membranes and valves in microfluidic devices, allow increased control over the local concentrations. As a result, integration of these components enables fast and accurate switching between different concentration gradients and stimulation patterns.

Microscale physiological stimulation is essential for studying biological phenomena such as directed cell migration [43]. For example, neutrophils, the fundamental component of the physiologic defense machinery, have been among the most widely studied cell phenotypes with regards to cell migration. Neutrophils' movements are regulated by complex phenomena involving continuously changing chemical signals. With the aid of advanced microfluidic systems, it has become possible to study their complex behavior [44]. By controlling the type and the temporal presentation of signaling molecules, the migratory patterns of neutrophils can be predicted. Additional stimulations (other than spatial and temporal gradients alone)

are necessary in a higher hierarchical fashion to fully encompass the complex behavioral responses of neutrophils *in vivo*.

COMPLEX SIGNALING OF *IN VIVO* BIOREACTORS

The bioreactors developed thus far have demonstrated capability to partially mimic native environment for *in vitro* culture. However, tissue generation is a complex process involving numerous interconnected pathways, cell types, and interactions needed for proper development [52,57]; thus, these complex physiological processes make it difficult to generate complete physiological mimics *in vitro*. One particular challenge is the generation of vasculature within engineered tissues. To this end, significant research has been directed to the development of *in vivo* bioreactors [51]. Most *in vivo* bioreactors are placed into highly vascularized, minimally functioning locations within the body [51,52,57]. Adequate nutrient supply, and the availability of angiogenic factors and different cell types, can facilitate the development of vasculature to support tissue-engineered constructs [48].

Despite the successes in creating complex tissues that are highly vascularized, shortcomings have prevented this technology from being broadly adopted. Firstly, only a limited number of locations in the body meet the conditions necessary for proper construct development [57]. Secondly, due to the high variability of processes *in vivo*, significant control of variables is lost and additional stimulation, such as mechanical or electrical, is difficult to incorporate [57]. However, continued work is ongoing to solve these problems while maintaining the extraordinary benefits of the *in vivo* bioreactors.

ADVANCED BIOREACTORS IN TISSUE ENGINEERING

In order to study the complex biological responses and generate native-like tissues *in vitro*, bioreactor designs have advanced with greater complexity. Despite these improvements, the bioreactors are maintaining user-friendly operation to provide effective experimental tools and reduce user errors. This section introduces advanced bioreactor systems that have been successfully used to progress their application of regenerative medicine, biological research and high-throughput screening.

Regenerative medicine

Generating functional tissue grafts of clinically relevant sizes *in vitro*, by using autologous or immunologically matching stem cells would change the way we currently treat tissue loss. Engineered grafts would overcome the combined limitations of autografts (lengthy surgical procedures, donor site morbidity), allografts (immune rejection, disease transfer, limited supply), and xenografts (immune rejection and disease transfer). However, the engineering of functional grafts matching the needs of the patient and the specific clinical condition remains a challenge to bioreactor design [58]. We discuss here two studies that have employed innovative bioreactor techniques in attempts to engineer functional tissues.

DECELLULARIZING AND RECELLULARIZING LIVER FOR TRANSPLANTATION

The most commonly used technique in liver transplantation is orthotopic transplantation, in which the native liver is removed and replaced by the donor organ in the same anatomic location as the original liver. Tissue engineering offers a promising substitute to allograft transplantation which would overcome the problem of donor liver shortage and the complications caused by donor mismatch. Notably, the difficulties in providing sufficient transport rates of oxygen and nutrients to metabolically active cells in large pieces of engineered liver tissue have limited the development of liver grafts.

Uygun et al. demonstrated a novel approach to generating transplantable liver grafts using a custom-designed perfusion bioreactor [59]. The bioreactor consisted of a simple perfusion chamber in which a whole rat liver was submerged in fluid and connected to the perfusion

loop by portal vein cannulation, with the inferior vena cava and superior vena cava left open for the fluid outlet. The bioreactor was connected via tubing to a pump and a reservoir. The liver was first decellularized by perfusion with Sodium Dodecyl Sulfate (SDS), an anionic detergent that lyses cells and solubilizes cytoplasmic components, Triton X and Phosphate Buffered Saline (PBS). This decellularization process generated a translucent acellular scaffold, which retained the gross anatomical shape of the liver (Fig. 14.2a–c). While cellular content was mostly removed, the technique retained 100% of the fibrillar collagen and ~50% of the glycosaminoglycans of native liver. Importantly, the structural components of the vascular network, consisting of the portal and venous circulation system all the way down to the microcirculatory branches, were largely preserved (Fig. 14.2d).

The same bioreactor was used to seed the decellularized livers with adult rat hepatocytes via portal vein perfusion-recirculation with high engraftment efficiency (> 95%) and viability (> 90%). After seeding, recellularized liver grafts were transferred into a new perfusion chamber (Fig. 14.2e), configured into a pouch between two hermetically sealed silicon sheets filled with culture medium, and cultured for five days. An important aspect of this approach is that the preserved vascular structure enabled connection of the recellularized liver to the host vasculature at the time of transplantation. The blood supply that was established immediately maintained viability of transplanted cells for 8 hours *in vivo*. This study demonstrates the utility of perfusion bioreactors for generating liver grafts by decellularizing and recellularizing whole rat livers, and for maintaining perfusion *in vivo* by preserving the vascular network and connecting it to the host vasculature.

BIOREACTOR FOR ENGINEERING FULLY CELLULARIZED, ANATOMICALLY SHAPED BONE GRAFTS

The use of bone autografts, allografts, and engineered bone substitutes all require pre-implantation shaping in order to match the anatomical features of the specific defect. The ability to generate functional bone grafts with precisely defined geometries would significantly reduce the time of surgery, improve grafting outcomes and eliminate a number of complications associated with the use of other grafts. Maintaining viability and function of the cells in clinically sized, anatomically shaped tissue constructs requires a well-controlled supply of nutrients and oxygen throughout the tissue constructs.

Recently, a custom-designed perfusion bioreactor was developed to support cultivation of clinically sized, anatomically shaped human bone grafts by bioreactor cultivation of bone marrow mesenchymal stem cells on scaffolds made of decellularized bone [4]. Human temporomandibular joint (TMJ) condyles were engineered by using two different bioreactor systems: mixing chambers for seeding scaffolds with cells, and perfusion chambers, anatomically shaped, to differentiate stem cells and assemble bone matrix. The seeding bioreactor was adapted from the spinner flask cultivating system, and contained three scaffolds fixed in place and submerged in cell suspension. Magnetic stirrers created a convective flow that propelled cell suspension deep into the scaffold pores permitting cell adhesion.

The cultivation bioreactor consisted of a specially designed bioreactor chamber connected via a perfusion loop to a medium reservoir and a peristaltic pump to recirculate medium and perfuse it through the scaffold within the chamber. The chamber consisted of a soft Polydimethylsiloxane (PDMS) block created as a negative mold that fully entrapped the scaffold, and an outside casing chamber that contained the inlet and outlet ports for culture medium perfusion (Fig. 14.2f–h). Both the scaffold and the bioreactor chamber were fabricated using a three-dimensional file created from clinical images of a human TMJ. By employing computer software to analyze fluid flow patterns, the locations for the medium inlet and outlets were optimized to ensure perfusion of all regions of the seeded scaffold (Fig. 14.2i). After five weeks of cultivation, tissue growth was evident by the formation of confluent layers of lamellar

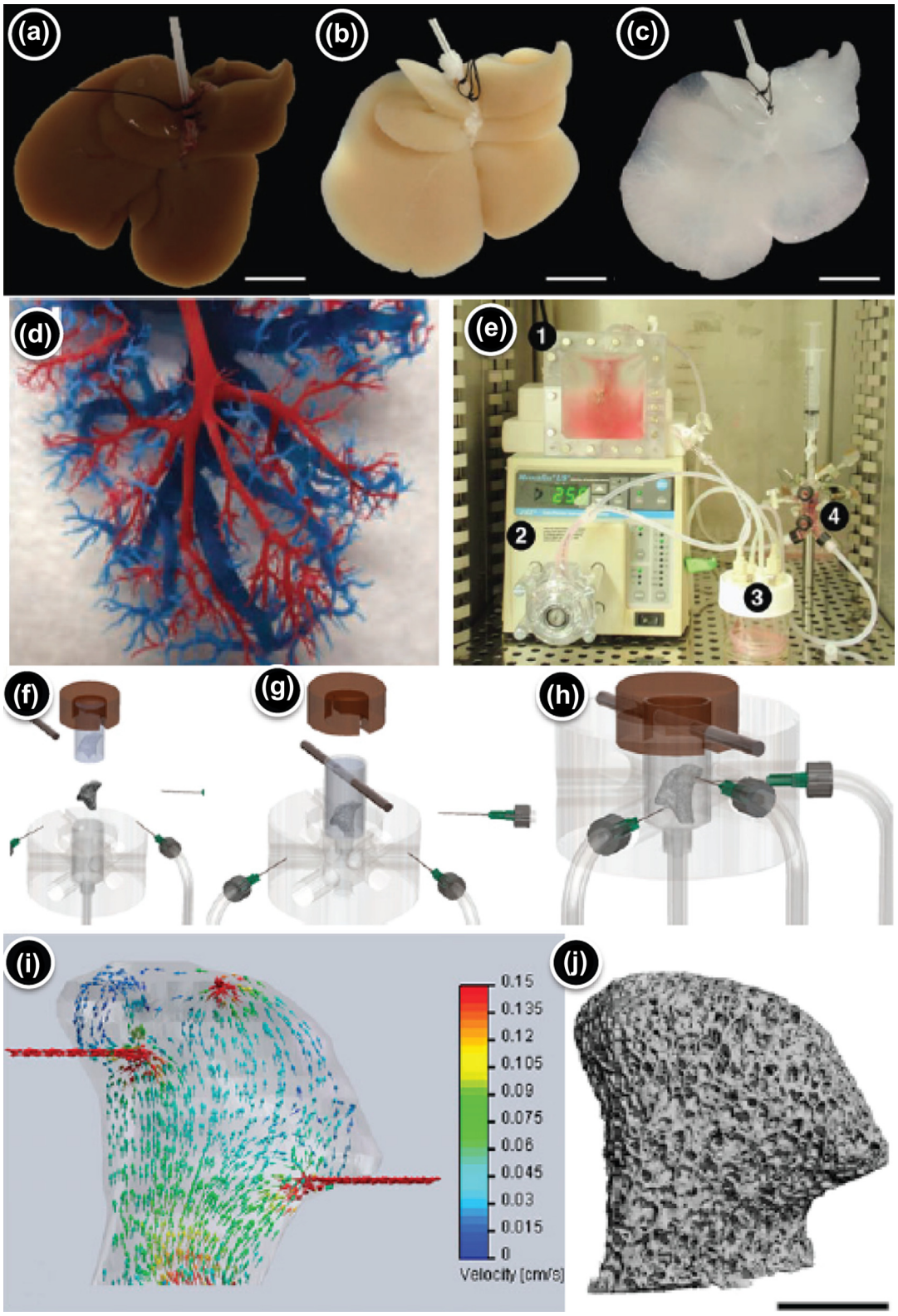

FIGURE 14.2

Bioreactor design and generation of tissue grafts for regenerative medicine. Liver tissue engineering (a—e). Ischemic rat livers during decellularization in a perfusion bioreactor at (a) 0 hr, (b) 48 hr, and (c) 72 hr. (d) Corrosion cast model showing preservation of vasculature within the left lobe of the decellularized liver matrix. (e) Perfusion system for in vitro culture of the recellularized liver graft: (1) perfusion chamber, (2) peristaltic pump, (3) oxygenator, and (4) bubble trap [59]. **Bone tissue engineering (f—j)**. (f—h) Bioreactor design and assembly for cultivation of anatomically shaped human bone grafts. (i) Computerized fluid flow model demonstrated total perfusion of medium throughout the complex-shape graft. Color-coded velocity vectors indicate the magnitude and direction of flow through the entire construct. (j) Architecture of the mineralized bone matrix after 5 weeks of cultivation in the perfusion bioreactor [4].

bone (by scanning electron microscopy), markedly increased volume of mineralized matrix (by microcomputerized tomography; Fig. 14.2j), and the formation of osteoids (histologically). The cells were present at a physiologic density and were fully viable, likely an important factor for graft function and successful integration with the host tissues.

These studies of two distinctly different and rather complex tissues — liver and bone — demonstrate that custom-designed bioreactors can be used in conjunction with biological scaffolds and cells to grow viable and functional tissues *in vitro* in clinically relevant sizes and shapes. It is expected that further advancements in bioreactor designs, such as incorporating additional regulatory signals (molecular, cellular, hydrodynamic, mechanical), will continue to drive tissue-engineering approaches towards clinical translation.

Biological research

During development and regeneration, tissues are formed from coordinated sequences of stem cell renewal, specialization and assembly that are orchestrated by cascades of regulatory factors. Standard *in vitro* techniques typically fail to replicate the complexities of the *in vivo* milieu, often resulting in unrealistic biological readouts that fall short of being physiologically predictive of the actual *in vivo* situation. In this context, there is a clear need for bioengineered environments that combine tissue-specific transport and cell signaling with multiple cell types, and 3D spatial-temporal control of molecular and physical signals, in ways resembling the contexts encountered *in vivo*. There is a growing recognition of how important microenvironmental control is in studying development, regeneration and disease in settings predictive of human situations.

CELL COMMUNICATION

Cell co-cultures are typically performed by either seeding different cell types one after another (into the same or different regions of the scaffold), or by direct seeding of mixed cell populations [37–39]. The spatial and temporal control of cell-cell interactions is as challenging as it is important for proper tissue development and function. One of the most elegant approaches developed thus far has demonstrated that dynamic regulation of cell-cell interactions can be achieved by direct manipulation of adherent cells and the resulting tissue organization with micrometer-scale precision (Fig. 14.3a) [36]. The device is composed of two interlocking comb fingers and an integrated snap-lock mechanism, which was fabricated using electromechanical microfabrication methods (Fig. 14.3a-*a*).

The silicon fingers are pre-coated with polystyrene, the tissue culture plastic material, and can be seeded individually with different cell types and re-assembled in the desired configuration (Fig. 14.3a-*b,c*). The parameters for cell-cell contact and communication between cell populations can be controlled separately and cultured simultaneously (Fig. 14.3a-*d*). For example, by setting a μm-scale separation between the plates, cell contacts can be eliminated while soluble signaling is maintained, in a way dependent on the separation distance. In addition, plates are interchangeable, enabling dynamic regulation of diverse signaling phenomena (cell contact-mediated and soluble signaling) without the need of interrupting culture or setting up multiple experimental runs.

This device enabled the investigators to deconstruct the dynamics of intercellular communication between hepatocytes and supporting stromal cells in co-culture. The preservation of hepatocyte viability and liver-specific functions depended on the complex dynamics of spatial and temporal signal sequences, resulting in the identification of key signals. The adaptability of this microbioreactor system is suitable for studying a broad spectrum of biological processes. The integration of microfluidic components for fluid delivery would enable recreation of the dynamically changing microenvironment of the *in vivo* niche, while preserving the cell-mediated signaling originating from the multiple cell types.

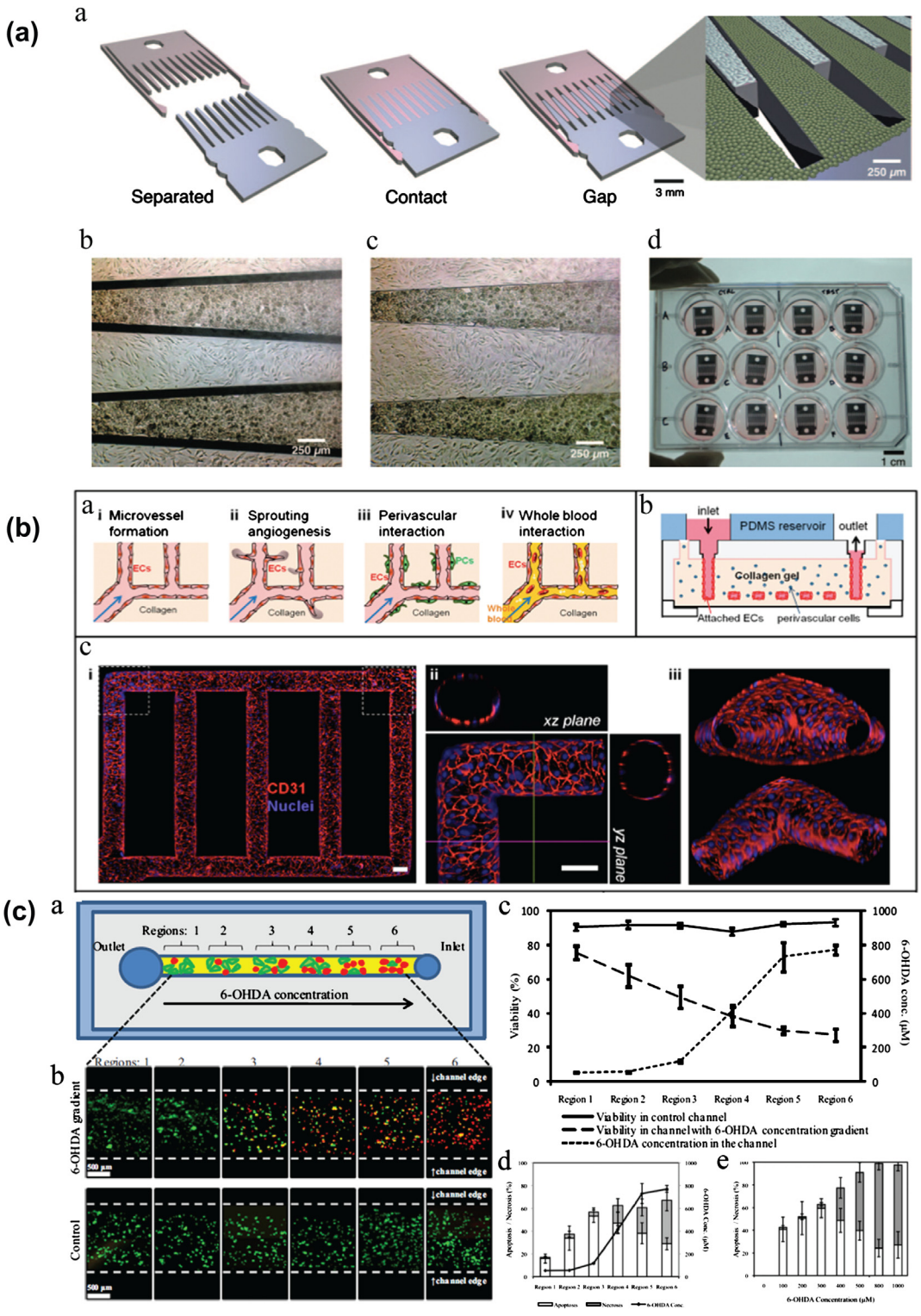

FIGURE 14.3

Microbioreactors for biological research. (a) Micromechanical control of cell communication. (a) Separable, microfabricated comb finger mechanism that allows micron-scale spatial control of co-cultured cells. Appropriate actuation can provide an interlocked configuration (center), or micron-scale gap separation (right). (b and c) Co-culture of hepatocytes (darker cells) and 3T3 fibroblasts demonstrating the different spatial configurations, gap (b) and interlocked (c). (d) Mechanism dimensions were designed to conform to a standard 12 well plate to allow simple cell culture and reproducibility [36]. **(b) Generation of complex microvessel networks.** (a) Schematic of the theoretical development of the microfluidic vessel networks achieved through sequential seeding of endothelium and subsequent development. (b) Schematic of the microfluidic bioreactor system, with arrows demonstrating fluid flow.

In summary, technologies capable of recreating and dissecting the complexity of cell communication might prove very useful in the studies of cell development, disease onset and progression, therapy development and cell niche investigation.

TISSUE DEVELOPMENT

Native tissues develop under tight spatial and temporal control of multiple factors, with synergistic and competing effects. Averaged tissue properties are important for determining the utility of engineered tissue grafts but are less instructive for defining the cell-scale phenomena. Therefore, more precise microscale systems are needed for appropriate cell-scale observations of relevant phenomena. A recent advance resulted in a network of endothelialized microfluidic vessels generated within collagen matrix that were fabricated via standard lithography and micromolding (Fig. 14.3b) [60]. Custom-designed microstructured PDMS constructs were used as templates to mold the microvessel structures, with sealed plexiglass frames used to define the thickness, size and shape. Collagen gel provided adequate mechanical stiffness and structural stability of the vessel microstructures while also enabling remodeling through cellular degradation and deposition of extracellular matrix (Fig. 14.3).

One important design characteristic of the bioreactor was the ability for sequential seeding, allowing different cell types to seed either the vessel channels or the extravascular collagen gel. Gravity-driven perfusion of culture medium was maintained for up to two weeks to form endothelium, which exerted a barrier-function to the transfer of solutes from the lumen into the surrounding matrix (Fig. 14.3).

Specific behaviors were identified depending on the different stimulations of the microvessel networks. For example, stimulation with a proangiogenic environment found in the vicinity of ischemic tissue or solid tumors resulted in less organized structures with loose cell-cell contacts, cell sprouting from the endothelium into the bulk collagen matrix and increased leaking into the surrounding areas. Remarkably, the *in vitro*-produced vascular endothelium was non-thrombogenic, and could be induced by inflammatory factors into a prothrombotic state. Following perfusion with whole human blood, 'healthy' vessels showed little or no platelet adhesion, while large aggregates of platelets formed immediately in an induced inflammatory environment. This bioreactor technology enabled investigation of the endothelial interactions with other cell types, for complex phenomena such as angiogenesis and thrombosis. This system holds great potential for modeling of tumor progression, inflammation, thrombosis, and many other pathological states.

DISEASE MODELS

Primary human cell cultures or animal cell lines are often used as *in vitro* models for disease and screening studies. However, these models often fail to represent the highly specialized characteristics of human tissues. The possibility of reconstructing *in vivo*-like niches resembling the natural architecture, biomechanics, soluble environment, spatial and temporal delivery of factors opens exciting prospects for studying disease physiology in organ-specific contexts.

In a recent study [47], microfluidic technologies were applied to an *in vitro* model of Parkinson's disease. A simple microfluidic channel was fabricated by soft lithography, seeded with cells, and used to generate concentration gradients of neurotoxins for 24 hours

(c) Confocal images of the precisely controlled development of the microfluidic vascular networks. i. the entire network, ii. varying projections of the corner regions, including luminal views, iii. varying projections of the branching segments [60]. **(c) Microfluidic device for disease modeling.** (a) Schematic of the microfluidic device that enables the study of the concentration related effect of the 6-OHDA neurotoxin on neuronal cells. Representative image of the live/ dead staining of PC12 cells is included in the schematic. (b) The upper row demonstrates the live/ dead images with a channel toxin concentration of 1000 μM. The lower demonstrates the live/ dead images with no added toxin. (c) Quantification of PC12 cell viability within the channel based on the region segmentation marked in part B. (d) Quantification of PC12 apoptosis/ necrosis of the experimental treatment along the channel following the region segmentation marked in part B. (e) Quantification of the apoptosis/ necrosis of PC12 cells exposed to varying concentrations of 6-OHDA performed in static culture [47].

(Fig. 14.3c). The viability, apoptosis and necrosis of cells in the channels were determined. Cell viability was inversely proportional to the concentration of neurotoxin. Interestingly, low drug concentrations mainly induced death by apoptosis, while higher concentrations resulted in cell necrosis. Such *in vitro* mimics of *in vivo* events will be crucial for developing novel therapeutic targets.

The integration of similar bioengineered environments with novel stem cell sources can open new perspectives in understanding the fundamental biology of disease, investigating the mechanisms of disease, and developing new treatment modalities. Recent advances have proven the feasibility of deriving patient-specific pluripotent stem cells (iPS cells) to be used for a multiplicity of purposes, including disease modeling studies. A series of diseases have already been investigated *in vitro* using iPS cells differentiated into disease-relevant cells. These advances represent a fundamental tool for developing high-throughput screens for candidate drugs and performing mechanistic studies of disease pathogenesis [61].

High-throughput screening

High-throughput screening methods are beginning to incorporate robotics, microspotting, data processing control software, liquid handling devices, and sensitive detectors. The integration of such components into miniaturized biological experimental systems allows researchers to more readily conduct vast chemical screenings. High-throughput screenings can dramatically improve the identification of active compounds, antibodies or genes which modulate biomolecular pathways and can help studying cell behavior in a combinatorial, efficient manner.

OPTIMIZING TISSUE CULTIVATION TECHNIQUES

Robotic fluid handling and microspotting facilitate high-throughput studies of different immobilized factors in microarrays, but require time-dependent stimulation to be applied simultaneously. Microfluidic platforms, which facilitate studies of diffusible growth factors with spatial and temporal control, have historically been difficult to scale-up into larger, individually manipulated culture chambers.

Versatile, fully automated microfluidic cell culture systems that create arbitrary media formulations in independent cell culture chambers can help address these requirements [40]. A classical example of this approach is an automated microfluidic platform with 96 individually addressable culture chambers, with a volume of 60nL each, that was capable of maintaining cell viability for weeks and was designed to screen for the effects of multiple conditions on cell behavior (Fig. 14.4a). Culture conditions could be customized in terms of cell seeding density, composition of culture medium, and feeding schedule, and each culture chamber could be imaged with time-lapse microscopy. Multiplexers and mixers (controlled by actuators and valves) allowed generation of broad ranges of medium compositions and accurately controlled the temporal feeding/washing patterns.

The automation was also extended to post-processing procedures such as fixing and staining of the cell samples, achieving improved accuracy and saving reagents. hMSCs were selectively stimulated to either proliferate or differentiate into the osteogenic or adipogenic lineages. This microfluidic platform, also designed to precisely study the effects of culture medium exchange rates, allowed exploration of different types of temporal gradients in the environmental conditions. As a case study, the authors tested eight different durations of osteogenic stimulation on the differentiation and motility of hMSCs. Bioreactor design incorporating automated and multiplexed devices demonstrated the ability to quantitatively and systematically determine the optimal cell and, thus, tissue cultivation method.

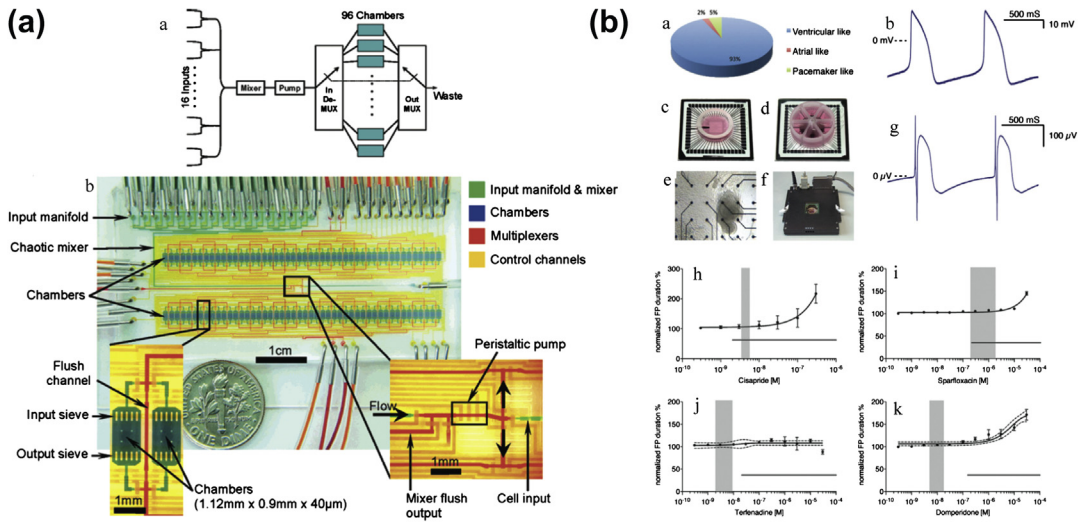

FIGURE 14.4

Bioreactors for high-throughput screening. (a) Fully automated, multiplexed platform for high-throughput screening. (a) Schematic of the medium fluidic path within the chip bioreactor. (b) Photograph of the chip bioreactor, with annotations detailing the important components. The left inset demonstrates two culture chambers with the integral components, and the right inset demonstrates the input multiplexer, with the necessary components for media change and cell seeding [46]. **(b) Drug screening studies on human cells.** (a) Analysis of the cardiac differentiation of HE53 cells. (b) Ventricular-like action potential measured by patch clamp electrophysiology. (c) Multichannel electrode array (MEA) with culture chamber and (d) MEA with a 6 well chamber. (e) Image of human embryonic stem cell (hESC) derived cardiomyocytes cultured on the MEA system. (f) Image of the amplifier used to digitize the recorded electrical signals. (g) hESC derived cardiomyocyte field potential. (h-k) Concentration-dependent effect of cardiac drugs, (h) Cisapride (i) Sparfloxacin (j) Terfenadine (k) Domperidone, on the field potential of hESC derived cardiomyocytes [48].

DRUG SCREENING

The integration of relevant stem cell sources with advanced technologies will also likely become a significant resource for preliminary drug screening, eliminating or greatly reducing the need for costly (and at times poorly predictive of the actual human physiology) animal experiments. A particularly important need for *in vitro* modeling of multi-organ systems is the prediction of toxicity. A novel 'organ-on-chip' concept [41] involves advanced engineered systems in which cells are cultured within microfluidic devices designed to recapitulate specific microenvironment of living organs. Such *in vivo*-like niches, which resemble the natural architecture, biomechanics, soluble environment, spatial and temporal delivery of factors, open exciting opportunities for studying disease physiology in organ-specific contexts. The additional capability of organ-to-organ communication would bring the study of toxicology and pharmacology to an unprecedented level, and could shorten time to market for novel therapies.

Human cardiomyocytes, derived from embryonic stem cells, were used as a renewable and scalable source for cardiac pharmacology assays. Patch clamp analyses and multichannel electrode arrays (MEA) allowed generation of field potential duration (FPD) values of the cardiomyocytes following exposure to different drugs [62]. The use of MEA allowed a dramatic increase in the throughput, obtaining large amounts of data using limited number of cells and reagents (Fig. 14.4). One of the most widely used criteria for cardiac drug toxicity is the prolongation of the QT interval (the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle. It's the portion of an ECG representing the time from the beginning of ventricular depolarization to the end of ventricular repolarization), which can signal ventricular arrhythmias. After having established a working model for cardiotoxicity, the authors demonstrated improved performance of their model in detecting QT prolongation/shortening and arrhythmia caused by various drugs. This method, measuring drug toxicity responses on *in vitro* models of human cardiac cells, proved to be reliable for preclinical

evaluation of new drugs. This success suggests it can be used in replacement of the current assays, which typically rely on tedious, operator dependent, single cell-based measurements (i.e., patch clamp). Patient-specific studies would also enable developing ad hoc, personalized therapies.

SUMMARY

The development of bioreactors for tissue culture applies engineering concepts to mimic native tissue environments and physiological functions. Previous studies have extensively demonstrated various types of natively mimicking bioreactor systems on tissue engineering and in the study of cellular responses. To date, the bioreactor designs have been increasingly progressed to the point which allows scientists and tissue engineers to generate fully cellularized functional tissue grafts, study complex biological responses *in vitro*, and improve tissue culture methods and pathological treatments. Engineers and scientists are continuously advancing bioreactor system in tissue engineering toward (i) developing complete tissue cultivation systems for generation of functional tissue grafts for clinical appplication, (ii) providing tools to study sophisticated biological responses which will open up novel direction for tissue regeneration and tissue engineering, and (iii) optimizing cellular cultivation techniques, especially in the field of stem cell research, which is the most clinically relevant cell source for regenerative medicine.

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CHAPTER 15

Regulation of Cell Behavior by Extracellular Proteins

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INTRODUCTION

The extracellular milieu is critical for the control of the behavior of every cell in all tissues. Many factors can contribute to the environment of a cell, for example, cell-cell contact, growth factors, extracellular matrix (ECM) proteins, and matricellular proteins. All of these components act together to regulate cell surface protein activity, intracellular signal transduction, and subsequent gene expression which leads to proliferation, migration, differentiation, and ultimately the formation of complex tissues. This chapter will focus on matricellular proteins as modulators of extracellular signals. The matricellular proteins are characterized as secreted, modular proteins that are associated with the extracellular matrix but do not act as structural constituents [1]. Presumably, the function of matricellular proteins is to provide a link between the extracellular matrix and cell surface receptors, or cytokines and proteases localized in the extracellular environment whose activity might be affected by this interaction [1]. Thrombospondin 1 and 2, tenascin-C, osteopontin, and SPARC are representatives of this class of proteins. A growing body of evidence points to these proteins as important mediators of growth factor, ECM, and cell signaling pathways (Table 15.1). Consequently, *in vitro* systems designed to mimic tissue conditions should consider the influence of matricellular proteins.

THROMBOSPONDIN-1

Thrombospondin-1 is a 450,000 dalton glycoprotein with seven modular domains [2]. To date, five different paralogs of thrombspondin have been identified, termed thrombospondin-1–5. This chapter will review the two more characterized forms, thrombospondin-1 and thrombospondin-2. At least five different ECM-associated proteins are able to bind to thrombospondin-1: collagens I and V, fibronectin, laminin, fibrinogen and SPARC [2]. Likewise, cell surface receptors for thrombospondin are numerous, and include the integrin family of extracellular matrix receptors [3]. Given the significant number and variety of thrombospondin-1-binding proteins, it is of little surprise that a wide variety of functions have been attributed to this protein, some of which appear to be contradictory. Many of these disparities may, however, actually reflect the dynamic interaction of thrombospondin-1 with

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TABLE 15.1 Matricellular protein interactions and activities

	Extracellular matrix interaction	Receptor	Adhesive (+) vs. counter-adhesive (–)	Growth factor modulation	Extracellular matrix formation
Thrombospondin-1	Col I & V Fn, Ln, Fg	Integrin CD-36 CD-47 LRP	(–)	HGF (–) TGFβ (+)	+
Thrombospondin-2	MMP-2	LRP CD-36	(–)	?	+
Tenascin-C	Fn SMOC1 Perlecan	Integrin Annexin II	(–) (+)	EGF (+) bFGF (+) PDGF (+)	+
Osteopontin	Fn, Col I, II,III, IV, & V	Integrin CD-44	(+)	?	+
SPARC	Col I, III, IV, & V	Stabilin-1 VCAM	(–)	bFGF (–) VEGF (–) PDGF (–) TGFβ (+)	+
Hevin/SPARC-like 1 (SPL1)	Col I	?	(–)	?	+

A summary of the activities for the matricellular proteins described in this chapter. This table is not a complete list of all activities and receptors for the proteins included in the table nor are all the matricellular proteins included.
For references refer to the text.
Abbreviations: Col: Collagen, Fn: Fibronectin, Ln, Laminin, Fg: Fibrinogen, LRP: Lipoprotein Receptor-Related Protein, SMOC: SPARC Related Modular Calcium-binding Protein, VCAM: Vascular Cell Adhesion Molecule. Growth factor abbreviations are defined in the text.

other extracellular factors which can influence cells in different ways to give rise to distinct cellular outcomes – a common theme in matricellular protein biology.

Several studies have established an anti-angiogenic function of thrombospondin-1 [3]. *In vitro*, aortic endothelial cells transfected with anti-sense thrombospondin-1 cDNA generate twice as many capillary-like structures on a gelled basement membrane in comparison to control cells that produce higher levels of thrombospondin-1 [4]. *In vivo*, increased expression of thrombospondin-1 by tumor cells results in decreased vascularization and an accompanied reduction in tumor progression by a number of different types of tumor-derived cells [5].

One mechanism by which thrombospondin-1 might influence angiogenesis is by promoting cell death in microvascular endothelial cells [6]. CD-36, a thrombospondin-1 cell surface receptor, has been shown to be required for caspase-3 mediated induction of apoptosis by thrombospondin-1. Anti-migratory effects elicited by CD-36 interaction with thrombospondin-1 in endothelial cells have also been demonstrated. Recently, CD36 complex formation with β1 integrins has suggested these cell surface receptors collaborate in thrombospondin-1 (and -2) cell signaling [7]. Another thrombospondin receptor, CD47, has been implicated in cardiovascular function as a potent regulator of nitric oxide (NO) [8]. Engagement of CD47 by thrombospondin-1 inhibits NO production. As NO is known to be significantly decreased in cardiovascular tissues in aging, the age-dependent increase in thrombospondin-1 has been implicated as mechanism that might account for deficient NO signaling in chronic diseases of aging. As the first natural inhibitor of angiogenesis, characterization of the mechanisms by which thrombospondin-1 functions has provided valuable insight into strategies to control blood vessel formation.

Thrombospondin-1, like many of the matricellular proteins, influences cytokine activity. For example, thrombospondin-1 has been shown to interact specifically with transforming growth factor (TGF) β [9]. This interaction leads to activation of the latent form of TGFβ, presumably

through a conformational change in the cytokine which allows interaction with cell surface receptors [9]. Consequently, the presence of thrombospondin-1 in the extracellular milieu might affect the activity of this potent, multi-functional cytokine. Generation of a thrombospondin-1 null mouse lends support to the importance of thrombospondin-1-mediated activation of TGFβ *in vivo*. The phenotype manifest in the absence of thrombospondin-1 expression mimics to some degree the phenotype of the TGFβ-null mouse [10]. Specifically, similar pathologies of the lung and pancreas are observed in both mice and, importantly, thrombospondin-1-null mice treated with thrombospondin-1 peptides show a partial restoration of active TGFβ levels and a reversion of the lung and pancreatic abnormalities toward tissues of wild-type mice. Importantly, thrombospondin-1 is a potent chemoattractant for inflammatory cells. Therefore, a reduction in active TGF-β levels in thrombospondin-1 null mice might be influenced by inflammatory cell recruitment.

The function of thrombospondin-1 in the activation of TGF-β appears to be of greater significance in some tissues in response to injury in adulthood rather than in development. For example, in the heart, thrombospondin-1 null mice have an altered response to both pressure overload (PO) induced hypertrophy and to myocardial infarction in comparison to the response in wild-type animals although uninjured adult hearts appear grossly normal. In hearts with PO, an increase in the number of myofibroblasts is found in thrombopsondin-1 null versus wild-type hearts in the absence of increases in fibrillar collagen [11]. As myofibroblasts are frequently associated with sites of robust collagen production, the lack of increased collagen suggests myofibroblast differentiation is impaired in the absence of thrombopsondin-1. In fact, thrombopsondin-1-null myofibroblasts from PO hearts are smaller and produce less collagen type I than those from wild-type counterparts. TGF-β is known to induce myofibroblast differentiation, hence the authors of this study conclude that inefficient TGF-β activity in the absence of thrombopsondin-1 likely influences myofibroblast phenotype in PO hearts.

Thrombospondin-1 influences cell adhesion and cell shape. For example, it will diminish the number of focal adhesions of bovine aortic endothelial cells and thus will promote a migratory phenotype [12]. Thrombospondin-1, therefore, is proposed to modulate cell:matrix interaction to allow for cell migration when necessary. An intermediate-stage of adhesion describes cell responses to counter-adhesive matricellular proteins and, as such, is predicted to promote cell motility [12]. Accordingly, thrombospondin-1 expression is observed during events such as dermal wound repair, when cell movement is required. Thrombospondin-1 is also expressed by many tumor cells and might facilitate metastatic migration [7].

THROMBOSPONDIN-2

Similar to thrombospondin-1, thrombospondin-2 inhibits angiogenesis *in vivo*. Recombinant thrombospondin-2 contained in pellets implanted in the rat cornea inhibited basic-fibroblast growth factor (bFGF)-induced vessel invasion to nearly the same degree as thrombospondin-1 [13]. Thus, the observation that the thrombospondin-2 null mouse appears to have a greater degree of vascularization in the skin is in accordance with the hypothesis that thrombospondin-2 might serve as an endogenous regulator of angiogenesis in mice [14]. In fact, the expression pattern of thrombospondin-2 is more consistent with the importance of this protein in vascular formation, as the mRNA for thrombospondin-2 is more closely associated with the vasculature in developing tissues, in comparison to that of thrombospondin-1 [15].

How might thrombospondin-2 mediate the inhibition of angiogenesis? Abrogation of thrombospondin-2 expression results in increased amounts of matrix metalloproteinase (MMP)-2 activity [16]. Thrombospondin-2 binds to MMP-2 and facilitates uptake by scavenger receptors on cell surfaces. Hence without thrombospondin-2, MMP-2 levels accumulate

in the extracellular milieu and result in significant decreases in cell adhesion. The increase in MMP-2 levels is correlated with decreased amounts of tissue transglutaminase on thrombospondin-2-null cell surfaces [17]. As transglutaminase is known to enhance integrin-mediated cell attachment and serve as a substrate of MMP-2, the increase in MMP-2 activity is proposed to elicit decreased cell adhesion in the absence of thrombospondin-2 by diminishing amounts of transglutaminase on cell surfaces. Hence, blood vessel formation appears to be sensitive to levels of MMP-2 in tissues. Likewise, tissue transglutaminase on cell surfaces might also contribute to angiogenic events.

Another observation from the thrombospondin-2 null mouse is that of altered collagen fibrillogenesis in the skin, relative to that seen in wild-type mice. The collagen fibrils in the null mice are larger in diameter, have aberrant contours, and are disordered [14]. Presumably this effect on collagen fibril formation contributes to lower tensile strength of the skin in thrombospondin-2-null mice compared to their wild-type counterparts. An intriguing relationship between aberrant collagen fibril morphology and decreased transglutaminase activity is suggested by the thrombospondin-2 null phenotype. Recent evidence also suggests that thrombospondin-2 is a pro-fibrotic molecule. In the absence of thrombospondin-2, less collagen deposition is found in a murine cardiac graft model [18], whereas increased thrombospondin-2 levels in scleroderma fibroblasts contribute to increased collagen production through upregulation of miR 7 [19].

Thrombospondins-1 and -2 can act as negative regulators of cell growth. In particular, endothelial cells are susceptible to an inhibition of proliferation by both proteins, resulting in their classification as inhibitors of angiogenesis. However, the variety of cell surface receptors for thrombospondins allows for diverse signaling events in different cell types; consequently, there might be situations when thrombospondin appears to support angiogenesis as well [20]. Thrombospondin-1 has been shown to modulate the activity of at least two cytokines, TGF-β and hepatocyte growth factor (HGF): diminished activity, in the case of HGF, or enhanced activity, as seen for TGF-β. Finally, thrombospondin-1 can alter cell shape to promote a migratory phenotype but can also inhibit migration. Such conflicting conclusions illustrate the importance of contextual presentation of matricellular proteins in various assays. Further characterization of the thrombospondins, including closer examination of the remaining family members (thrombospondins-3–5), will no doubt yield fascinating insight into this multi-functional gene family.

TENASCIN-C

Tenascin-C is a matricellular protein with a widespread pattern of developmental expression, in comparison to a restricted pattern in adult tissues. In addition to tenascin-C, three other, less-characterized forms of tenascin have been identified: tenascin-R, tenascin-X, and tenascin W [21]. This review will focus on tenascin-C, as the best characterized of the tenascin gene family. Tenascin-C consists of six subunits (or arms) linked by disulfide bonds to form a 2,000 kDa molecule that can associate with fibronectin in the ECM [22]. Like thrombospondin-1, a number of different functions have been attributed to tenascin-C and, accordingly, a number of cell surface receptors appear to mediate distinct properties of this matricellular protein. Cell surface receptors for tenascin-C include annexin II and at least five different integrins receptors, of which $\alpha_9\beta_1$ demonstrates the highest affinity for tenascin-C [23]. Whereas the integrins appear to support cell adhesion to tenascin-C, annexin II is thought to mediate the counter-adhesive function attributed to this protein. Hence, tenascin-C can act as either an adhesive or as a counter-adhesive substrate for different cell types, dependent upon the profile of receptors expressed on the cell surface.

Tenascin-C has also been shown to modulate the activity of growth factors: specifically, it promotes epidermal growth factor (EGF)-dependent and bFGF-dependent cell growth [24,25]. In fact, Jones et al. (1997) have shown that smooth muscle cells plated in a collagen gel secrete

MMPs that degrade the collagen to expose integrin receptor binding sites. Engagement of these receptors induces tenascin-C expression; tenascin-C is subsequently deposited into the extracellular matrix and can itself serve as an integrin ligand. The deposition of tenascin-C leads to cell shape changes initiated by a redistribution of focal adhesion complexes concomitant with a clustering of EGF receptors on the cell surface. Presumably, clustering of the EGF receptors facilitates EGF signaling and thereby enhances the mitogenic effect of EGF. Conversely, when MMP activity is inhibited, tenascin-C expression is decreased and the cells become apoptotic [25]. Thus, tenascin-C is able to modulate EGF activity such that the presence of this matricellular protein supports cell growth and its absence induces programmed cell death.

Similarly, tenascin-C supports tumor metastatic colonization by breast cancer cells through suppression of cell apoptosis [26]. Tenascin-C expression by stromal cells as well as by metastasis-initiating breast cancer cells appears to protect tumor-derived cells from apoptotic stresses and thereby faciliates establishment of metastatic colonization in some tissues [27,28]. A function of tenascin-C in hematopoiesis following myeloablation in adult mice is also reported. Tenascin-C expression in the bone marrow niche microenvironment is required for regeneration of hematopoiesis after ablation but is not required to maintain steady-state conditions [29].

Given the widespread expression of tenascin-C in the developing embryo, the lack of an overt phenotype in the tenascin-C-null mouse is surprising [30]. In particular, the high level of tenascin-C expression in the central and peripheral nervous system had indicated that the absence of this protein might lead to neuronal abnormalities. Although no histological differences could be detected in the brains of adult tenascin-C-null mice, they displayed behavioral aberrations including reduced anxiety and enhanced novelty-induced activity [31,32]. In addition, altered numbers of embryonic central nervous system stem cells are noted in the absence of tenascin-C expression, an observation confirming that the composition of the extracellular matrix is an important factor in cell differentiation [33].

The genetic background of the tenascin-C-null mouse is likely to be a major factor in the identification of tissues in which tenascin-C might be functionally important. For example, Nakao et al. (1998) use three different congenic mouse lines to study the effect of Habu-snake venom-induced glomerulonephritis in a tenascin-C-null background. Although the disease is worse in all tenascin-C-null mice in comparison to wild-type controls, each line exhibits a different level of severity. Induction of the disease in one strain, GRS/A, results in death from irreversible renal failure [34]. Moreover, mesangial cells cultured from tenascin-C-null animals do not respond to cytokines such as platelet-derived growth factor (PDGF) unless exogenous tenascin-C is included in the culture medium. Hence, tenascin-C can also modulate the activity of this growth factor as observed previously for EGF. Tenascin-C provides another example of a matricellular protein able to affect growth factor efficacy.

Although tenascin-C shows a limited pattern of expression in the adult, an induction of tenascin-C is seen in many tissues undergoing wound repair or neoplasia [35]. Thus tenascin-C, like the other matricellular proteins, is ideally suited to act as a modulator of cell shape, migration, and growth. One mechanism by which tenascin-C influences cell behavior is through the modulation of fibronectin interaction with cells. Tenascin-C decreases cell adhesion to fibronectin through competition with a heparan sulfate proteoglycan, syndecan 4 [36]. Syndecan 4 is required for efficient cell attachment to fibronectin and for tenascin-C inhibition of adhesion to fibronectin. Hence, a scenario in which tenascin-C competes for syndecan 4 binding to fibronectin is consistent with these results. Certain proteolytic fragments of tenascin-C interfere with fibronectin assembly although full-length tenascin-C does not demonstrate inhibition of fibronectin fibrillogenesis [37]. Given the high levels of proteolytic activity in wounds, for example, cleavage of tenascin-C might be one mechanism by which regulation of fibronectin assembly is achieved.

The paucity of developmental abnormalities manifested in the tenascin-C-null mouse points to the greater importance of tenascin-C in remodeling events which take place in response to injury or transformation. For example, tenascin-C has been shown to regulate cardiac neovascularization by bone-marrow-derived endothelial progenitor cells in response to angiogenic stimuli in adult mice [38]. In addition, as mentioned, high levels of tenascin-C expression are frequently associated with several types of malignancies including tumors of the brain and breast [21].

OSTEOPONTIN

As the name implies, osteopontin was originally classified as a bone protein. A more thorough examination, however, reveals a widespread expression pattern for this protein with multiple potential functions [39]. Osteopontin associates with the extracellular matrix, as it binds to fibronectin and to collagens I, II, III, IV, and V. Osteopontin also affects cellular signaling pathways by virtue of its capacity to act as a ligand for multiple integrin receptors as well as Cluster Designation (CD)-44 [39]. Thus osteopontin, like most of the matricellular proteins, is able to act as a bridge between the extracellular matrix and the cell surface. Since matricellular proteins might be synthesized, secreted, and incorporated into the extracellular matrix with greater ease than more complex secreted proteins that must be incorporated into fibrils and assembled into a network, a bridging function might be useful during remodeling events in the organism when rapid conversion of the cellular substrata is required for cell movement.

In support of this concept, Weintraub et al. (1996) report that transfection of vascular smooth muscle cells with anti-osteopontin cDNA reduces adhesion, spreading, and invasion of three-dimensional collagen matrices. Addition of osteopontin to the collagen gel restores the capacity of these cells to invade the gel [40]. Osteopontin also appears to be susceptible to modification by extracellular proteases which have revealed cryptic integrin binding sites within the sequence. The protease thrombin cleaves osteopontin at the N-terminal domain and exposes binding sites for integrin $\alpha 9\beta 1$ and $\alpha 4\beta 1$ [41]. In addition, Senger et al. (1996) report that endothelial cells treated with vascular endothelial growth factor (VEGF) increase their expression of the integrin $\alpha v\beta 3$, another osteopontin cell surface receptor, concomitantly with an increase in osteopontin. These investigators also show an increase in the amount of thrombin-processed osteopontin in tissues injected with VEGF and radiolabeled osteopontin. The significance of this result lies in the enhanced support of endothelial cell migration *in vitro* by thrombin-cleaved versus full-length protein. Since the migratory activity of the endothelial cells is blocked partially by an anti-$\alpha v\beta 3$ antibody, the remainder of the activity might be attributed to $\beta 1$-integrins or to other cell surface receptors [42].

In pulmonary hypertension, adventitial fibroblasts from pulmonary arteries demonstrate an activated phenotype characterized by high rates of proliferation and migratory potential. High levels of osteopontin expression are correlated with this activated phenotype [43]. Furthermore, silencing of osteopontin expression significantly reduces proliferation and migration in hypertensive fibroblasts whereas increased osteopontin activity stimulated proliferation and invasion by normal fibroblasts [43]. Thus osteopontin is predicted to be a primary determinant of the activated phenotype characteristic of adventitial fibroblasts in hypertensive lungs. Increased levels of osteopontin secreted by senescent fibroblasts also promote migration of mammary epithelial cells, a process linked to neoplastic progression and tumor development in aging tissues [44].

The capacity of osteopontin to influence cell migration might be linked to matrix metalloproteinase activity. Osteopontin is a member of the recently classified SIBLING (Small Integrin Binding LIgand N-linked Glycoprotein) family of proteins that have been shown to bind to and regulate the activity of MMPs [45]. Osteopontin binds to recombinant proMMP-3

and active MMP-3. In addition, a decrease in MMP-9 activity has been reported in osteopontin-null myofibroblasts and vascular smooth muscle cells [46].

The promotion of cell survival is another property ascribed to osteopontin. Denhardt and Noda (1998) have reported that human umbilical vein endothelial cells plated in the absence of growth factors will undergo apoptosis. If these cells are plated on an osteopontin substrate, however, apoptosis is inhibited [47]. Furthermore, rat aortic endothelial cells subjected to serum withdrawal undergo programmed cell death, a response inhibited by an osteopontin substratum. In fact, it is the ligation of integrin αvβ3 by osteopontin at the cell surface that induces nuclear factor-kappa B (NF-κB) a transcription factor that controls a variety of genes through direct binding to their promoters. Thus osteopontin and other αvβ3 ligands can protect cells from apoptosis [48].

Osteopontin is involved in inflammatory responses. Expression of osteopontin is found to increase during intradermal macrophage infiltration, and purified osteopontin injected into the rat dermis leads to an increase in the number of macrophages at the site of administration. Importantly, anti-osteopontin antibodies inhibit macrophage accumulation in a rat intradermal model after a potent macrophage chemotactic peptide is used to induce an inflammatory response [49]. Osteopontin expression is required for development of an effective T_H1 immune response [50]. Interestingly, a recently described intracellular form of osteopontin has been implicated as the mediator of interferon-α production in plasmacytoid dendritic cells [51].

The phenotype of the osteopontin-null mouse supports the hypothesis that osteopontin affects macrophage activity. Although the number of macrophages does not appear to differ significantly in incisional wounds of wild-type and osteopontin-null mice, the amount of cell debris was higher in wounds of the latter animals. Because macrophages are thought to be primary mediators of wound debridement, osteopontin could be important in the regulation of macrophage function [52]. Collagen fibril formation in the deeper dermal layers of the wounded osteopontin-null mice also appear to be affected. Osteopontin-null mice have smaller collagen fibrils compared to wild-type controls. Similar to thrombospondin-2, osteopontin might affect collagen fibrillogenesis especially at wound sites, as no differences are seen in the size of collagen fibrils in unwounded skin [52].

The pro-inflammatory and profibrotic properties of osteopontin suggest that osteopontin might contribute to human diseases in which inflammation and fibrosis contribute to pathological processes such as in systemic sclerosis (SSc). In fact, high levels of circulating osteopontin are present in patients with SSc. Osteopontin-null mice, when challenged with bleomycin to induce dermal fibrosis, exhibit significantly reduced levels of collagenous extracellular matrix and fewer macrophages [53]. Similarly, in a renal model of fibrosis, significantly less interstitial fibrosis and inflammatory infiltration occur in osteopontin-null mice in comparison with wild-type animals [54].

An additional function of osteopontin as a negative regulator of calcification is supported by a number of different studies [55]. Osteopontin inhibits apatite crystal formation *in vitro* and facilitates resorption of cellular minerals. As shown in an *in vivo* model of ectopic calcification, the capacity of osteopontin to abrogate calicification is dependent upon phosphorylation of osteopontin and the presence of the integrin-binding RGD sequence in osteopontin [56].

Osteopontin and its repertoire of cell surface receptors represent components of a pathway used by cells in need of rapid movement or migration. In addition, the ligation of osteopontin by certain cell surface receptors supports cell survival and thus provides a mechanism for a given tissue to protect a subset of cells, expressing the appropriate receptor, from apoptosis. Hence, osteopontin is a useful protein in events which require cell movement and cell survival, such as wound healing and angiogenesis, and has the potential to contribute to cancer progression.

SPARC

SPARC (secreted protein acidic and rich in cysteine; BM-40; osteonectin) was first identified as a primary component of bone but has since been shown to have a wider distribution [57]. Increased expression of SPARC is observed in many tissues undergoing different types of remodeling. For example, SPARC is found in the gut epithelium, which normally exhibits rapid turnover, and in healing wounds. An increase in SPARC is observed during glomerulonephritis, liver fibrosis, and in association with many different tumors. Like the other matricellular proteins, SPARC interacts with the extracellular matrix by binding to collagens I, III, IV, and V [57].

SPARC has also been shown to bind to a variety of growth factors present in the extracellular space [58]. For example, SPARC binds to PDGF-AB and BB and prevents their interaction with PDGF cell surface receptors. PDGF-stimulated mitogenesis is inhibited by the addition of SPARC [59]. SPARC can also prevent VEGF-induced endothelial cell growth, as it binds directly to the growth factor and thereby prevents VEGF receptor stimulation of the mitogen-activated protein kinases, Erk-1 and Erk-2 [60]. *In vivo*, regulation of VEGF activity by SPARC via VEGF receptor 1 occurs during choroidal neovascularization after injury [61].

Interestingly, a variety of mitogenic stimulators are inhibited by SPARC in culture, some of which do not necessarily associate physically with this protein. For example, SPARC is thought not to bind to bFGF, but SPARC inhibits bFGF-stimulated endothelial cell cycle progression [58]. Apparently, the effect of SPARC on cell proliferation is complex and could occur through; i) a direct prevention of receptor activation, and/or; ii) a pathway mediated by a cell surface receptor recognizing SPARC. To date, cell surface receptors for SPARC include stabilin-1 on macrophages and VCAM-1 on endothelial cells [62,63]. SPARC also affects integrin engagement on a number of different cell types. Furthermore, SPARC interaction with integrin heterodimers appears to be cell-type dependent [58].

Another significant effect of SPARC on cells in culture is its capacity to elicit changes in cell shape. Many cell types plated on various substrata retract their filopodia and lamellopodia and assume a rounded phenotype after exposure to SPARC. Bovine aortic endothelial cells, for example, are prevented from spreading in the presence of SPARC [58]. Clearly cell rounding could contribute to the inhibition of cell cycle mentioned previously; however, these two effects of SPARC appear to be independent. Motamed and Sage (1998) have shown that inhibition of tyrosine kinases reverses the counter-adhesive function of SPARC but has no effect on cell cycle inhibition [64]. Thus, at least in endothelial cells, SPARC appears to mediate two aspects of cell behavior through different mechanisms.

The targeted inactivation of the SPARC gene in mice has provided insight into SPARC activity in tissues. In support of SPARC as a regulator of cell proliferation, primary mesenchymal cells isolated from SPARC-null mice proliferate faster than their wild-type counterparts [65]. The majority of SPARC-null phenotypic abnormalities, however, are seen as aberrant extracellular matrix assembly. Early onset cataractogenesis reported in two independently-generated SPARC-null mice appears to be caused by inappropriate basement membrane synthesis by lens epithelial cells [66]. In the absence of SPARC, collagen IV, a SPARC ligand, is not localized to the outer border of the lens capsule, in contrast to its distribution in wild-type capsules [67]. In skin, collagen fibrils are small and uniform in diameter in comparison to those of wild-type dermis. In fact, fibrosis in SPARC-null mice in response to a variety of stimuli is decreased in several tissues including, lung, kidney, and skin [66]. Hence a growing body of evidence supports a function of SPARC in assembly and stability of fibrillar collagen and basement membranes.

Two studies have examined SPARC interaction with collagen I to define the SPARC-binding site(s) on fibrillar collagen heterotrimers [68,69]. Interestingly, SPARC shares the identical fibrillar collagen-binding site with that of Discoidin domain receptor (DDR) 2 and von

Willebrand factor. Down-stream signaling pathways activated by DDR2 engagement of collagen include increased cell proliferation and enhanced cell migration. Thus, engagement of collagen I by SPARC is predicted to prevent collagen engagement by DDR2 (and by von Willebrand factor) and perhaps diminish DDR2-dependent cell activities, such as cell proliferation, in certain cells and tissues.

In addition to its effect on cell cycle, recent evidence shows that SPARC influences TGF-β-dependent pathways in some cell types. Mesangial cells isolated from SPARC-null mice show a decrease in collagen I expression accompanied by a decrease in the levels of the cytokine TGFβ-1, in comparison to wild-type cells [70]. TGFβ is a known positive regulator of extracellular matrix synthesis. Addition of recombinant SPARC to SPARC-null mesangial cultures restores the levels of collagen I and TGFβ nearly to those of wild-type cells [70]. SPARC also promotes the TGFβ signal transduction pathway in epithelial cells [71]. In pericytes, the absence of SPARC increased TGF-β-mediated inhibition of migration, an effect dependent upon the TGF-β receptor endoglin and integrin α_v [72]. Thus it appears that SPARC can act as a regulator of TGF-β activity and, perhaps by extension, influences collagen I production.

Once again, we see SPARC as an example of a multi-functional protein able to regulate cell shape and modulate growth factor activity. In addition, SPARC appears to be a key factor in extracellular matrix assembly in both basal lamina and interstitium. Consistent with these functions, a homolog of SPARC termed SPARC-like protein 1 (hevin) was recently shown to be counter-adhesive and modulatory of extracellular matrix structure [57].

CONCLUSIONS

In addition to the proteins described here, others are also potential candidates for inclusion in the family of matricellular proteins. These include small proteoglycans such as the matrix-associated protein, decorin, which has been shown to be an endogenous regulator of TGFβ activity, as well as certain members of the recently classified CCN gene family [73]. The CCN proteins are secreted, modular, and exhibit functions based, at least in part, on integrin-mediated mechanisms. Whereas CCN1 (CYR 61) enhances apoptosis in fibroblasts, endothelial cell adhesion to CCN1 promotes cell survival [73]. Periostin and other SPARC family members are also recognized as matricellular proteins [57,74]. Further analysis of these proteins and their actions will expand our comprehension of matricellular proteins and the functions they serve in regulating cell interaction with components of their immediate environment.

In any cellular environment, numerous extracellular signals are in place to control cell behavior. In adult tissues, injury and disease both lead to a wide-scale release of multiple factors, either secreted from cells or sequestered in the extracellular matrix, that are capable of eliciting potent cellular responses. Appropriately, an increase in the expression of many matricellular proteins is associated with pathological events. Hence, the matricellular proteins appear to be ideally suited to act as modulators of these extracellular signals. They are able to serve as bridges between the extracellular matrix and cell surface receptors, such that cell shape changes or cell movements can be initiated prior to matrix breakdown or synthesis. At least three matricellular proteins, thrombospondin-2, osteopontin, and SPARC, appear to participate in matrix synthesis, either through the promotion of collagen fibrillogenesis or the enhancement of collagen production. Matricellular proteins can either inhibit or potentiate specific growth factor signal transduction pathways. Thus, different growth factor effects may be amplified or subdued by the presence or absence of these proteins. The fact that multiple receptors exist for most of the matricellular proteins allows for diverse functional consequences for different cell types in a complex tissue in response to a matricellular ligand. A given repertoire of cell surface receptors can stimulate (or inhibit) a particular pathway in a cell-type dependent manner. Apparently, evolution has fine-tuned these proteins to serve as specialized mediators of extracellular signals that provide a coordinated, efficient resolution of tissue injury.

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CHAPTER 16

Growth Factors

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INTRODUCTION

Tissue remodeling is an essential process in the maintenance and survival of all organisms throughout life. Tissue remodeling occurs throughout the entire span of injury, repair and wound healing. Early studies clarified the sequence and time course of appearance of different cell types and their changing phenotypes in healing wounds [1–3]. However, the mechanisms that regulate the tissue remodeling that constitutes the wound healing process remain incompletely understood. An essential component of the regulatory processes that drive tissue remodeling includes the different growth factors and the cytokines that dictate cell type and tissue specificity of responses that drive tissue remodeling. The regulation of their gene expression is the critical component that establishes the timing and sequence of appearance of the tissue remodeling that leads to the healed wound. Coordination of these properties effectively defines the wound healing process.

A major step forward came from experiments in which different growth factors/cytokines were applied directly to experimental wounds. These experiments defined in greater detail the process of tissue remodeling *in vivo*. They established that specific factors not only accelerated wound healing, but appeared to faithfully recapitulate the normal wound healing process [2–22].

These studies later proved valuable to better understand the tissue-remodeling characteristic of different malignancies. The different growth factors/cytokines proved to be oncogenes when inappropriately expressed in different cell types. Constitutively expressed growth factors/cytokines were found to be pathogenic in different models of malignant neoplasms both *in vivo* and *in vitro*. In these models, the phenotypes of the experimental tumors were profoundly influenced by the constitutively expressed growth factor/cytokine that functioned to transform the cells used in these different cancer models. These factors also are likely to be major contributors in the remodeling characteristic of atherosclerosis and other inflammatory states. In all these instances, the deregulation of growth factors and other cytokines may have serious consequences for the host; tight regulation of their activities is needed for normal function.

WOUND HEALING

Normal wound healing proceeds in a clear sequence through different overlapping stages, ending with the healed wound. Common features of all wounds are clumps of activated platelets that are seen at sites of injury where they release granule products. Also products of the coagulation process are deposited locally. The sequential migration of neutrophils begins immediately, and later in time, monocytes, and fibroblasts follow. Wound macrophages (derived from circulating monocytes) and fibroblasts in turn become activated, initiating a

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cascade of new gene expression leading to the *de novo* synthesis of growth factors and other cytokines, synthesis of extracellular matrix proteins (including collagens and elastin), and proliferation of fibroblasts. Later, tissue remodeling results in active collagen turnover and cross-linking that lasts from two weeks to one year post-wounding.

Factors that arise locally were thought to account for the cell migration and activation of fibroblasts in wounds [23–25]. However, circulating platelets are invariably associated with wounds and are now known to release factors into wounds originally stored within intracellular compartments such as the platelet granules. These factors attract neutrophils, monocytes, fibroblasts, and other (tissue-specific) cells, such as the smooth muscle cells. Platelets thus are important mediators in the initiation processes of inflammation and subsequent tissue remodeling leading to repair. Platelet release is initiated by products of coagulation, such as thrombin, and by platelet exposure to foreign surfaces, such as collagen. Platelet release occurs within seconds of platelet activation. Synthesis of other potent molecules, such as the prostaglandins and leukotrienes, also influence intracellular responses and extracellular activities which contribute significantly to early events within wounds. These factors also initiate the transcriptional activation of genes that encode proteins with other signaling roles, serving to attract inflammatory cells locally. Because inflammatory cells are in close proximity to platelets in a number of models of inflammation (immune complex disease and atherosclerosis), it is likely that platelets are important in the earliest stages of different inflammatory states. Platelets and inflammatory cells appear to 'talk' to each other in essential ways, by releasing cytokines and growth factors to attract additional inflammatory cells, and to stimulate the transcription of factors that regulate or encode additional mediators of inflammation and wound repair. Studies with platelet-derived growth factor (PDGF) in the *in vivo* contexts noted above support these functional roles for PDGF and other platelet secretary proteins released at the time of platelet activation.

Platelet-derived growth factor (PDGF)

PDGF was initially described as a potent mitogen and many of its critically important properties have been identified [26–35]. However, PDGF directs cell migration, activates diverse cell types, and upregulates expression of genes unique to different cell types that otherwise are quiescent and not normally expressed at significant levels. Gene induction in response to PDGF establishes gene activation pathways that provide the temporal sequence of expression of genes in diverse pathways of central importance in tissue remodeling and repair. Experiments alluded to above, in which PDGF was directly applied to wounds, demonstrated that PDGF accelerates the normal healing process; it does not distort it, indicating PDGF together with its downstream signaling molecules upregulates the genetic programmes needed for wound healing in a precise order. However, there was little if any information to establish the relative contributions of PDGF and the different growth factors to the healing of normal wounds, and thus experimental strategies continue to focus on *in vitro* properties of growth factors. Additional models and reagents are needed to directly test these results *in vivo*.

PDGF was originally identified as a potent mitogen in serum for fibroblasts [26,27]. Purified human PDGF is a heterodimeric molecule consisting of two separate but highly related polypeptide chains (A and B chains) [12]. The B chain of PDGF is ~92% identical with the protein product of the v-sis gene, the oncogene of simian sarcoma virus, an acute transforming retrovirus, and thus it is a proto-oncogene [32,33]. The study that showed the relationship of the v-sis gene and the B-chin of PDGF was the first to demonstrate the function of a proto-oncogene. It also was the first 'Eureka' discovery that came from comparative sequence coning.

Both BB and AA homodimers of PDGF also have been isolated from natural sources. They are expressed in many cell types of diverse origins, including macrophages, endothelial cells, both smooth and skeletal muscle cells, fibroblasts, glial cells and neurons. These cells secrete molecules with PDGF-like activity. The detection of these molecules has been associated with

the expression of the mRNAs for A and B chains that can be specifically associated with chain isoforms of PDGF. However, identification of the specific isoform(s) at the level of the protein has been difficult because reagents to detect each isoform specifically were not available in early studies. Multiple PDGF-AA transcripts have been detected in cells that are alternative splice variants of the single seven-exon gene [36,37]. Three splice variants result in short and long processed proteins, 110 and 125 amino acids [36–38] in length. These different length PTGF A chain products are important but have not yet been fully worked out. There is differential regulation of the transcripts. They bind with different affinities to the extracellular matrix [17,39]. The transcripts for both proteins are widely expressed in multiple cell types.

Each of the three isoforms (BB, AB, and AA) binds to the PDGF-α receptor, whereas only the BB isoform binds with significant affinity to the type of PDGF receptor-β [40–42]. Both receptor types are highly homologous trans-membrane tyrosine kinases and are expressed in a cell type-specific manner, not only governing cell target specificity of the PDGF response [43], but also adding both complexity and thus diversity to the potential roles of PDGF in inflammation and wound healing. The functions of PDGF *in vivo* thus are regulated by the need for specific ligand-receptor interactions to transduce the signals required for various functions of PDGF and the upregulation of other genes that propagate the cascade of events initiated by PDGF.

Growth factors and cytokines as early mediators of the inflammatory process

To better understand the roles of growth factors and other cytokine mediators of inflammation, tissue remodeling, and wound healing, experimental strategies were designed to first isolate and characterize these factors and to analyze their properties *in vitro*. Other studies determined the sites and levels of expression of these factors *in vivo*. Reagents were then developed to test the properties of these factors in different experimental models to analyze normal functions of growth factors and other cytokines in the intact animal.

Early work focused on the roles of PDGF as a mitogen. However, in addition to the striking potency of PDGF as a mitogen, PDGF also is strongly chemotactic for human monocytes and neutrophils, a property of PDGF that may be more important in early phases of inflammation. Optimal concentrations of PDGF that are required for maximum chemotactic and mitogenic activity are 20 and 1 ng/ml, respectively [44], concentrations of PDGF that are well below those measured in human serum (~50 ng/ml) [44]. Because platelets invariably aggregate and release platelet granule products at wound sites, the concentrations of PDGF at these sites may be substantially higher than those in serum. PDGF also is a chemoattractant for fibroblasts and smooth muscle cells but requires a somewhat higher concentration [45,46], suggesting PDGF also functions in later phases of wound healing through its influence on fibroblast and smooth muscle cell movement [46].

In contrast to the PDGF AB isoform, the chemotactic potential of PDGF AA has been controversial. This controversy was important to resolve because the A chain homodimers of PDGF (PDGF AA) are widely expressed in normal and transformed cells. However, PDGF AA has been independently established as a strong chemoattractant for human monocytes, granulocytes, and fetal bovine ligament fibroblasts. Relevant to the roles of monocytes/tissue macrophages in the wound healing process, it was shown that highly purified (>98%) monocytes require the addition of lymphocytes or interleukin 1 (IL-l) for chemotactic responsiveness to PDGF AA, but not for the chemotactic activity of formyl-methionyl-leucyl-phenylalanine (fMLP) or C5a. This apparent specificity of monocytes for activation before becoming responsive to PDGF as a chemoattractant indicates the striking complexity of the different cellular responses to cytokines. It also indicates that regulation of the chemotactic response of the monocyte to PDGF AA by the lymphocyte or cytokines may be another regulatory level of importance in the ultimate pathway to tissue remodeling and wound healing *in vivo* [47].

Other platelet secretary products than PDGF also are active in the process of tissue remodeling during wound healing for example, Platelet factor 4 is a potent chemoattractant [48–51].

β-thromboglobulin (TG-β) is a highly active chemotactic factor for fibroblasts [46]. Apart from platelet secretary proteins released immediately at injured sites, different lymphokines, a peptide derived from the fifth component of complement, collagen-derived peptides, fibronectin, tropoelastin, and elastin-derived peptides are wound-associated factors that also are potent chemo attractants. These different chemo attractants are functional with different cell types and thus exhibit different cell type specificity [52–55].

In addition to its roles as a mitogen and in cell migration, PDGF also activates the polymorphonuclear leukocyte [56] and is capable of inducing monocyte activation responses, as evaluated by generation of superoxide anion (O_2) from the membrane-associated oxidase system, release of granule enzymes, and enhanced cell adherence and cell aggregation. Superoxide anion release in monocytes is observed at 10 ng/ml and the levels of PDGF-dependent release are comparable to release induced by 10^{-7} ml/liter fMLP. The potency of PDGF to induce this response in monocytes is of the same magnitude as that observed in polymorphonuclear leukocytes (PMNs). Similarly, lysozyme release and monocyte adherence are increased in a dose-dependent manner in response to PDGF and achieve maximal responses at 40 ng/ml PDGF. The PDGF concentrations required to achieve maximal monocyte aggregation was twofold (60 ng/ml) that found for PMNs and, at this concentration of PDGF, it is likely to occur only in the immediate vicinity of platelet aggregates. PDGF thus induces the full sequence of cell activation events in human monocytes, similar to that in human PMNs. These properties may well be of major significance resulting in the release of enzymes and other factors required to begin the process of tissue breakdown required for remodeling.

Other early cellular effects important to inflammation and repair also may be initiated by the release of PDGF from platelets and cells within the wound. For example, collagenase expression was studied in normal human skin fibroblasts that were cultured for 24 hr in the presence of PDGF. Collagenase release is required for the breakdown of collagen necessary for the later remodeling of collagen and for tissue repair [10,57]. A dose-dependent, saturable increase in collagenase activity in culture media was observed with paralleled increases in immunoreactive collagenase protein, suggesting that the enhanced synthesis of an immunologically unaltered enzyme. The specificity of this effect was demonstrated by collagenase stimulatory effect with that on total protein synthesis and DNA synthesis. Under *in vitro* conditions that produced a 2.5-fold increase in collagenase synthesis, there was a ~20% increase in total protein synthesis and no detectable change in DNA synthesis. In addition, as a second control, platelet factor 4, another platelet-derived protein, caused a <20% increase in collagenase expression. In time course studies, stimulation of collagenase synthesis was first observed 8–10 hr after exposure to the growth factor. Furthermore, when cells were exposed to PDGF for ~24 hr, an increase in the rate of collagenase synthesis were seen for ~6 hr after PDGF was removed, after which the rate reverted to control levels. Growth factors-regulated expression of collagenase may be of unique importance to tissue remodeling, because it occurs somewhat later than the most immediate responses to PDGF at the time collogen degradation may be essential for progression of remodeling and tissue repair [57].

Patients with Werner's syndrome, an autosomal recessive disorder, undergo an accelerated aging process and premature death. Fibroblasts from such patients typically grow poorly in culture but have a markedly attenuated mitogenic response to platelet-derived growth factor and fibroblast growth factor (FGF). In contrast, these cells have a full mitogenic response to fetal bovine serum. The Werner's syndrome cells express high constitutive levels of collagenase *in vitro*. However, induction of collagenase was not seen to occur in the Werner's syndrome fibroblasts in response to PDGF. The failure of one or more PDGF-mediated pathways in Werner's syndrome cells coupled with the phenotype of this disorder provides important support for roles of PDGF and other cytokines in tissue remodeling [58].

Inflammatory response mediators activate transcription of quiescent genes

Another potentially highly important role of PDGF and other growth factors and cytokines in wound healing is the upregulation of early-intermediate response genes in cells activated by PDGF. In response to PDGF and other cytokines, fibroblasts and other cells initiate transcription and expression of genes that are dormant in the absence of stimulation. The products of some of these genes are important in the intracellular propagation of the mitogenic signal but others are important since they propagate intercellular communication, direct cell migration, and activation of cells of different types. The products of these genes [10,58–60] thus serve to coordinate diverse responses designated to combat wound infection and to initiate and propagate the healing process.

The PDGF early/early-intermediate response genes *JE* and *KC* were first isolated by differential colony hybridization [58]. Only later were they identified as cytokines belonging to a newly described superfamily of small inducible genes (SIGs) [61] (now known as the chemokine family). This family includes platelet granule proteins, including the platelet basic protein and platelet factor 4. These PDGF-induced cytokines regulate pathways of gene activation that account in part for cell type specificity and the temporal responses of wound healing. The *JE* gene encodes a basic (PI = 10.4) 148-amino acid polypeptide [60, 61] that shows 82% amino acid identity with the murine *JE* gene [62]. Both rat and murine *JE* gene products contain N-terminal hydrophobic leader sequences, virtually identical alternating hydrophobic and hydrophilic domains, a single N-linked glycosylation site at position 126 that is conserved in both the murine and rat *JE* genes, and identical intron-exon splice junctions.

The importance of the *JE* gene product was suggested when the human homolog of the rodent *JE* gene was identified as monocyte chemotactic protein-l (MCP-1) [63]. MCP-l is identical with monocyte chemotactic and activating factor (MCAF) [64,65], HC11 [66], and with the smooth muscle cell chemotactic factor (SMC-CF) [67]. Both the MCP-l and rodent *JE* genes are induced in fibroblasts by PDGF [also serum, phorbol myristate acetate (PMA), double-stranded RNA, and interleukin-l] and each cross-hybridizes to the same bands on Southern blots [68]. Antibodies raised to the murine *JE* cross-react with MCP-l. MCP-l is a potent factor chemotactic for monocytes but not for neutrophils [69], a specificity of cell type recognition that is not seen with many of the other chemotactic factors that have been characterized [70], suggesting it has a unique role in the inflammatory process. Other cytokines with sharply defined functional activity also have been identified in the chemokine family. MCP-l/SMC-CF or related molecules appear to be the major chemotactic factor(s) released by a number of tumor cell lines as well [67]. This relationship calls to attention the surprising parallels that have been found between tumor cells and cells which are activated such as the fibroblast.

The genes of this family are normally not expressed at detectable levels but can be induced in the presence of the appropriate stimuli. Remarkably, each of the inducers has an important role in cell growth, inflammation, or immune responses, thus, linking downstream effectors as common endpoints of multiple different stimuli. Increasing knowledge of this family of genes and their products has suggested an enlarging role for PDGF and other cytokines in the sequential and cell type-specific development of inflammation and the evolution of the wound healing process [71].

BIOLOGICAL PROPERTIES OF THE SIG (CHEMOKINE) FAMILY MEMBERS

Neutrophil activation protein/IL-8 (NAP-l/IL-8) is a highly cell type-specific activator and chemotactic factor for neutrophils but not for monocytes [73]. NAP-l/IL-8 was identified in conditioned medium of lipopolysaccharide-stimulated monocytes [72,73] and its gene (*3–10C*) was cloned independently as a staphylococcal enterotoxin A-induced gene from

peripheral blood leukocytes [74]. NAP-l is also chemotactic for T lymphocytes and basophils [75,76] and is the N-terminal processed form of endothelial-derived leukocyte adhesion inhibitor (LAI), an inhibitor of neutrophil adhesion that protects endothelial cells from neutrophil-mediated damage [77,71].

MCP-l is chemotactic for monocytes. It was purified from the culture media of human glioma cell line U-105MG [69], THP-l cells [78], phytohemagglutinin-stimulated mononuclear cells [79], and double-stranded RNA [poly(rI).poly(rC)]-stimulated fibroblasts [71,80].

Macrophage inflammatory protein-l (MIP-l) was purified in two forms (MIP-l, MIP-2) from lipopolysaccharide-stimulated RAW264.7 cells. It is chemotactic for polymorphonuclear cells [81]. MIP-l induces an oxidative burst and degranulation in neutrophils and promotes inflammatory reactions. MIP-l is also a prostaglandin-independent pyrogen [82]. MIP-2 is a potent chemotactic factor for neutrophils and causes neutrophil degranulation, but does not induce an oxidative burst [71].

Platelet factor 4 (PF4) is a platelet granule protein that is chemotactic for both neutrophils and monocytes [83]. It has immunoregulatory activity [84] and can inhibit angiogenesis [85]. Platelet basic protein, connective tissue-activating peptide III (CTAP-III), β-thromboglobulin, and neutrophil-activating peptide-2 (NAP-2) are N-terminal proteolytic processed forms of a single gene product. CTAP-III is mitogenic for human dermal fibroblasts [86]. NAP-2 is induced in monocytes when they are exposed to lipopolysaccharides and can stimulate the release of elastase from human neutrophils [71,72].

Melanoma growth stimulatory activity (MGSA) is a mitogen for cultured melanoma and pigmented (nevus) cells [87]. Primary melanoma cells secrete MGSA and, for this reason, it has been suggested to be an autocrine regulator of growth. The protein product of MGSA is identical to the *gro-α* gene isolated by subtractive hybridization of mRNA from transformed hamster CHEF/16 cells. The rat analog, cytokine-induced neutrophil chemoattractant (CINC), is chemotactic for neutrophils [88]. The murine analog (*KC*) is a PDGF-inducible gene [58,71].

Regulation of *JE* gene expression by glucocortocoids

The role of PDGF and other cytokines in the induction of otherwise unexpressed genes has resulted in additional insights into the wound healing process. The addition of dexamethasone and other steroid drugs to PDGF- and serum-stimulated BALB/c 3T3 fibroblasts prevents the PDGF and serum-dependent induction of the *JE* gene in a dose-dependent manner. Furthermore, induction follows the rank order of potency expected for glucocorticoid receptor-mediated anti-inflammatory activity. The inhibition appears to be highly specific for inflammatory mediators, suggesting that the role of PDGF in the induction of selective SIG family members may be a highly important step at which glucocorticoids negatively influence both inflammation and repair [71].

GROWTH FACTORS AND ACCELERATED HEALING

Much new information about the roles of growth factors and other cytokines has resulted from the direct application of PDGF to experimental wounds in animals. Morphometric methods and histologic analysis were used to compare wounds treated with supra-physiological concentrations of growth factors to untreated wounds. This approach, coupled with the *in vitro* approaches cited above, has greatly clarified roles of PDGF-enhanced tissue repair and remodeling. It has stimulated similar experiments with other cytokines as well.

In one set of experiments, exogenous PDGF was applied locally, and once only, to incisional wounds in rats and rabbits. PDGF accelerated tissue repair in a dose-dependent manner [7,16]. A single application of PDGF to rat incisions enhanced the strength required to 'break' the healing wound by 150 to 170% over a 3-week time course [7]. PDGF accelerated healing by

4–6 days over the first 2 weeks and by 5–10 days between 2 and 7 weeks post-wounding [9]. At 89 days post-wounding, both PDGF-treated and control wounds had achieved similar wound strength (90% of unwounded dermis). The PDGF doses therefore induced a long-lasting enhancement in the rate of healing, supporting the view that PDGF and other growth factors initiate events required for normal healing of wounds and also enhance the normal rate of wound healing and tissue remodeling.

In these wounds, PDGF significantly increased the rate of increase of cellularity and granulation tissue formation. The early appearance of wounds treated with PDGF is that of a highly exaggerated inflammatory response. An increased neutrophil influx was seen at 12 hrs, and shortly thereafter a marked increase in influx of macrophages followed. Fibroblast infiltration was found from day 2 onwards, persisting through 21 days post-wounding [7]. Furthermore, enhanced cellular function of fibroblasts could be established in treated wounds. Increased numbers of procollagen-containing fibroblasts were strikingly apparent as early as 2 days post-wounding. At 28 days, it was not possible to distinguish activated from non-activated fibroblasts in treated and untreated wounds. These results indicate that PDGF accelerates the healing response.

Furthermore, within limits of detection, PDGF-stimulated wounds also result in a healed wound that cannot be distinguished from its untreated counterpart [9]. PDGF stimulation of wounds thus is multifunctional in diverse ways, perhaps through the recruitment and activation of macrophages and fibroblasts and through upregulation of expression at different cytokine genes whose functions and cell type specificity provide additional diversity to the normal healing process.

Another polypeptide growth factor that is stored in the granules of platelets, transforming growth factor β (TGF-β), appears also to be very important in tissue repair [89]. Both PDGF and TGF-β increase collagen formation, DNA content, and protein levels in wound chambers implanted in rats [4,90]. TGF-β enhances the reversible formation of granulation tissue when injected subcutaneously into newborn mice [91]. A single application of human TGF-β at the time of wounding advanced by 2–3 days the breaking strength required to rupture the incision margins after when assayed at 1 week. The marked augmentation of wound healing using both human PDGF (hPDGF) and recombinant *c-sis* homodimers (rPDGF-B) revealed a unique pattern of response when compared with the results obtained with TGF-β [7,92].

Circular excision wounds also were placed through the dermis of the rabbit ear to the level of underlying cartilage, a model that is important since it does not allow contraction from the wound margins [16]. In-growth of extracellular matrix and granulation tissue was measured by histo-morphometric techniques [16]. In that model, a single application of PDGF increased granulation tissue by ~200% at 7 days, in association with a predominance of fibroblasts and collagen-containing extracellular matrix [16]. The healed wound again was indistinguishable from the control, non-PDGF-treated, healed wound. Interestingly, PDGF also nearly doubled the rate at which re-epithelialization occurred and neo-vascularization was prominent in granulation tissue. These unexpected findings in view of the known cell type specificity of PDGF suggested upregulation either of PDGF receptors or of other cytokine genes stimulating cell type-specific epithelial and/or endothelial cell functions. Whereas again the mechanisms of the PDGF effect are not established, the influence of a single application of a growth factor remarkably accelerated the inflammatory response and the subsequent tissue remodeling to result in a healed wound of normal appearance.

In this model also, PDGF accelerated deposition of matrix largely composed of glycosaminoglycans and later of collagen [93]. The normal sequence in repair and the acceleration of wound healing are, to all appearances, identical to those in an untreated wound, supporting the view that the role of PDGF is to accelerate normal wound healing in human wounds. It has been demonstrated in human wounds that PDGF AA expression is increased

within healing pressure ulcers. The accumulation of PDGF AA is accompanied by activated fibroblasts, extracellular matrix deposition, and active neovessel formation. However, far less PDGF AA is present in chronic non-healing wounds. Thus, upregulation of PDGF AA may be important in the normal repair process as well [94–96].

PDGF BB, bFGF, and epidermal growth factor (EGF), applied locally at the time of wounding, cause a twofold increase in complete re-epithelialization of treated wounds, whereas TGF-β significantly inhibits re-epithelializarion. Both PDGF BB and TGF-β increased the depth and area of new granulation tissue, the influx of fibroblasts, and the deposition of new matrix into wounds. Explants from treated wounds remained metabolically more active than controls, incorporating 473% more [[3]H]-thymidine into DNA and significantly more [^{3}H]-leucine and [^{3}H]-proline into collagenase-sensitive protein [16]. The different response to growth factors underscores the importance of tissue and cell type specificity of the growth factors to tissue repair, and perhaps also the need for exquisite timing of induction of different factors in the orderly progression of the healing process.

The use of these wound healing models provided the basis to suggest at least one mechanism whereby PDGF may function *in vivo* [10,97]. Wounds in animals that were pretreated with glucocorticoids or total body irradiation to reduce circulating monocytes to non-detectable levels had a sharply reduced influx of wound macrophages. PDGF was tested in these animals. It was shown that the PDGF-induced acceleration of incision repair was abrogated in glucocorticoid-treated animals [10]. However, PDGF attracted a significant influx of fibroblasts into the compromised wounds of animals. The fibroblasts lacked procollagen type I, suggesting the requirement of the wound macrophage to support stimulation of fibroblast procollagen type I synthesis. PDGF alone does not stimulate procollagen type I synthesis when added directly to fibroblasts *in vitro* [98,99]. However, PDGF-activated macrophages synthesize TGF-β [10], a potent activator of the fibroblast procollagen type I gene [165,99]. It is suggested that TGF-β may be a second signal messenger to induce collagen formation in PDGF-treated wounds. This suggestion is supported directly in experiments in which macrophages and fibroblasts in PDGF-treated incision wounds were shown to contain increased levels of intracellular TGF-β [10].

The results with wound healing models and PDGF have illustrated much about inflammation, tissue remodeling, and the process of wound healing. Other *in vivo* models of PDGF-treated wounds also indicate that PDGF stimulates normal and reverses deficient dermal repair [4,6,100], and have suggested roles for this growth factor in inflammation [101], uterine smooth muscle hypertrophy [102], lens growth and transparency [163] and the central nervous system [103]. What may be the most important conclusions are that PDGF has the potential to initiate and accelerate the process, and to sustain it to an end point that is effectively identical to untreated wounds. The most important mechanisms of its activity may be the consequences of its potent chemotactic activity and its ability to induce multiple autocrine loops that lead to an endogenous cascade of new gene activation and cell-specific cytokine synthesis within the healing wound. Clinical trials have now established the efficacy of PDGF in advancing the healing of diabetic ulcers. PDGF is now 'in the clinic'.

ROLE OF BASIC FIBROBLAST GROWTH FACTOR AND ANGIOGENESIS

Normal wound healing can be divided into three phases: inflammation, fibroplasia, and maturation provoked by liberated angiogenic factors. Vessel-dense granulation tissue is central to the process of tissue repair. The formation of new blood vessels provides a route for oxygen and nutrient delivery, as well as a conduit for components of the inflammatory response. Endogenous bFGF, like PDGF, is also found in the wound site and is presumed to be a necessary part of natural wound healing. Mechanically damaged endothelial cells and burned wound

tissue release significant amounts of bFGF into the wound area, suggesting that damaged endothelial cells at the cut edges of blood vessels, as well as other damaged cells at the site of a wound, may provide an early source of bFGF [104–106]. A slightly later and more sustained supply of bFGF at the wound site is delivered by the invading activated macrophages [107–109]. Moreover, recombinant bFGF has been shown to enhance healing, if added exogenously to a wound site [110]. It is now clear that bFGF can act as an angiogenic factor *in vitro* [111] and *in vivo* [112], directing endothelial cell migration and proliferation, and that bFGF is a mitogen to endothelial cells. It also upregulates expression of plasminogen activator and the αvβ3 integrin that promotes both migration of endothelial cells and luminal formation [18,113]. The effects of stimulating endothelial cell growth, capillary differentiation, and connective tissue cell growth by bFGF contribute to wound healing and tissue regeneration [106,166].

Wound healing involves the interactions of many cell types, and is controlled in part by growth factors. Intercellular communication mediated by gap junctions is considered to play an important role in the coordination of cellular metabolism during the growth and development of tissues and organs. Basic FGF, known to be important in wound healing, has been found to increase gap junctional protein annexin 43 expression and intercellular communication in endothelial cells and cardiac [114]. It has been proposed that an increased coupling is necessary for the coordination of these cells in wound healing and angiogenesis, and that one of the actions of bFGF is to modulate intercellular communication. In a variety of animal models, exogenous bFGF has been shown to accelerate wound healing by speeding granulation tissue formation and increasing fibroblast proliferation and collagen accumulation at the site of a subcutaneously implanted sponge in rats [115–116]. Fresh wound tensile strength has also been increased [117]. However, the greatest benefit from the application of exogenous bFGF can probably be obtained in cases in which wound healing is impaired. Research on skin flaps indicates that bFGF has the potential to increase viability by accelerating flap revascularization when administered in a sustained release manner [164]. This may have applications to open or nonunion fractures with impaired wound healing. Despite encouraging experimental results to date coming from studies showing that treatment of genetically diabetic mice with recombinant bFGF not only increased fibroblast and capillary density in the wound, but also accelerated wound closure, clinical application of recombinant human bFGF has fallen short of expectations. In a randomized double blind placebo-controlled study, direct application of bFGF to non-healing ulcers of diabetic patients did not accelerate wound healing [118]. In a subsequent randomized placebo-controlled trial to study the effect of recombinant bovine bFGF in the healing of burned tissues, all patients treated with bFGF had faster granulation tissue formation and epidermal regeneration, compared with those in the placebo group. Superficial and deep second-degree burns treated with bFGF healed in a mean of 9.9 days (SD 2.5) and 17.0 days (SD 4.6), respectively, compared with 12.4 (2.7) and 21.2 (4.9) days. No adverse effects were seen locally or systematically with bFGF. It is indicated that bFGF effectively decreased healing time and improved healing quality. Clinical benefits for use of bFGF in burn wounds would be shorter hospital stays and the patients' skin quickly becoming available for harvesting and grafting [119].

PLEIOTROPHIN REMODELS TUMOR MICROENVIRONMENT AND STIMULATES ANGIOGENESIS

Pleiotrophin (PTN) is a PDGF-inducible, heparin-binding cytokine that functions as a downstream effecter of PDGF. Pleiotrophin was first purified as an 18 kD, heparin-binding, mitogenic polypeptide from bovine uterus [120] and as neurite outgrowth promoting activity from neonatal rat brain [124]. The *Ptn* cDNA encodes a lysine-rich, highly basic protein of 168 amino acids with a 32 amino acid signal peptide that is nearly 50% identical in amino acid sequence with the protein product of *Mk* [121], a gene expressed in early stages of retinoic-acid-induced differentiation in mouse embryonal carcinoma cells [122].

Pleiotrophin and MK are the only known members of this family. Pleiotrophin lacks homology with members of the heparin-binding growth factor (HBGF) family of structurally related polypeptides [123].

The *Ptn* gene is expressed during neuroectodermal and mesodermal development but not in endoderm, ectoderm, or trophoblasts [125]. Expression levels of *Ptn* peak in late embryonic development and the immediate postnatal period and correlate with peaks of growth and differentiation in both neoectoderm and mesoderm [121,124]. In adults, PTN expression is limited to selected cell populations and is constitutive; however, *Ptn* gene expression is upregulated after injury and during repair. In cultured cells, its expression is modulated by growth factors, including PDGF and steroid hormones [126–128].

Pleiotrophin normally signals a diversity of responses that suggest its potential importance in deregulated growth and tumor angiogenesis when it is inappropriately expressed in the cancer cell. PTN has mitogenic activity for fibroblasts [120], for brain capillary endothelial cells [129], and for SW-13 (adrenal carcinoma) cells in soft-agar culture [130]. Pleiotrophin also stimulates angiogenesis *in vivo* and *in vitro* [129]. Furthermore, the *Ptn* gene is a proto-oncogene; *Ptn* transformed NIH 3T3 cells develop into rapidly growing and highly vascular tumors in flanks of nude mice [131,132]. Importantly, it is highly expressed in many human breast cancers [130,133]. Introduction of the exogenous *Ptn* gene into 'premalignant' SW13 (adrenal carcinoma) cells or MCF-7 (human premalignant breast carcinoma) stimulates highly malignant subcutaneous tumor growth at sites of implantation in flanks of nude mice [134] Chang and Deuel Submitted Zuka and Deuel Submitted). The highly malignant phenotype of human malignant breast cancer cells and other cells with constitutive activation of the *Ptn* gene is reversed when a dominant negative inhibitor of PTN signaling or a *Ptn* gene targeted ribozyme is introduced [134–136] (Chang and Deuel), thus establishing that a single gene mutation that stimulates activation of the *Ptn* gene alone is fully sufficient to 'switch' the premalignant phenotype to a highly malignant phenotype. These findings may be highly significant, since many different human malignancies of different origins express *Ptn* at readily detectable levels [135,137–141]. Importantly, in those cell lines studied, expression of the endogenous *Ptn* gene is constitutive and PTN signaling in those instances which have been studied PTN has been found to be a major contributor to be a major contributor to the highly malignant phenotype [135,137–140].

The pleiotrophin receptor is the transmembrane receptor protein tyrosine phosphatase (RPTP) β/ζ [142]. RPTPβ/ζ was the first transmembrane tyrosine phosphatase receptor for which a ligand was discovered. Pleiotrophin inactivates the tyrosine phosphatase activity of RPTPβ/ζ leaving unchecked the persistent activity of a tyrosine kinase(s) that phosphorylate the tyrosine otherwise dephosphorylated by RPTPβ/ζ [142]. Through inactivation of RPTPβ/ζ, the tyrosine phosphorylation levels of the substrates of RPTPβ/ζ are sharply increased. As noted above, pleiotrophin is the first natural ligand to be discovered for any of the RPTPs [142]. Anaplastic Lymphoma Kinase (ALK) also has been proposed as a receptor for PTN [143], but, subsequent studies effectively ruled out ALK as a PTN receptor. Syndecan 3 also has been suggested to function as the PTN receptor. The downstream targets of PTN/RPTPβ/ζ signaling pathway are β-catenin [142], β-adducin [144] and Fyn [145]. β-catenin, β-adducin, and Fyn also are substrates of RPTPβ/ζ; the steady state levels of tyrosine phosphorylation of each of these proteins is sharply increased in PTN-stimulated cells. However, each of these proteins is phosphorylated in non-PTN-stimulated cells as well, suggesting their steady state levels of tyrosine phosphorylation are maintained by the endogenous PTN/RPTPβ/ζ signaling pathway. Each of the downstream targets of PTN signaling are important regulators of cytoskeletal structure [142,144] and destabilization of cytoskeleton and loss of cell-cell adhesion is the immediate response of cells stimulated by PTN [142], suggesting that a major target of exogenous PTN is de-stabilization of cytoskeletal elements and cell-cell adhesion through the coordinated increase in tyrosine phosphorylation of these substrates of RPTPβ/ζ. This finding

is substantiated since pleiotrophin also has been found to induce an epithelial-mesenchymal transition.

Collagens and elastin are the major fibrillar elements in the cardiovascular system and thus are major determinants of vascular tone, distendability, and other properties of aorta and other vessels. Midkine (MK, *Mk*) is a growth factor which shows ~50% amino acid identity to PTN [145]. Analysis of the aorta of *Ptn*−/− and *Mk*−/− mice uncovered the previously unknown but remarkable findings that PTN and MK are major regulators of the transcripts of the catecholamine biosynthesis pathway [146,147] and the transcripts of the renin-angiotensin pathway [146,148] in mouse. Since procollagen and elastin synthesis is known to be regulated through both the catecholamine and the angiotensin II signaling pathways, the striking downregulation of the elastin precursor gene in aorta of *Ptn* −/− and *Mk* −/− mice define PTN and MK as critical regulators of elastin expression. Furthermore, tumors of MMTV-*PyMT* transgenic mice in which expressed very high levels of the MMTV-*Ptn* transgene have strikingly increased synthesis of collagen and elastin interspersed with activated stroma, which is also seen in wound healing process [149]. These data, collectively, suggested that pleiotrophin plays an important role in remodeling the microenvironment of cells both in normal and disordered conditions.

OTHER ROLES OF GROWTH FACTORS AND CYTOKINES

A growing appreciation for roles of growth factors in many normal and abnormal processes is emerging from numerous other investigations of a diverse nature. For example, TGF-α and TGF-β are expressed with high specificity in developing mouse embryo [150,151], PDGF may mediate normal gliogenesis [87]; maternally encoded FGF, TGF-β, and PDGF have been implicated as important in the developing Xenopus embryo [152–154]; and bFGF is a potential neurotrophin during development [155]. PDGF also has been identified within plaques and is a potent vasoconstrictor and thus has been implicated in the genesis of atherosclerosis [156,157]. Furthermore, PDGF is secreted from endothelial cells and arterial smooth muscle cells [37,158–162] and activated monocytes/macrophages [160]. Elevated levels of PDGF receptors are found in synovial cells in patients with rheumatoid arthritis [162]. In each instance, however, when the *in vitro* studies are considered in the context of roles of the growth factors in inflammation and tissue repair, it seems likely that common roles of growth factors are associated with normal development and the abnormal remodeling is associated with disease states, indicating the importance of attention to mechanisms that regulate cell type and temporal levels of expression of the growth factors and their cognate receptors.

CONCLUSIONS

Much needs to be learned concerning the roles of growth factors and cytokines if we are to establish fully how they function in both normal and de-regulated tissue remodeling. Remarkable progress over the past several years has resulted from the identification, isolation, cloning, and characterization of the properties of these molecules and their use in wound healing models. The most important lesson learned from their use appears to be that the growth factors and cytokines are important to initiate and accelerate the normal processes involved from injury to repair. The growth factors are the 'trigger' that opens sequential activation of downstream pathways of critical importance to the progressive tissue remodeling that leads to the healed wound.

Questions for future avenues of investigation should address mechanisms by which these factors initiate and propagate the processes and the regulatory signals that govern cell type specificity, differentiation responses in cells migrating into and dividing within wounds, and the temporal sequences needed for orderly progression to a healed and ultimately functional tissue. Answers to these questions also will do much to advance the phenotype of malignant cancers, atherosclerosis, and other proliferative diseases.

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CHAPTER 17

Mechanobiology, Tissue Development and Organ Engineering

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INTRODUCTION

Tissue engineering has as its main goal the fabrication of artificial tissues for use as replacements for damaged organs and complex body structures. Great advances have been made in terms of developing prosthetic devices that can repair structural defects (e.g., vascular grafts) and even replace complex mechanical behaviors (e.g., artificial joints). However, the challenge for the future is to develop tissue substitutes that restore the normal biochemical functions of living tissues and organs, in addition to their structural features. To accomplish this feat, we must first establish precise design criteria for tissue engineering and develop new fabrication approaches to create complex organomimetics that reconstitute the tissue-tissue interfaces and physical microenvironments of living organs. These design features should be based on a thorough understanding of the molecular, cellular and biophysical basis of tissue regulation. In particular, they must take into account the important role that insoluble extracellular matrix (ECM) scaffolds and mechanical stresses play in control of cell phenotypes or 'fates' (e.g., growth, differentiation, motility, apoptosis, different stem cell lineages) during tissue formation, maintenance, and repair. This latter point is critical, since the spatial organization of cells and the mechanical constraints imposed on them as they grow actively regulate tissue development [1], as well as tissue and organ function throughout adult life [2].

The goal of this brief chapter is not to provide an extensive review of literature in the field of ECM biology or tissue development. Rather, it will summarize the known functions of ECM and describe some insights we have made relating to how ECM regulates cell growth and function as well as tissue morphogenesis. Our analysis of this regulatory mechanism should be of particular interest to the tissue engineer, because it has led to the identification of critical chemical and mechanical features of ECM that are responsible for control of cell fate switching in developing tissues. In addition, the reader will be introduced to some unanswered puzzles in developmental biology which, if deciphered, could provide powerful new approaches to tissue regeneration and repair. Finally, recent advances will be described in terms of micro-engineering human 'organs-on-chips' that recapitulate complex structures and functions of living human organs, which have opened new avenues of investigation for drug screening as well as modeling complex disease processes *in vitro*.

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EXTRACELLULAR MATRIX STRUCTURE AND FUNCTION

Composition and organization

One of the most critical elements of tissue engineering is the ability to mimic the ECM scaffolds that normally serve to organize cells into tissues. ECMs are composed of different collagen types, large glycoproteins (e.g., fibronectin, laminin, entactin, osteopontin), and proteoglycans that contain large glycosaminoglycan side chains (e.g, heparan sulfate, chondroitin sulfate, dermatan suflate, keratan sulfate, hyaluronic acid) [see chapter 10 by Olsen for more details]. While all ECMs share these components, the organization, form, and mechanical properties of ECMs can vary widely in different tissues depending on the chemical composition and three-dimensional (3D) organization of the specific ECM components that are present. For example, interstitial collagens (e.g., types I, III) self assemble into 3D lattices which, in turn, bind fibronectin and proteoglycans. This type of native ECM hydrogel forms the backbone of loose connective tissues, such as dermis. In contrast, basement membrane collagens (types IV, V) assemble into planar arrays; when these collagenous sheets interact with fibronectin, laminin, and heparan sulfate proteoglycan, a planar ECM results (i.e., the 'basement membrane'). The ability of tendons to resist tension and of cartilage and bone to resist compression, similarly result from local differences in the organization and composition of the ECM.

In vivo foundation for cell anchorage

The first and foremost function of ECM in tissue development is its role as a physiological substratum for cell attachment. This feature is easily visualized by treating whole tissues with ECM-degrading enzymes (collagenase, proteases); cell detachment and loss of cell and tissue morphology rapidly result. Cells that are dissociated in this manner can reattach to an artificial culture substrate (e.g., plastic, glass). However, adhesion is again mediated by cell binding to ECM components that are either experimentally immobilized on the culture surface, deposited *de novo* by the adhering cells, or spontaneously adsorbed from serum (e.g., fibronectin, vitronectin) [3–9]. In fact, standard tissue culture plates are actually bacteriological (non-adhesive) plastic dishes that have been chemically treated using proprietary methods to enhance adsorption of serum- and cell-derived ECM proteins. To summarize, cells do not attach directly to the culture substrate (i.e., plastic or glass), rather they bind to intervening ECM components that are adsorbed (or derivatized) to these substrates. For this reason, cell adhesion can be prevented by coating normally adhesive culture surfaces with polymers that prevent protein adsorption, such as poly(hydroxyethylmethacrylate) [9,10].

Spatial organizer of polarized epithelium

Living cells exhibit polarized form as well as function (e.g., basal nuclei, supranuclear Golgi complex, apical secretory granules in secretory epithelia). Dissociated cells fail to orient in a consistent manner when cultured on standard tissue culture substrata or on interstitial connective tissue. In the case of epithelial cells, the normal polarized form is often restored if the cells synthesize and accumulate their own ECM, or if they are cultured on exogenous basement membrane (i.e., the specialized epithelial ECM) [11–14]. These types of studies suggest that basement membrane normally serves to integrate and maintain individual cells within a polarized epithelium. Clearly, there are many intracellular and intercellular determinants of polarized cell form and function (e.g., cytoskeletal organization, organelle movement, junctional complex formation). However, anchorage to ECM appears to provide an initial point of orientation and stability on which additional steps in the epithelial organization cascade can build. ECM may regulate the orientation of other cell types (e.g., chondrocytes, osteoclasts) as well.

Scaffolding for orderly tissue renewal

All tissues are dynamic structures that exhibit continual turnover of all molecular and cellular components. Thus, it is the maintenance of tissue pattern integrity that is most critical to the survival of the organism, and regeneration of normal tissue architecture is one of the main goals of tissue engineering. Maintenance of specialized tissue form requires that cells that are lost due to injury or aging must be replaced in an organized fashion. Importantly, orderly tissue renewal depends on the continued presence of insoluble ECM scaffoldings which act as templates that maintain the original architectural form and assure for accurate regeneration of pre-existing structures [15]. For example, when cells within a tissue are killed by freezing or treatment with toxic chemicals, all of the cellular components die and are removed; however, the basement membranes often remain intact. These residual ECM scaffoldings assure the correct repositioning of cells (e.g., cell polarity) and restore different cell types to their correct locations (e.g., muscle cells within muscle basement membrane, nerve cells in nerve sheaths, endothelium within vessels, etc.), in addition to promoting the cell migration and growth which are required for the repair of all the component tissues. Conversely, loss of ECM integrity during wound healing results in disorganization of tissue pattern, and thus, scar formation. Uncoupling between basement membrane extension and cell doubling also leads to disorganization of tissue morphology during neoplastic transformation [16].

Establishment of tissue microenvironments

ECMs often establish a physical boundary between neighboring tissues. For example, the basement membrane normally restricts mixing between the epithelium and underlying connective tissue, and compromise of basement membrane integrity is indicative of the onset of malignant invasion when seen in the context of tumor formation [16]. The ECM boundary also may regulate macromolecular transport between adjacent tissues given that the basement membrane forms the semi-permeable filtration barrier in the kidney glomerulus [17]. However, little is known about this potential function of the ECM in other local tissue microenvironments.

Sequestration, storage, and presentation of soluble regulatory molecules

ECMs may modulate tissue growth and morphogenesis through their ability to bind, store, and eventually release soluble regulators of morphogenesis. For example, the soluble mitogen, basic fibroblast growth factor (FGF) exists in an immobilized form in ECMs deposited by cells cultured *in vitro* [18], as well as in normal tissues (e.g. corneal basement membranes) *in vivo* [19]. In fact, many cytokines (e.g., FGF, VEGF, HGF, insulin-like growth factor, hematopoietic growth factors, etc.) are normally stored bound to heparin, heparan sulfate or other specific binding proteins within natural ECMs [20,21]. The low growth rate observed in most normal tissues may result from sequestration of mitogens by ECM, whereas release of these stored factors ('stormones') due to injury or hormonally-induced changes in ECM turnover may help to switch growth on locally. Binding of other types of regulatory molecules to the endothelial basement membrane (e.g., plasminogen activator inhibitor [22]) also may play a role in tissue physiology (e.g., blood coagulation, cell migration).

Regulator of cell growth, differentiation, and apoptosis

Most normal (non-transformed) cells only grow when attached and spread on a solid substrate [10]. Cells attach and spread *in vitro* either by depositing new ECM components or by binding to exogenous ECM [3–9]. In fact, cell spreading and growth can be suppressed by inhibiting ECM deposition *in vitro* using drugs [4–6]. Cell growth stimulated by soluble mitogens has also been shown to vary depending on the type of ECM component used for cell

attachment (e.g., collagen versus fibronectin [3,8,23]) as well as on the mechanical properties of the ECM (e.g., malleable gel versus rigid ECM-coated dish) [24–28].

Interestingly, ECM substrates that promote growth tend to suppress differentiation and vice versa. For examples, many cells proliferate and lose differentiated features when cultured on attached type I collagen gels that can resist cell tension and promote cell spreading. In contrast, the same cells cease growing and increase expression of tissue-specific functions (e.g. albumin secretion in hepatocytes, milk secretion and acinus formation by mammary cells, capillary tube formation by endothelial cells) if cultured on the same gels that are made flexible by floating them free in medium or on attached ECM gels that exhibit high malleability (e.g., basement membrane gels, such as Matrigel). Under these conditions, the cells exert tension across their adhesions, resulting in contraction of the ECM gel and cell rounding which, in turn, shuts off growth and turns on differentiation-specific gene functions. The differentiation-inducing effects of these malleable ECM substrates can also be suppressed by making the gels rigid through chemical fixation [24,27], thus confirming the critical role of cell-generated mechanical forces in this response.

While local changes in ECM turnover may promote tissue remodeling, large-scale breakdown of the ECM may force the same growing tissues to undergo involution. Many cultured cells rapidly lose viability and undergo programmed cell death (i.e., apoptosis) when detached from ECM and maintained in a round form [29,30]. Loss of basement membrane integrity is also observed in regions of tissues that are actively regressing [31,14] and growing tissues (e.g., capillaries, mammary gland) can be induced to involute using pharmacological agents (e.g., proline analogues) that inhibit ECM deposition and lead to basement membrane dissolution *in vivo* [14,32,33]. Recent transgenic mice studies confirm that growing tissues can be made to involute by shifting the endogenous proteolytic balance such that total ECM breakdown results [34]. These findings suggest that local changes in ECM composition and flexibility may regulate cell sensitivity to soluble mitogens, and thereby control cell growth, viability, and function in the local tissue microenvironment.

PATTERN FORMATION THROUGH ECM REMODELING

Mesenchymal control of epithelial pattern

Probably the greatest insight into the role of ECM in tissue development comes from analysis of embryogenesis. In the embryo, genesis of a tissue's characteristic form (e.g., tubular versus acinar) and deposition of ECM are both controlled by complex interactions between adjacent epithelial and mesenchymal cell societies. The epithelial cell is genetically programmed to express tissue-specific (differentiated) functions and to deposit the insoluble basement membrane which functions as a common attachment foundation that both separates adjacent tissues and stabilizes tissue form [35,36]. However, while production of tissue-specific cell products (cytodifferentiation) is determined by the epithelium, tissue pattern (histodifferentiation) is usually directed by the surrounding mesenchyme. For example, when embryonic mammary epithelium is isolated and combined with salivary mesenchyme, the mammary epithelial cells take on the form of the salivary gland although they still produce milk proteins [37]. However, the specificity of these epithelial-mesenchymal interactions can vary widely from organ to organ. For instance, embryonic salivary epithelia specifically require salivary mesenchyme for successful development, while pancreatic epithelia will undergo normal cytodifferentiation and histodifferentiation in response to mesenchyme isolated from a variety of embryonic tissues [38].

Tissue patterning through localized ECM remodeling

The complex tissue patterns that are generated through epithelial-mesenchymal interactions result from the establishment of local differentials in tissue growth and expansion in a

microenvironment that is likely saturated with soluble mitogens. The classic work on salivary gland development by Bernfield and coworkers revealed that the epithelium imposes morphological stability through production of its basement membrane, whereas the mesenchyme produces local changes in tissue form, specifically by degrading basement membrane at selective sites [35,39–42]. An increased rate of cell division is observed in the tips of growing lobules where the highest rate of ECM breakdown and re-synthesis (i.e., highest turnover rate) is also observed. At the same time, the mesenchyme slows basement membrane turnover and suppresses epithelial cell growth in the clefts of the glands. This is accomplished by secretion of fibrillar collagens which slow ECM degradation locally, and thereby promote basement membrane accumulation in these regions. Similar local coupling between ECM turnover, cell growth rates and tissue expansion is observed in many other developing tissues, including growing capillary blood vessels [43].

It is important to note that increased ECM turnover involves enhanced rates of matrix synthesis as well as degradation. In fact, net basement membrane accumulation (i.e., increased area available for cell attachment) must result for epithelial tissues to grow and expand laterally, and thus the local rate of ECM synthesis must be greater than that of degradation in these high turnover regions. If the rate of ECM degradation is significantly greater than synthesis, then net basement membrane dissolution along with cell retraction and rounding results. As described above, this would lead to cell death and tissue regression rather than expansion.

Role of mechanical stresses

Before ending discussion of the role of ECM in pattern formation, it is critical to emphasize that while chemical regulators mediate tissue morphogenesis, the signals that are actually responsible for dictating tissue pattern are often mechanical in nature [2]. The pattern-generating effects of compression on bone, shear on blood vessels, and tension on muscle are just a few examples. Mechanical stresses are also important for embryological development; however, internal cell-generated forces appear to play a more critical role [1]. For example, mechanical tension that is generated via actomyosin filament sliding within the cytoskeleton of the cells that compose the embryo plays a key role during gastrulation [44] as well as during tissue morphogenesis [45]. In fact, the pattern of development can be experimentally altered by applying external stresses to the embryo using micropipettes [46], or by altering the level of cytoskeletal tension [47]. Mechanical compression of mesenchyme associated with the 'condensation' of mesenchyme that commonly precedes formation of new organ rudiments has been recently shown to be sufficient to induce whole tooth organ formation during embryogenesis [48]. The pattern-generating capabilities of mesenchyme isolated from different developing tissues also correlates with differences in their ability to exert mechanical tension on external substrates (e.g., reference [49]). Local changes in ECM turnover may drive morphogenetic patterning of tissues, in part, by altering the mechanical compliance of the ECM and thereby, changing cell shape or cytoskeletal tension [49a,50], as will be described below.

MECHANOCHEMICAL SWITCHING BETWEEN CELL FATES

Given the pivotal role that ECM plays in tissue development, many studies have been carried out to analyze how changes in cell-ECM interactions act locally to regulate cell sensitivity to soluble mitogens, and thereby establish the differentials of growth, differentiation, apoptosis and motility that are required for tissue morphogenesis. To accomplish this, simplified *in vitro* model systems have been developed which retain the minimal determinants necessary for maintenance of the physiological functions of interest (i.e., cell growth and differentiation) [9,51,52]. For example, to determine the effects of varying cell-ECM contacts directly, we pre-coated bacteriological petri dishes that were otherwise non-adhesive with different densities of purified ECM molecules, such as fibronectin, laminin, or different collagen types. Quiescent,

serum-deprived cells were plated on these dishes in a chemically-defined medium that contained a constant and saturating amount of soluble growth factor.

When capillary endothelial cells were studied, DNA synthesis and cell doubling rates increased in an exponential fashion as the density of immobilized ECM ligand was raised and cell spreading was promoted [9]. When higher cell plating numbers were used to promote cell-cell interactions as well as cell-ECM contact formation, the capillary cells could be switched between growth and differentiation (capillary tube formation) in the presence of saturating amounts of soluble mitogen (FGF) simply by varying the ECM coating density [51]. Specifically, when plated on a high ECM density (e.g., >500 ng/cm^2 fibronectin), the cells attached, spread extensively, formed many cell-cell contacts, and organized into a planar cell monolayer. When the same cells were plated on a low ECM density (<100 ng/cm^2, the cells attached but they could not spread, and thus only cell clumps or aggregates were observed. When the same capillary cells were plated on a moderate density, cells first attached, spread, and formed cell-cell contacts as they did on the higher ECM densities. However, the tensile forces generated by the cells appeared to overcome the resistance provided by their relatively weak ECM adhesions and thus, the cell aggregates began to retract over a period of hours until a mechanical equilibrium was attained. Under these conditions, formation of an extensive network comprised of interconnected capillary tubes resulted. Many of these capillary tubes became elevated above the culture surface, although the network remained adherent to the culture dish at discrete points through interconnected multicellular aggregates. The importance of mechanical forces for this switching between growth and differentiation was confirmed by demonstrating that similar capillary tube formation could be induced on the high ECM density that normally promoted spreading and growth, simply by increasing the cell plating numbers and thereby amplifying the level of cell tension.

The same system was used to demonstrate similar shape (stretch)-dependent switching between growth and differentiation in other cell types. For example, the growth and differentiation of primary rat hepatocytes could be controlled independently of cell-cell contact formation by varying cell-ECM contacts and cell spreading using the method described above [52]. Additional studies confirmed that ECM exerts its regulatory effects at the level of gene expression [54,53] and that these effects are mediated at least in part through modulation of the cytoskeleton [55]. These results are consistent with those from other laboratories, which demonstrate that malleable ECM gels (e.g., Matrigel, native collagen gels) that promote cell rounding also induce differentiation and suppress growth — whereas the opposite effects are produced when these gels are fixed and made rigid [24–27].

Altering cell-ECM contacts by varying ECM coating densities influences cell function via two distinct but integrated mechanisms. First, increasing the local density of immobilized ECM ligand promotes clustering of transmembrane ECM receptors on the cell surface which are known as 'integrins' [56]. Integrin clustering, in turn, activates a number of different chemical signaling pathways (e.g., tyrosine phosphorylation, inositol lipid turnover, Na+/H+ exchange, MAP kinase) that are also utilized by growth factor receptors to alter cellular biochemistry and gene expression [57–61]. Activation of these signaling pathways likely plays an important role in control of cell differentiation and survival; however, integrin-dependent chemical signaling alone is not sufficient to explain how cells are induced to enter S phase and proliferate [9,30,53,62,63]. A second mechanism that involves tension-dependent changes in cell shape and cytoskeletal organization, or changes in the level prestress (isometric tension) within the cell and linked ECM, also comes into play.

The importance of tension-dependent changes of cell shape and cytoskeletal organization was demonstrated directly by developing a model system in which cell distortion was varied independently of the local density of immobilized ECM molecule by controlling the spatial distribution of ECM anchors that can resist cell-generated traction forces. This was made possible by adapting a technique for forming spontaneously assembled monolayers (SAMs) of

alkanethiols [64] to create micropatterned surfaces containing adhesive ECM islands with defined surface chemistry, shape and position on the cell (micrometer) scale, separated by non-adhesive regions [30,65]. The method involves the fabrication of a flexible elastomeric stamp that exhibits the particular surface features of interest using photolithographic techniques. The topographic high points on the stamp (e.g., 40 x 40 μm square plateaus raised above recessed intervening regions) are coated with an alkanethiol ink and the stamp is then apposed to a gold-coated surface. The alkanethiol forms SAMs covering only the regions where the stamp contacts the surface (i.e., 40 x 40 μm squares). Then the surrounding uncoated regions are filled with a SAM composed of similar alkanethiols that are conjugated to poly(ethylene glycol) (PEG) that prevents protein adsorption. The result is a chemically-defined culture surface that is completely covered with a continuous SAM of alkanethiols, however, the local adhesive islands of defined geometry support protein adsorption, whereas the surrounding boundary regions coated with PEG do not. Thus, when these substrates are coated with a high density of purified ECM protein, such as laminin or fibronectin, adhesive islands of defined shape and position, coated with a saturating density of matrix molecule result. Using this technique, cell position and shape can be precisely controlled because the cells only attach to the ECM-coated adhesive islands. In fact, even square and rectangular shaped cells exhibiting 90° corners can be engineered using this approach [30,65].

This micropatterning method was first used to ask the question: if cells are restricted to a small size similar to that produced on a low ECM coating concentration, but the local density of immobilized integrin ligand is increased 1000-fold, which is the critical determinant of cell function — the ECM density or cell shape? The answer was that it was cell shape. Primary hepatocytes remained quiescent on small adhesive islands coated with a high ECM density, even though the cells were stimulated with high concentrations of soluble growth factors, and cell growth (DNA synthesis) increased in parallel as the size of the adhesive island was increased [65]. Similar results also were obtained with capillary endothelial cells [30]. Moreover, inhibition of hepatocyte growth on the small islands also was accompanied by a concomitant increase in albumin secretion. In the case of endothelial cells, the cells were similarly induced to differentiate into capillary tubes when cultured on linear patterns that supported cell-cell contact as well as a moderate degree of cell distension whereas the cells were induced to undergo apoptosis (cellular suicide) when cultured on the smallest islands that fully prevented cell extension [30,66]. Thus, cells shape and function can be engineered simply by altering the geometry of the cell's adhesive substrate.

Intracellular signals elicited by integrin receptor clustering due to ECM binding have been shown to be critical for control of cell growth and function [56–58,61]. Thus, one could argue that cell shape and mechanical distortion of the cytoskeleton (CSK) are not important. Instead, it might be the increase in the total area of cell-ECM contacts and the associated enhancement of integrin binding that dictates whether cells will grow, differentiate or die on large versus small adhesive islands. To explore this further, substrates were designed in which a single small adhesive island (which would not support spreading or growth) was effectively broken up into many smaller islands (3–5 μm in diameter) that were separated by non-adhesive barrier regions [30]. When capillary cells were plated on these substrates, their processes stretched from island to island and the cells exhibited an overall extended form similar to cells on large islands. However, the total area of cell-ECM contact was almost identical to that exhibited by non-growing cells on the smaller islands. These studies revealed that in the presence of optimal growth factors and high ECM binding, DNA synthesis was high in the cells that spread over multiple small islands whereas apoptosis was completely shut off [30], thus confirming that cell shape distortion is the critical governor of this response.

Importantly, cell distortion also impacts cell migration as well as stem cell lineage switching. For instance, when cells on square islands are stimulated with motility factors, the cells preferentially extend new motile processes (e.g., lamellipodia, filopodia, microspikes) from

their corners, whereas they extend in all directions along the edge of round cells on circular islands [67]. Cells on polygonal ECM islands also prefer to form new lamellipodia from corners with acute angles rather than obtuse ones [83]. In contrast, when mesenchymal stem cells are cultured on different sized ECM islands, those on small islands switch on the fat cell lineage, whereas the spread cells on larger ECM islands become bone cells [68]. Thus, taken together, these results suggest that microfabrication methods might lead to new approaches to tissue engineering using microfabricated substrates. With this approach, it may be possible to direct cell migration, growth and differentiation of stem cells in specific locations by modifying the surface chemistry and topography of artificial materials, instead of creating gradients of soluble chemokines.

The effects of shape distortion on cell fate switching may, in part, be mediated by changes in the level of isometric tension or 'prestress' within the cytoskeleton. For example, both flexible and poorly adhesive culture dishes that inhibit growth and induce differentiation dissipate prestress, whereas substrates that stimulate cell spreading and growth (e.g., rigid dishes) support high levels of cytoskeletal tension. Small ECM islands that inhibit cell spreading also prevent pulmonary vascular smooth muscle cells from responding to vasconstrictors, such as endothelin-1 (as measured by increases of myosin light chain phosphorylation), and similar effects are produced by culturing cells on flexible ECM substrates that dissipate tensional prestress in the ECM and in the interconnect cytoskeleton (i.e., linked through transmembrane integrins) [68a,69]. In addition, cells preferentially differentiate on ECM substrates that are compliance-matched with their cytoskeleton and that have a mechanical stiffness similar to that of their natural tissues [70–72]. Cells also migrate up gradients of ECM stiffness, a process known as durotaxis [73]. These observations suggest that tissue formation may be controlled by changing the physical properties of either the ECM or of the cell, or by altering tension generation in the cytoskeleton.

Regional variations of cell shape distortion or of cytoskeletal prestress similarly drive tissue patterning in the embryo. For example, during tissue morphogenesis, only a subset of cells must respond to soluble growth factors by proliferating locally and sprouting or budding outward relative to neighboring non-growing cells. This process is repeated along the sides of the newly formed sprouts and buds, and the whole process reiterates over time; this is how the fractal-like patterns of tissues develop. This process is mediated by regional changes in ECM structure: the ECM thins in regions where new buds or sprouts will form due to local enzymatic degradation [39]. Because tissues are prestressed (due to the action of cytoskeletal contractile forces), a local region of the tensed ECM may thin more than the rest like a 'run' in a woman's stocking. Cells anchored to this region will also stretch or spread, whereas the shape of neighboring cells on intact ECM would remain unchanged. If cell spreading promotes growth, then local cell growth differentials would result.

This possibility that tissue morphogenesis may be controlled through changes in the mechanical force balance between the cytoskeleton and the ECM is supported by recent experimental studies in embryonic and adult systems. Analysis of the effects of cell shape on cell cycle progression *in vitro* have revealed that these effects are mediated by altering signal transduction through the small GTPase Rho and its downstream target, Rho-associated kinase (ROCK) that controls cytoskeletal tension generation [62,63]. Importantly, when cytoskeletal contractility was suppressed in whole embryonic lung rudiments by inhibiting Rho or ROCK, both epithelial branching morphogenesis and angiogenesis were inhibited, and this was accompanied by decreased basement thinning (as if there was no tension in the stocking) [47]. In contrast, stimulating the Rho pathway had the opposite effect (i.e., it increased morphogenetic branching) at moderate levels of activation and caused complete organ contraction and inhibition of tissue growth at very high levels of stimulation [47]. Altering the cellular force balance by modulating ECM rigidity also can alter expression of the angiogenic factor receptor VEGFR2 (via control of nuclear transport of opposing transcription factors) and

thereby, regulate angiogenesis both *in vitro* and *in vivo* [74]. Thus, local changes in ECM structure and cell shape caused by altering cytoskeletal prestress may govern how individual cells respond to chemical signals in their microenvironment *in vivo*, just as experimental studies using microfabricated ECM islands have demonstrated *in vitro*. This mechanism for establishing local growth differentials may play a critical role in morphogenesis in all developing systems [50,74a].

MECHANOBIOLOGY SUMMARY

In summary, our work has shown that the development of functional tissues, such as branching capillary networks, requires both soluble growth factors and insoluble ECM molecules. The ECM appears to be the dominant regulator, however, since it dictates whether individual cells will proliferate, differentiate, undergo directional motility or die locally in response to soluble stimuli. This local control mechanism is likely critical for the establishment of the local differentials in cell growth, motility, differentiation, apoptosis and stem cell commitment that mediate pattern formation in all developing tissues.

Analysis of the molecular basis of these effects has revealed that ECM molecules alter cell growth via both biochemical and biomechanical signaling mechanisms. ECM molecules cluster specific integrin receptors on the cell surface and thereby activate intracellular chemical signaling pathways [57–60], stimulate expression of early growth response genes (e.g., c-fos, jun-B) [53], and induce quiescent cells to pass through the G_0/G_1 transition. However, in addition, the immobilized ECM components must physically resist cell tension and promote changes of cell shape [9,30,33,52,65] and cytoskeletal tension [55,62,63,75] in order to promote full progression through G_1 and entry into S phase. In fact, studies with living and membrane-permeabilized cells confirm that changes in cell shape result from the action of mechanical tension which is generated within microfilaments and balanced by resistance sites within the underlying ECM [69,76]. Local changes in ECM structure or mechanics will therefore alter the ECM's ability to resist cell tractional forces exerted on integrins, and thereby produce changes in cytoskeletal organization. This will, in turn, alter the level of isometric tension (prestress) in the cytoskeleton, as well as modulate the activity of various signal transduction pathways signal inside the cell [2]. Taken together, this work suggests that the pattern-regulating information that the ECM conveys to cells is both chemical and mechanical in nature. Thus, design of future artificial ECMs for tissue and organ engineering applications must take into account the critical role that mechanical forces and these mechanotransduction mechanisms play in biology.

THE FUTURE

Early tissue-engineering efforts by reconstructive surgeons and material scientists started with knowledge of the clinical need and of the mechanical behavior of connective tissues on the macroscopic scale, and worked backwards. The long-term goal for the field is to design and fabricate tissue and organ substitutes starting from first principles. This includes incorporating biologically inspired mechanical and architectural principles, as well as an in depth understanding of the molecular and biophysical basis of tissue regulation. Clearly, given the potent and varied functions of the ECM, fabrication of artificial ECMs will play a central role in all of these future efforts. We and others have already begun to explore the utility of synthetic bioerodible polymers as cell attachment substrates [77,78] and the potential usefulness of immobilized synthetic ECM peptides for controlling cell growth and function [53,52,79]. These materials offer a major advantage in terms of biocompatibility since the artificial substrates completely disappear over time, and thus the implanted donor cells become fully incorporated into the host. They also provide great chemical versatility, as well as the potential for large-scale production at relatively low cost. In addition, use of synthetic chemistry reduces the likelihood of batch to batch variation during large-scale production, a problem which can

potentially complicate use of purified ECM components. For these reasons, polymer chemistry and novel fabrication techniques will likely lead to development of more effective tissue substitutes.

However, if we understood the fundamental principles that guide ECM remodeling and pattern formation in tissues, perhaps tissue engineering might take a different approach in the future. For example, one could envision entirely new methods of clinical intervention if we understood how tissue-specific mesenchyme generates tissue pattern; how ECM turnover is controlled locally; how cell-generated contractile forces contribute to tissue repair and remodeling; or how compressing or pulling tissues alter their growth and form. This knowledge could lead to methods for identifying and isolating relevant 'pattern-generating' cells; for developing pharmacological modifiers of ECM remodeling that may be incorporated in local regions of implants to promote or suppress tissue expansion locally; and for fabricating 'biomimetic' scaffolds that mimic the mechanical and architectural features of natural ECMs necessary to switch on or off the function of interest (e.g., growth vs. differentiation or apoptosis) at a particular time or place. These are the just a few of the challenges for the future.

Finally, one glimpse of the future comes from our recent work that focuses on the development of human 'organs-on-chips' in which microscale-engineering technologies are used to create microfluidic devices lined by cultured living human cells that recapitulate the physiological microenvironments of whole living organs. The goal is to develop organomimetic microdevices that enable study of complex human physiology in an organ-specific context. Our first success in this area was the development of a human breathing 'lung-on-a-chip' that reproduces key structural, functional, and mechanical properties of the fundamental functional unit of the living human lung — the alveolar-capillary interface. We did this by microfabricating a microfluidic system containing two closely apposed microchannels separated by a thin (10 μm) porous flexible membrane made of poly(dimethylsiloxane) (PDMS), which was coated with ECM. Human alveolar epithelial cells were cultured on one side of the membrane with air flowing above their apical survace, and human pulmonary microvascular endothelial cells were cultured on the other with flowing medium containing human white blood cells to mimic blood flow. Breathing movements were reproduced in this engineered microsystem by applying cyclic suction to neighboring hollow microchambers; this rhythmically deforms the central porous membrane and attached cell layers much as occurs at the living alveolar-capillary interfaces during breathing.

The lung-on-a-chip was shown to replicate the complex organ-level responses of living human lung, including the inflammatory response triggered by bacteria or cytokines introduced into the air space [80]. The endothelial cells rapidly become activated in response to these cues, and increase their expression of ICAM-1, which induces adhesion of primary human neutrophils flowing in the capillary channel; this is followed by their transmigration across the capillary-alveolar interface and into the alveolar space where the white blood cells engulf the bacteria. Because the PDMS has high optical clarity, the entire human inflammatory response can be visualized in real-time within this biochip.

Importantly, we found that the breathing motions and rhythmic distortion of the cells are absolutely critical for them to mimic organ-level physiology and pathophysiology. For example, we found that physiological breathing movements greatly accentuate toxic and inflammatory responses of the lung to silica nanoparticles (12 nm) that were introduced into the air channel to mimic airborne particulates [80]. Mechanical strain also enhanced epithelial and endothelial uptake of nanoparticulates and stimulated their transport into the underlying microvascular channel. Importantly, similar effects of physiological breathing on nanoparticle absorption are observed in whole mouse lung. Moreover, we have recently extended this work by developing a microengineered human disease model of pulmonary edema [81], which closely mimics this life-threatening side effect that is induced by the cancer chemotherapic interleukin-2 (IL-2) in human patients, and is characterized by fluid

accumulation and fibrin deposition in the alveolar airspace. Again, we found that physiological breathing motions are responsible for the majority of the effects of IL-2 on pulmonary vascular leakage, and we confirmed this in an animal model. Finally, when we adapted the same microengineering approach to produce a human 'gut-on-a-chip' lined by human intestinal epithelial cells, we also found that by mimicking peristaltic-like mechanical motions and the trickling fluid flows of the living gut, we obtained levels of differentiation and functionality never seen before *in vitro* [82]. These included spontaneous formation of intestinal villi, restoration of epithelial barriers, and gaining the ability to co-culture intestinal bacterial (microbiome) on the human epithelium without compromising tissue viability or function.

Human organ-on-chips models could provide a potential new approach to enhance our fundamental understanding of complex disease processes and to enable more rapid, accurate, cost-effective and clinically relevant testing of drugs, as well as cosmetics, chemicals and environmental toxins. On-chip human disease models also could help to facilitate the translation of basic discoveries into effective new treatment strategies.

But most importantly, these results demonstrate the power of combining insights into mechanobiology with advanced engineering approaches as we seek to confront future challenges in tissue and organ engineering.

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PART 3

In Vivo Synthesis of Tissues and Organs

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CHAPTER 18

In Vivo Synthesis of Tissues and Organs

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INTRODUCTION

The goal of achieving *in vivo* induced regeneration for a variety of tissue and organs following severe injury remains at the forefront of current tissue-engineering investigations. Typically, an analog of the extracellular matrix is utilized as a template that, when properly formulated, induces regeneration of lost or damaged tissue. Currently, successful regeneration has been induced in the skin and peripheral nerves [1], while progress has been made in developing appropriate extracellular matrix analogs to alter the typical organismic response to injury in a variety of tissues, including kidney, cartilage, bone, central nervous system, and brain dura. These investigations, active for the previous three decades, have primarily focused on identifying the optimal extracellular matrix analog components to block organized wound contraction and scar tissue formation, while inducing regeneration of physiological tissue [1]. Although many tissue engineering investigations currently focus on developing techniques appropriate for synthesis of tissues and organs *in vitro*, such products must eventually be implanted in the appropriate anatomical site of the host. Since implantation of an organ construct is almost always preceded by a surgical procedure that generates a severe wound, it is essential to master the evolving methodology of *in vivo* wound healing in order to synthesize appropriate neo-organ constructs *in vitro* for eventual implantation. The goal of this chapter is to inform the reader about the salient features of the organismic response to injury, the historical underpinnings of current studies of induced tissue regeneration, induced tissue regeneration as a technique to treat severe injuries to a variety of tissue and organs, and the future directions of research in this field.

MAMMALIAN RESPONSE TO INJURY

Defect scale

Treatment options for organ injury depend significantly on the scale of the defect. Microscopic defects can be treated using a wide variety of soluble factors (i.e., herbs, potions, pharmaceuticals, vitamins, hormones and antibiotics). However, organ-scale defects present a significantly larger wound site, require considerably different treatment practices, and constitute the focus of this article. These defects, primarily created by disease or by an acute or chronic insult that result in millimeter or centimeter scale wounds, cannot be treated with drugs because the problem is the failure of a mass of tissue including cells, soluble proteins

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and cytokines, and insoluble extracellular matrix (ECM). Significant loss of function in the affected tissue or organ, termed the 'missing organ', leads to consequences such as lack of social acceptance in cases of severe burns and facial scars, loss of mobility and sensory function in the case of neuroma, and life-threatening symptoms in cases such as cirrhotic liver, large-scale severe burns, and ischemic heart muscle.

REGENERATION VERSUS REPAIR

Certain organisms have the ability to regenerate significant portions of damaged tissue. An example is the amphibian newt, which regenerates functional limbs following amputation. The mammalian fetus has displayed the ability to regenerate damaged organs and tissue spontaneously up to the third trimester of gestation; however, adult mammals do not typically exhibit spontaneous regeneration following severe organ injuries [1,2]. Instead, the adult mammal response to severe injury is closure of the wound by contraction and scar tissue formation, a process termed repair. Cell-mediated contraction of the wound site is observed in many different species in varying degrees, at many organ sites [1]. Distinct from repair, regeneration is characterized by synthesis of physiological (normal, functional) replacement tissue in the wound site that is structurally and functionally similar to the original tissue. Based on these observations, for the remainder of this text the term 'early fetal' refers to the fetal response to injury that leads to regeneration, while 'adult' refers to all mammals (adult as well as late fetal) that respond to injury via repair.

The contractile fibroblast phenotype, termed the myofibroblast, plays a critical role in determining the nature (repair or regeneration) of mammalian wound healing. During adult repair, myofibroblast-mediated organized wound contraction and scar tissue synthesis is observed [1]. During early fetal healing, differentiation of myofibroblasts has not yet occurred and regeneration of damaged tissue and organs occurs in the absence of contraction. The data suggest that induced organ regeneration in the adult may be encouraged by developing techniques to stimulate partial reversion to early fetal healing. Additionally, the transforming growth factor-β (TGF-β) family of molecules has been implicated in this ontogenetic transition between fetal regeneration and adult repair response to injury. TGF-βs are multifunctional cytokines with widespread effects on cell growth and differentiation, embryogenesis, immune regulation, inflammation, and wound healing [3]. In terms of their relationship with repair processes, TGF-β1 and TGF-β2 are known to promote scarring, while TGF-β3 may reduce scarring [4,5]. As such, deficient levels of TGF-β1 and β2 and increased levels of TGF-β3 are observed in early gestational ('fetal' regenerative healing response) compared to late gestational ('adult' repair healing response) mice. These results implicate increased TGF-β1, β2, and decreased TGF-β3 expression along with myofibroblast activity in late gestation and post-partum fetal scar formation [6]. The available evidence suggests future experiments utilizing procedures for control of TGF-β in conjunction with other tissue-engineering constructs that modify myofibroblast behavior, such as bioactive scaffolds, to induce regeneration of tissues that are known to be non-regenerative.

Tissue triad

There are three distinct tissue types, termed the tissue triad, which together define the structure of most organs: the epithelial layer, the basement membrane layer, and the stroma (Fig. 18.1) [1,7]. Developmental and functional similarities between this triad in a variety of tissues and organs such as skin and peripheral nerves have been observed, suggesting that it can be used as an illustrative device to understand injury response in other organs as well [1]. A layer of epithelial cells (the epithelial layer) covers all surfaces, tubes, and cavities of the body; this layer is cell-continuous and avascular; unlike the basement membrane and stroma, the epithelial layer does not comprise a significant amount of ECM. The basement membrane is an acellular, avascular, continuous layer of ECM components separating the epithelial layer and

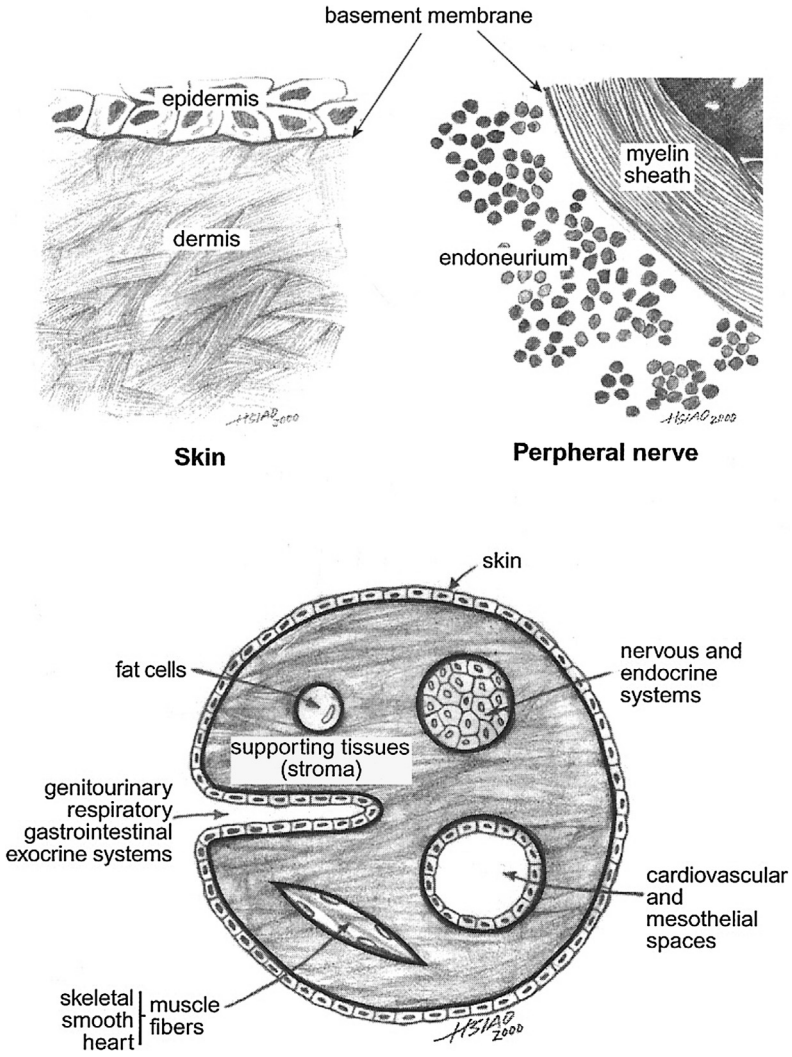

FIGURE 18.1

Schematics of the tissue triad structure observed in mammalian tissue. *Top*: Tissue triad of skin and peripheral nerves. The basement membrane is a thin extracellular matrix tissue located between the cellular and non-vascular epithelia (epidermis, myelin sheath) and the cellular, vascularized stroma (dermis, endoneurium). *Bottom*: Diagram of the distribution of epithelial, basement membrane, and stromal tissues in the mammalian system. Examples of stromal tissues are bone, cartilage, and their associated cell types as well as elastin and collagen. Examples of epithelial tissues are those covering the surface of the genitourinary, respiratory, and gastrointestinal tracts as well as surfaces of the mesothelial cells in body cavities, muscle fibers, fat cells, and endothelial cells in the cardiovascular system [1].

the stroma. The stroma is cellular, contains ECM and connective tissue components, is heavily vascularized, and provides a reservoir for nutrient uptake to and waste removal from the basement membrane and epithelia.

Following injury to a variety of tissues, such as the skin, peripheral nerves, blood vessels, lung, kidney, and pancreas, the epithelial and basement membrane layers regenerate spontaneously when the stroma remains intact, while the damaged stoma heals through repair-mediated contraction and scar formation processes [1,8–18]. Understanding the injury response of the tissue triad has suggested a paradigm for inducing regeneration in non-regenerative tissues: the repair mechanisms appear to be activated by disruption of the stromal architecture, and proper replacement of the stromal layer is critical for any regeneration to occur. As such, development of materials to act as analogs of the ECM to replace the lost stromal architecture has been a primary focus of studies of regenerative medicine over the past two decades.

METHODS TO TREAT LOSS OF ORGAN FUNCTION

Six approaches have been used to restore some level of functionality to a damaged tissue or organ: transplantation, autografting, implantation of a permanent prosthetic device, use of stem cells, *in vitro* synthesis of organs, and induced regeneration. The last three techniques are often collectively referred to as 'tissue engineering' [19]. *In vivo* induced regeneration is the only methodology to date which has modified the adult mammalian wound healing response to induce regeneration. All six techniques will be briefly discussed in the following sections; a more detailed description has been previously published [1].

Transplantation

Transplantation is widely used to replace complex tissues and organs, but is limited by two significant factors. While transplantation of a select few tissues such as the eye and testis occur without rejection, a significant challenge facing transplantation is the immunological barrier between the donor and host. After transplantation, the donor organ is attacked and rejected by the host's immune system. The primary clinical method for preventing such rejection is the use of immunosuppressive drugs for the remainder of the host's life to suppress their immune system. However, immunosuppression also makes the host vulnerable to infections [20]. A second major obstacle is the difficulty in finding immunocompatible donors and the shortness of supply of suitable organs [21].

Autografting

With autografting, the donor and the recipient are the same individual; a fraction of the tissue or organ is harvested from an uninjured site and grafted at the non-functional site [22]. Autografting removes issues related to immune response, but necessitates the creation of a second wound site (donor site), subjecting the patient to a second severe trauma and additional loss of functionality. Therefore, autografting is utilized only when sufficient autograft tissue is available and when the loss of functionality or morbidity at the primary wound site outweighs that at the harvest site, giving it limited applicability. Typical applications of autografting follow severe burns and peripheral nerve injuries in the hand.

Permanent prosthetic device

Permanent, prosthetic devices are typically fabricated from biologically inert materials such as metals, ceramics, and synthetic polymers that do not provoke the immune response problems inherent to many transplanted tissues. Even though these devices are fabricated from bio-inert materials, interactions between the prosthesis and the surrounding biological environment still lead to a number of unfavorable physical and biological manifestations. Specific examples are the formation of a thick, fibrous scar tissue capsule around the implant [23], stress-shielding of the surrounding tissue [24], platelet aggregation to implanted surfaces [25], and accumulation of wear particles both at the site of implantation and in the lymphatic system [26]. The spontaneous remodeling process of the tissues surrounding the implant can also be significantly altered, leading to further tissue degradation [27]. These often-serious side effects illustrate the difficulty of replacing bioactive tissues with bio-inert implants fabricated from materials possessing drastically different material and mechanical properties.

Stem cells

The pluripotent nature of stem cells offers a multitude of therapeutic possibilities [28,29]. Current efforts in stem cell research have focused on understanding stem cell plasticity, aging, and ways of controlling differentiation [30–33]. Mesenchymal [34], epithelial [35], and neural stem cells [36] have been grown *in vitro* and studied. More recently, experimental investigations of stem cells have also focused on utilizing hematopoietic and embryonic stem cells. In particular, techniques for harvesting and identifying them [37,38], expanding and

differentiating them in culture [30,39–45], and re-implanting them at an injury site [42,46] have been at the forefront of stem cell research. Importantly, many of these studies have made the transition to exploring biomaterial-mediated effects on basic cell bioactivity [47,48] and regenerative potential [49–54]. An improved understanding of stem cell behavior and the development of stem cell-based technologies also raise a number of important ethical questions [55], consideration of which will play a significant role in the development of stem cell-based tissue-engineering solutions.

In vitro synthesis

In vitro synthesis requires the growth of a functional volume of tissue *in vitro*. It allows total control over the culture environment, including soluble regulator content (i.e., growth factors, cytokines), insoluble regulator content (i.e., ECM proteins), and a variety of cell culture media and loading conditions. In order to develop large (critical dimension >1 cm), bioactive scaffolds, it is important to metabolically support the cells within these constructs. There are two mechanisms available for the transport of metabolites to and waste products from cells in a scaffold: diffusion, and with *in vivo* applications, transport through capillary networks formed in the scaffold via angiogenesis. While angiogenesis becomes the limiting factor *in vivo*, significant angiogenesis is not observed for the first few days after implantation, and is not present at all *in vitro*. As a result, current tissue engineering constructs are size limited (<1 cm) due to diffusion constraints. Improving metabolite influx is critical for larger, more complex scaffolds. Additionally, the complexity of biological systems, specifically cytokine, growth factor, and intercellular signaling needs throughout the volume of developing tissue have to date precluded, with a few exceptions such as *in vitro* culture of replacement heart valves [56] and epithelial sheets for severe burn patients [57], the formation of complex tissues *in vitro*.

Induced *in vivo* organ synthesis (induced regeneration)

Induced organ synthesis *in vivo* relies on the processes inherently active in the wound site to regenerate lost or damaged tissue. A highly porous analog of the ECM, also termed a scaffold, is utilized to induce regeneration at a wound site where the organism would normally respond via repair processes. Induced organ synthesis was made possible by the development of fabrication techniques to produce ECM analogs with well-defined pore microstructure, specific surface area, chemical composition, and degradation rate [1,13]. Its first application was the use of a collagen-glycosaminoglycan (CG) scaffold (termed dermal regeneration template – DRT) that induced skin regeneration following severe injury. The DRT displayed high biological activity when implanted into a full-thickness skin wound and was capable of inducing regeneration of the underlying dermal layer of skin as well as the epidermal and basement membrane layers [13,58]. Efforts using decellularized matrices have presented a new avenue for generating material platforms for tissue regeneration such as in the area of urogenital and intestinal repair [59–63].

For the remainder of this review, we will focus on studies on the structure and function of ECM analogs used in tissue-engineering applications as well as an in-depth discussion of the tissue-engineering approaches utilized to induce regeneration of a number of tissues, namely skin, peripheral nerves, and orthopedic tissues.

ACTIVE EXTRACELLULAR MATRIX ANALOGS

Fundamental design principles for tissue regeneration scaffolds

Porous, three-dimensional scaffolds have been used extensively as biomaterials in the field of tissue engineering for *in vitro* study of cell-scaffold interactions and tissue synthesis and *in vivo* induced tissue regeneration studies. These scaffolds, analogs of the ECM, act as a physical support and, more importantly, as an insoluble regulator of biological activity that affects cell processes such as migration, contraction, and division. For the remainder of this review, the

term active or bioactive ECM analog will refer to scaffolds that induce regeneration of normally non-regenerative tissues following severe injury. It has been hypothesized that these active ECM analogs induce regeneration by establishing an environment that selectively inhibits wound contraction by preventing the organized contractile response and blocking scar synthesis, the two processes normally responsible for closing a wound following severe injury [1]. Recent efforts suggest that porous collagen scaffolds enhance regeneration both in injured adult skin and peripheral nerves by disrupting the formation of a contractile cell capsule at the edges of the wound. Notably, capsules or clusters of contractile cells were hypothesized to impose a universal mechanical barrier during wound healing which, if disrupted appropriately, enhances the quality of induced regeneration in a wider range of organs [64].

Four physical and structural properties must be controlled to critical levels for the fabrication of an active ECM analog: the degradation rate which defines the template residence time, the chemical composition, the pore microstructure (mean pore size, shape, and orientation), and the scale of the scaffold that is defined by the critical cell path length [1]. These characteristics and any governing models that help to describe cell behavior in active ECM analogs will be discussed briefly in the following section.

Template residence time

The length of time that the scaffold remains insoluble in the wound site (residence time) is critical in defining its bioactivity. For physiologic tissue to be synthesized at a wound site, the scaffold must initially support cell migration, proliferation, and organization, but the scaffold must then degrade in such a manner that it does not interfere with the native tissue synthesis and remodeling processes. These considerations require a scaffold residence time with an upper and lower bound, a concept that has been formalized as the isomorphous tissue replacement model: the scaffold residence time must be approximately equal to the time required to synthesize a mature tissue via regeneration at the specific tissue site under study [65]. In the case of a full-thickness skin wound, for example, this healing time is approximately 25 days, and the degradation kinetics of the active ECM analog that induces skin regeneration was optimized for that time period [13]. Alternatively, the healing time of a peripheral nerve injury is dependent on the gap length: peripheral nerve trunks grow unidirectionally, from the proximal toward the distal stump, at a rate of approximately 1 mm/day [66]. Accordingly, during induced regeneration of peripheral nerves across a gap, a scaffold in contact with the nerve stumps (either the tube into which the stumps are inserted or the scaffold structure in the tube lumen) must remain in an insoluble state over a period approximately equal to the time, of order 10−20 days, during which axon elongation proceeds from the proximal to the distal stump.

An intact scaffold cannot diffuse away from the wound bed; therefore the simplest method for achieving isomorphous tissue replacement requires the insoluble scaffold structure to be degraded by enzymes in the wound bed into low molecular weight fragments. The lifetime of the scaffold is defined by the degradation time constant (t_d) and can be compared to the normal healing process time constant for a wound at the anatomic site of interest (t_h) [67]. When the scaffold remained in the wound bed as a non-degradable implant ($t_d/t_h > 1$), dense fibrous tissue similar to scar was synthesized underneath the scaffold [1,13,68]. When the scaffold degraded rapidly ($t_d/t_h < 1$), wound healing was marked by wounds contraction and scar synthesis similar to the reparative healing process observed in an ungrafted wound and no regeneration was observed [67]. Because different wound sites and even the same wound site in different species may have different time constants for healing (t_h), it is necessary to adjust the degradation rate of the ECM analog for each wound site and species in order to satisfy the isomorphous tissue replacement requirement and induce regeneration.

Scaffold resistance to degradation can be increased by increasing the relative density (solid content) of the scaffold. However, a more elegant option that does not involve changing the

structural characteristics of the scaffold is also available: scaffold degradation resistance increases with increasing crosslink density between the fibers that make up the scaffold structure [13,69–71]. In the case of collagen-based scaffolds, degradation in the wound bed is accomplished primarily by native collagenases. Decreased degradation rates for collagen-based scaffolds has been achieved by introducing glycosaminoglycans (GAGs) into the collagen mixture, which results in the formation of additional crosslinks, and by further crosslinking the resultant scaffold using a multitude of physical and chemical crosslinking processes. Dehydrothermal (DHT) crosslinking is a physical technique where the scaffold is exposed to a high temperature under vacuum, leading to the removal of water from the scaffold. Drastic dehydration (<1% water content) of the scaffold leads to the formation of inter-chain amide bonds through condensation [72]. DHT crosslinking is adjustable; exposure to higher temperatures or longer lengths of time produces a higher crosslink density and slower degradation rate [70,73]. UV treatment is a second physical crosslinking technique that can create crosslinks between collagen fibers due to the effects of radiation [74]. Chemical crosslinking treatments, such as glutaraldehyde [73] or carbodiimide [75] exposure can also be utilized to induce covalent bonds between collagen fibers. These crosslinking techniques are considerably more powerful, resulting in significantly higher crosslink densities and slower degradation rates. However, chemically crosslinked scaffolds must be extensively washed to remove all traces of the typically cytotoxic chemicals. Additionally, some chemical crosslinkers integrate a portion of the chemical compound into the crosslink; degradation of these scaffolds releases cytotoxic agents into the wound site so these techniques must be used with care [76,77]. While described in detail for collagen-based scaffolds, these crosslinking tools and techniques can be applied to a multitude of scaffolds, fabricated from both natural and synthetic materials.

Chemical composition

The chemical composition defines the ligands displayed on the scaffold surface. Cell behaviors such as attachment, migration, proliferation, and contraction are all mediated by interactions between the focal adhesions and integrins expressed on the cell surface and the ligands available on the scaffold surface. An active ECM analog must be fabricated in a manner, and from specific materials, that leads to expression of a chemical environment conducive to cell-scaffold interactions that prevent organized wound contraction and scar synthesis and that instead induce regeneration.

A multitude of different materials have been used to fabricate scaffolds for many tissue-engineering applications including studies of induced *in vivo* regeneration. Several synthetic, non-degradable polymers such as (poly)dimethyl siloxane have been occasionally utilized; these polymers, which parenthetically violate the principle of isomorphous tissue replacement, do not express ligands on their surface and have not induced regeneration. Degradable synthetic polymers such as poly(L-lactide) and poly(lactide-co-glycolide) variants have been fabricated to satisfy isomorphous tissue replacement and with surfaces that have been modified chemically to display appropriate ligands; however, these materials have not been observed to prevent contraction and scar formation nor to induce regeneration of stromal tissue. A chemical composition that has been used successfully to induce regeneration is a graft copolymer of type I collagen and a sulfated glycosaminoglycan (GAG) [1,13]. Collagen is a significant constituent of the natural ECM, and collagen-based scaffolds have been used in a variety of applications due to many useful properties: low antigenic response and a high density of ligands that interact specifically with integrin receptors in fibroblasts (the cell type predominantly responsible for cell-mediated contractions processes during repair). Particular collagen scaffolds have been observed to promote cell and tissue attachment and growth as well as to induce regeneration of tissue following injury [1,13,78–81]. However, in order to induce regeneration, the periodic banding (~64 nm) of the collagen fiber structure must be selectively abolished to prevent platelet aggregation that leads to repair [1]. A number of other natural protein-based scaffolds (i.e., hyaluronate-based, fibrin-based, and chitosan-based

scaffolds) have also shown great promise in the field of tissue engineering. These natural polymers are also capable of facilitating cell binding similar to that observed with collagen scaffolds in part due to their expression of natural ligand binding sites, but these materials have not been developed to the point of inducing regeneration at this point.

Template pore microstructure

The biological activity of any ECM analog also depends significantly on its three-dimensional pore microstructure. Having migrated into the scaffold, the cell interacts with the surrounding scaffold environment, making use of its cell surface receptors to bind to specific ligands on the scaffold surface. The first critical component of ECM analog microstructure to consider is the open- or closed-cell nature of the scaffold. An open-cell pore microstructure exhibits pore interconnectivity which a closed-cell microstructure exhibits membrane-like faces between adjacent pores, effectively sealing the environment of one pore from its neighbors. Pore interconnectivity is critical for scaffold bioactivity because cells to be able to migrate through the construct and to interact with other cells in a manner similar to that observed *in vivo*. A second critical structural feature is the relative density (R_d) or porosity of the ECM analog. The relative density of the scaffold is calculated as the ratio of the scaffold density to the density of the solid from which the scaffold is made. Porosity, a measure of how porous the scaffold is, is the pore volume fraction of the scaffold. Additionally, the relative density and porosity are inversely related: scaffold porosity can also be defined as $(1 - R_d)$. These variables both define the amount of solid material in the scaffold; when the pores are closed, too small, or when the relative density is too large, cells are not able to migrate through the scaffold, a significant impediment for a tissue-engineering scaffold. An active ECM analog must possess an open-cell pore structure with a relative density below a critical value that is characteristic of each application, but is typically significantly $<10\%$ (bioactive porosity typically $>90\%$). This structural criteria, determined from the results of a number of experiments studying cell-scaffold interactions, suggest that a critical number of cells are required within a bioactive scaffold [1].

The mean pore size of the ECM analog significantly influences its bioactivity; hence the effect of mean pore size will now be discussed in conjunction with the application of cellular solids modeling and a discussion of scaffold specific surface area. To describe even a simple cell-scaffold interaction, a highly detailed model describing the number of receptors utilized per bound cell and the nature of the binding and receptor sites needs to be developed. However, a more generic explanation can be used to describe the complexity of the cell-scaffold interaction and the significant influence the pore size has on its bioactivity. At first pass, it is apparent that there is a minimum pore size requirement for a bioactive ECM analog: the pores must be large enough to allow cells to fit through the pore structure and populate the analog. There is also an observed upper bound to pore size [1,13]; it has been hypothesized that this upper bound in mean pore size if due to the effects of scaffold specific surface area. To further test this hypothesis, cellular solids modeling techniques have been integrated to better describe scaffold microstructure using a quantitative framework. The complex geometry of foams (and of scaffolds) is difficult to model exactly; instead, dimensional arguments that rely on modeling the mechanisms of deformation and failure in the foam, but not the exact cell geometry, will be used [82]. Scaffold relative density (R_d) and mean pore size (d) together define the scaffold specific surface area (SA/V), the total surface area of pore walls available for cell attachment. A cellular solids model has been developed and experimentally validated to accurately predict SA/V of collagen-based scaffolds [83]:

$$\frac{SA}{V} = \frac{10.17}{d}(R_d)^{1/2} \tag{1}$$

Increasing mean pore size in a series of constructs and keeping R_d constant decreases the overall SA/V, while increasing R_d and keeping the mean pore size constant increases SA/V. The

primary feature of this structural analysis to consider is that a change in the construct SA/V indicates a change in the available area to which cells bind. This calculation and experimental result suggests the significance of the scaffold SA/V in defining its bioactivity. If the SA/V is too small (i.e., due to a relatively large mean pore size), an insufficient number of cells will be able to bind to the scaffold and the cells that remain free will contribute to the spontaneous repair mechanism. As previously discussed, there is also a minimum mean pore size, defined by the characteristic dimension of the cell: approximately 10−50 μm for most cells. When the scaffold pore size is smaller than this critical dimension, cells will be unable to migrate through the scaffold whereas when the pore size is too large an insufficient specific surface area will be available. These upper and lower bounds of the scaffold mean pore size, mediated by cell size and specific surface requirements, have been determined experimentally for each cell type for tissues where regenerative templates have been used [1].

The shape of the pores that make up the porous scaffold must also be considered. Cells have been observed to be exquisitely sensitive to the mechanical properties of the underlying substrate [84], and slight changes in the mean shape of the pores can result in significant variations in the extracellular mechanical properties [82,85] and overall construct bioactivity [86]. Changes in mean pore shape may also play a role in defining the areas of the scaffold available or unavailable for binding and the predominant direction of cell migration as well as in the geometrical organization of cells within the scaffold.

Critical cell path length

Successful migration of cells into an active ECM analog and their initial survival is critically important for successful regeneration. While the effect of the structural characteristics (i.e., pore size, shape, relative density) on the bioactivity of an ECM analog has been discussed, there is another important characteristic to consider: an adequate source of metabolites (i.e., oxygen, nutrients). There are two mechanisms available for transport of metabolites to and waste products from the cells: diffusion to and from the surrounding wound bed or transport along capillaries that have sprouted into the scaffold as a result of angiogenesis. While angiogenesis becomes the limiting factor for long-term cell survival and growth, significant angiogenesis is not observed for the first few days after implantation. Therefore, early cell survival inside the scaffold is defined solely by diffusion. The critical scaffold thickness, the maximum scaffold thickness than can be supported by metabolite diffusion, has been observed empirically in to be on the order of a few millimeters [1].

A quantitative model of cell metabolic requirements and nutrient diffusion characteristics that defines the critical cell path length for cell migration into a scaffold has been developed to describe the salient features of this process [1,87,88]. Here, the complexity of the nutritional requirements of the cell is simplified by generically considering a critical nutrient that is required for normal cell function; such a nutrient is assumed to be metabolized by the cell at a rate R in moles/mm^3/sec. The nutrient is pictured being transported from the wound bed, where the concentration of nutrient is assumed to be a constant, C_o, over a distance L via the exudate until it reaches the cell within the scaffold. Immediately following implantation of the scaffold, nutrient transport is performed exclusively via diffusion that can be modeled using the scaffold diffusivity D in mm^2/sec. Dimensional analysis readily yields the cell lifeline number (S):

$$S = RL^2/DC_o \tag{2}$$

The cell lifeline number characterizes the relative ratio of the rate of nutrient supply to nutrient consumption by the cell. If the rate of consumption of the critical nutrient exceeds greatly the rate of supply, $S >> 1$, the cell will soon die. At steady state ($S = 1$) the rate of consumption of nutrient by the cell equals the rate of transport via diffusion over a distance L; at steady state, the value of L is the critical cell path length, L_c, the longest distance away from the wound bed that the cell can migrate without requiring nutrient in excess of that supplied by diffusion. For

many cell nutrients of low molecular weight, L_c is of order a few hundred micrometers to a few millimeters, a distance short enough to suggest the need for very close proximity between wound bed and implant and to indicate that *in vivo* regeneration of large tissue or organs requires incorporation of special promoters to angiogenesis [1,67].

As a result, recent experimental work has focused on understanding the relationship between scaffold pore microstructure and permeability; scaffold permeability controls diffusion-based metabolite and waste transport to and from the scaffold and influences the final hydrostatic pressure distribution in the scaffold. Both of these parameters can significantly influence cell behavior and overall scaffold bioactivity. Cellular solid modeling tools that quantitatively describe scaffold permeability in terms of salient microstructural features have been utilized in this analysis. Both scaffold pore size and compressive strain can vary significantly with different applications, making them the primary features to characterize in terms of scaffold permeability. An open-cell foam, cellular solids model has been developed to accurately model scaffold permeability (K) in terms of scaffold mean pore size (d), percent compression (ε), a system constant (A), and scaffold relative density (ρ^*/ρ_s) was developed [89]:

$$K = A \cdot d^2 \cdot (1-\varepsilon)^2 \cdot \left(1 - \frac{\rho^*}{\rho_s}\right)^{3/2} \tag{3}$$

The cellular solids model (Eq. 3) of scaffold permeability suggests that scaffold permeability increases with increasing pore size and decreases with increasing compressive strain, a result that has also been observed experimentally for a series of collage-based scaffolds. The excellent comparison between experimentally measured permeability and that which is predicted from the cellular solids model suggests that such predictive modeling tools can be used to describe scaffold permeability for many different scaffold architectures under a variety of physiological loading conditions.

Active collagen-glycosaminoglycan scaffolds

Collagen-glycosaminoglycan scaffolds have been observed to have a high degree of bioactivity and be able to induce regeneration of non-regenerative tissues at a variety of anatomical sites. Three specific wounds and the appropriate ECM analogs have been studied in our laboratory: skin regenerated via the DRT [1,13,90], peripheral nerves regenerated by the nerve regeneration template (NRT) [71,86,91,92], and the conjunctiva regenerated by a modified DRT [93]. As predicted, the bioactivity of the DRT and NRT has been found to be closely related to specific physical parameters of the scaffold. Scaffold mean pore size (Fig. 18.2) [13], degradation rate (Figs. 18.3, 18.4) [13,71], chemical composition [13], pore orientation [86], and the pore volume fraction have all been shown to have a significant effect on the quality of regeneration and it appears that only a narrow range of structural features satisfies the criteria for bioactivity [1].

BASIC PARAMETERS FOR *IN VIVO* REGENERATION STUDIES: REPRODUCIBLE, NON-REGENERATIVE WOUNDS

In vivo regeneration of injured or excised tissue can be modeled as a process taking place within a bioreactor that is surrounded by a reservoir with constant properties; the bioreactor symbolizes the entire organism with its complex homeostatic mechanisms. From an engineering standpoint, the bioreactor must have a defined and consistent anatomical and physicochemical environment for quantitative study of any biological process. In order to study induced *in vivo* regeneration, it is therefore critical to standardize the wound site where studies are performed. Without a standardized, reproducible wound site, it is impossible to accurately and statistically assess differences between ECM analogs within a single laboratory or to compare results from different, independent laboratories. Billingham and Medawar [94] introduced the concept of an anatomically constant wound for the study of massive skin

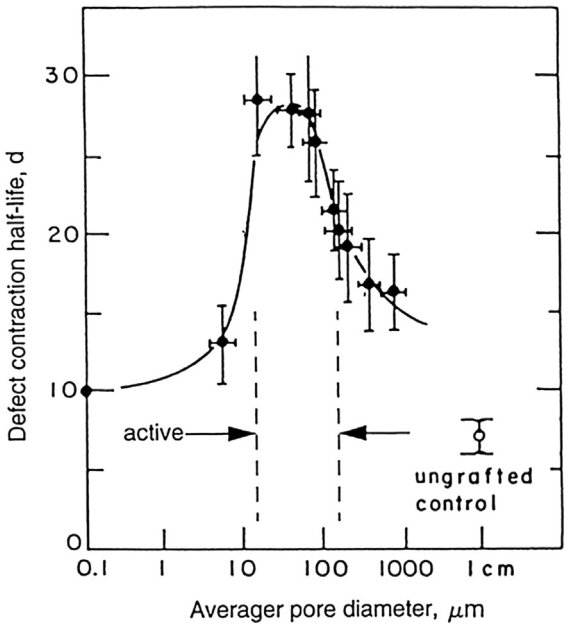

FIGURE 18.2
Bioactivity of ECM analog variants for skin regeneration versus scaffold mean pore diameter [13].

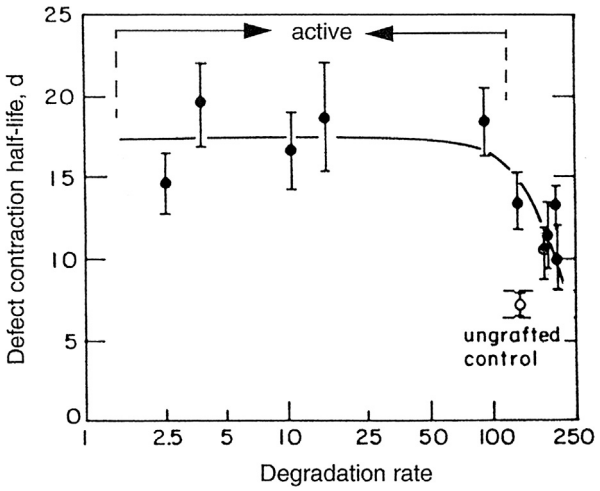

FIGURE 18.3
Bioactivity of ECM analog variants for skin regeneration versus scaffold degradation rate [13].

injuries. This concept has been amplified and standardized; it is referred to as the anatomically well-defined wound [1] and has been applied to the study of injuries in skin, peripheral nerves and a variety of other wound sites. The anatomically well-defined wound for studies of skin regeneration is the full-thickness skin wound where all tissue (epithelia, basement membrane, stoma) is removed down to the underlying fascia [1]. For peripheral nerve regeneration studies, the standardized wound is complete transection (total or full axotomy) of the nerve trunk, typically at the midpoint of the sciatic nerve [1]. For studies of cartilage repair following injury, the standardized wound model is complete removal of the cartilage down to the underlying subchondral bone [95]. These models standardize the experimental wound environment, making it possible to obtain statistically significant results that can be compared meaningfully. However, in clinical cases, the nature of the wounds typically varies on a case-by-case basis, and it is important to understand the applicability of each treatment methodology to the range of injuries that are encountered. For example, while a tubular scaffold can be used to study peripheral nerve regeneration using the full axotomy model, a tubular implant would be difficult to implement clinically in the case of severe crush injuries that do not result in complete nerve trunk transection.

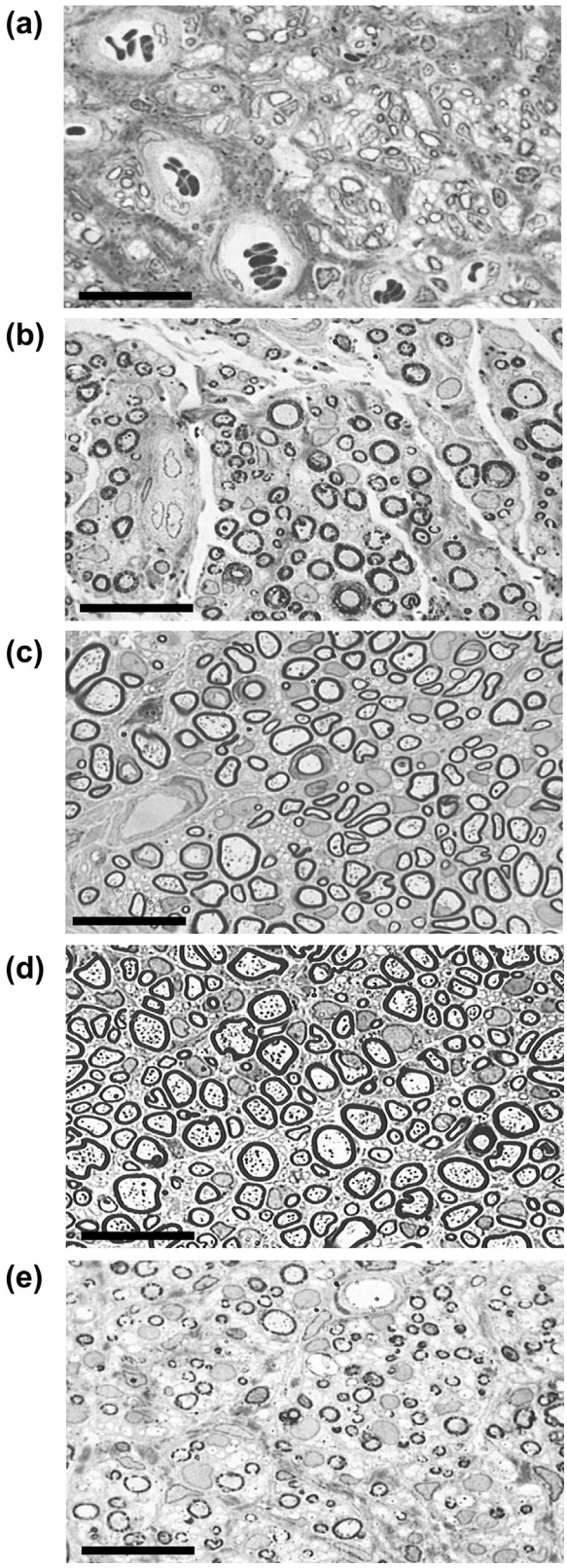

FIGURE 18.4
Histomorphometic, cross-sectional images of the regenerated nerve trunk from the five collagen tubes with distinct degradation rates, devices a to e. The devices are arranged in order of lowest to highest crosslink density, or most rapid to slowest degradation rate from a to e. Nerves trunks regenerated in devices c and d, characterized by intermediate levels of the crosslink density and degradation rate (device half-life: 2—3 weeks), showed superior morphology, with significantly larger axons, a more well-defined myelin sheath, and a significantly larger N-ratio. Light micrograph images of toluidine blue stained cross-sections of regenerated axons taken from the midpoint of the 15 mm gap. Scale bar = 25 μm [71].

EXAMPLES OF *IN VIVO* ORGAN REGENERATION

Skin regeneration

Patients exhibiting skin wounds with loss of a substantial fraction of total body surface area (TBSA) face an immediate threat to their survival, originating primarily from the loss of their epidermis. One result of this loss is an increase by an order of magnitude of the moisture evaporation rate, which if left uncorrected, leads to excessive dehydration and shock. Another result is a sharp increase in risk of massive bacterial infection, which if allowed to progress, frequently resists treatment and leads to sepsis. Even when patients manage to survive these immediate threats, there is a residual serious problem of quality of life, originating from the occurrence of crippling contractures and disfiguring scars due to the physiological repair process. Conventional treatment is based on use of autografting, which yields excellent results at the treatment site but which is burdened by the trauma caused at the donor site as well as by the unavailability of donor sites when the TBSA exceeds about 40–50%.

Of the three major tissue types that together comprise skin, the epidermis and basement membrane layers regenerate spontaneously following injury provided there is a dermal substrate (stromal layer) underneath. Complete regeneration of the damaged epithelia and basement membrane is typically observed following first and second degree burns, small cuts and scrapes, and blisters. However, complete skin regeneration is not observed in the case where substantial damage to the underlying stroma occurs; such cases include third-degree burns, deep cuts, and scrapes. In these cases, similar to the full-thickness, anatomically well-defined wounds created for studies of skin regeneration, organized wound contraction and scar synthesis is observed. A more in-depth description of the skin tissue triad, salient anatomical features, and its regenerative capacity has been previously published [1].

In addition to the clinical success of the collagen-based DRT, there are four other technologies developed to induce skin regeneration that have achieved variable levels of success that will be briefly discussed here to motivate further thought about scaffold-based options for treating severe injuries. Since its launch, this device (Integra Dermis Regeneration TemplateR) has been used with over 200,000 patients suffering skin loss around the world (Trasca T., Integra LifeSciences, Plainsboro, NJ). In the following section, the DRT, Cultured Epithelial Autograft (CEA), Living Dermal Replacement (LDR), Living Skin Equivalent (LSE), and the Naturally Derived Collagen Matrix (NDCM, Alloderm) will be discussed.

The DRT is a collagen-glycosaminoglycan scaffold whose microstructural and materials properties have been optimized to produce a bioactive ECM analog that, when implanted, induces sequential regeneration of the underlying dermis and resultant regeneration of the basement membrane and epithelial layers. The effectiveness of the DRT has been demonstrated clinically with a population of massively burned patients [1,58] and in animal experiments utilizing a full-thickness (anatomically well-defined) skin wound [1,13,68].

The DRT is typically used as an acellular implant that induces regeneration of the dermis, thereby providing the essential substrate for spontaneous regeneration of the epidermis and basement membrane layers. Following dermal regeneration by the unseeded DRT, epidermal cells from the wound edges migrate into the center of the wound and form a mature epidermis and basement membrane in a process termed sequential regeneration [58,96–99]. The resultant regeneration of appropriate tissue layers (tissue triad) along with associated structures (i.e., rete ridges) has indicated that the DRT is capable of inducing regeneration of mature skin in a full-thickness skin wound model (Fig. 18.5) [100].

Clinically, the DRT is used as an acellular, bilayer device consisting of an inner layer of the active ECM analog and an outer layer of elastomeric poly(dimethyl siloxane). The layer of silicone is removed about two weeks after grafting the device, having served the important

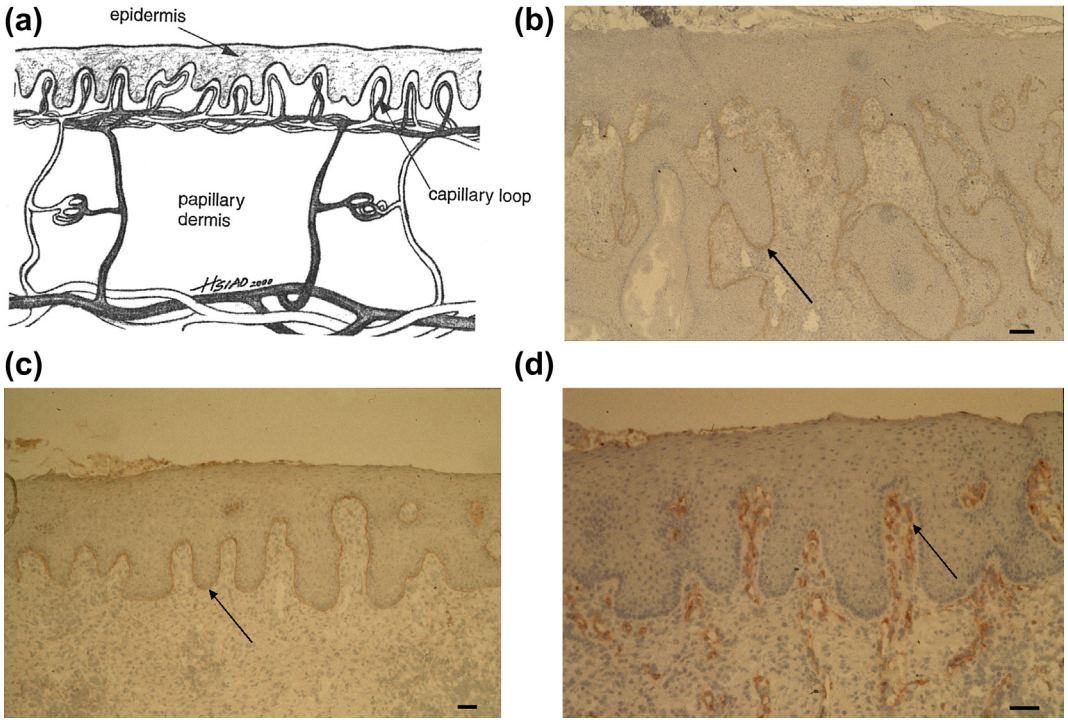

FIGURE 18.5
Diagram of normal skin showing the characteristic rete ridges at the dermo-epidermal junction and the vascular network (capillary loops) that populates the subepidermal region (a) [1]. As early as 12 days after grafting a full-thickness skin wound with a keratinocyte-seeded DRT, anchoring fibrils were observed in the regenerating basement membrane (arrow). The basal surface epithelium and the periphery of the epithelial cords are labeled with type VII collagen immunostaining, identifying the anchorage structures at the dermo-epidermal interface. Bar: 150 μm (b). As early as 35 days after grafting a full-thickness skin wound with a keratinocyte-seeded DRT, a confluent hemidesmosomal staining pattern is observed at the dermo-epidermal junction (arrow) by immunostaining for the $\alpha_6\beta_4$ integrin. The pattern observed in the regenerating skin is identical to that observed in physiologic skin. Bar: 100 μm (c). A full-thickness skin wound grafted with a keratinocyte-seeded DRT was observed to regenerate many of the structure observed in normal skin. Immunostaining for Factor VIII 35 days after grafting revealed that capillary loops had formed in the rete ridges of the regenerated dermis (arrow) similar to those observed in physiologic skin. Bar: 75 μm. (d) [100].

temporary role of controlling moisture flux and bacterial invasion at the site of organ synthesis. The DRT is fabricated from collagen-glycosaminoglycan copolymer with a 98:2 ratio of microfibrillar, type I collagen to chondroitin 6-sulfate. The microstructure of the DRT has been optimized with both a lower and upper pore size bound of 20 ± 4 μm and 125 ± 35 μm, respectively. Additionally, the biodegradation rate of the DRT has been optimized with lower and upper bounds of residence time in the wound bed of 5 and 15 days, respectively [1,13]. Clinical use of the DRT has emphasized the treatment of patients with massive burns, as well as those who require resurfacing of large or small scars from burns. The DRT is responsible for regenerating the underlying dermal architecture, providing the appropriate stromal layer upon which native regenerative processes of the basement membrane and epithelial layer can take place. For wounds of relatively small characteristic dimension, e.g., 1 cm, epithelial cells migrating at speeds of order 0.5 mm/day from the wound edges can provide a confluent epidermis within 10 days. In such cases, the unseeded DRT fulfills all the design specifications set for successful *in vivo* skin regeneration. However, the wounds incurred by a massively burned patient are typically of characteristic dimension of several centimeters, often more than 20–30 cm. These wounds are large enough to preclude formation of a new epidermis by cell migration alone within a clinically acceptable time frame. Current clinical protocol favors harvesting and use of a very thin autoepidermal graft from another part of the patient's body, thin enough not to leave behind a scarred donor site, to cover the neodermis [58,101,102], or

application of a CEA (described below) on top of the DRT following removal of the poly(dimethyl siloxane) membrane.

A keratinocyte-seeded DRT has been used in guinea pig and swine animal models; the keratinocyte-seeded DRT induces simultaneous formation of a dermis as well as the epidermal and basement membrane layers and removes the requirement for epithelial cell-mediated migration from the wound edges for successful skin regeneration. Although the keratinocyte-seeded DRT induces simultaneous regeneration of skin in the guinea pig model, an animal model characterized by extensive organized wound contraction that is significantly more severe than that observed with humans, the sum total of the results of studies using the DRT suggest that *in vivo* synthesis of skin in the clinical setting does not require anything more than the DRT [1].

CEAs are an epidermal graft formed from a sheet of keratinocytes grown *in vitro* and then implanted into the wound site [103–105]. A small epidermal sample is removed from the patient, dissociated, and the resultant cells are then cultured *in vitro* until they form an epithelial membrane. However, in the case of full-thickness skin wounds where there is no underlying dermis to support the epidermal sheet, rapid CEA degradation is typically observed and long-term clinical applicability has not been observed; in particular, one persistent problem is the formation of blisters under large areas of the graft (avulsion). However, in the case of partial-thickness wounds where a significant portion of the stroma remains, the 'take' of the CEA graft has been very good. As such, the CEA has been used to cover significant areas, as large as 50% of the TBSA, for both partial-thickness and as a temporary solution for full-thickness wounds to prevent immediate wound dehydration and infection. Additionally, regardless of whether the graft was placed on a partial- or full-thickness skin wound, the resulting CEA graft exhibited mechanical fragility due to a lack of the 7-S domain of type IV collagen, anchoring fibrils, and rete ridges [57]. These structures, required for the formation of a physiological dermo-epidermal junction, as well as the formation of a physiological collagen and elastin fiber architecture observed in the normal, adult dermis are not formed after grafting of the wound site with the CEA. Without these structures, the CEA cannot be used as a permanent skin replacement; instead, the CEA is often used as a temporary coverage as part of a more substantial treatment regimen such as autografting or DRT implantation.

The LDR is a polyglactin-910 nylon surgical mesh scaffold fabricated from a copolymer of 90 wt% glycolic acid and 10 wt% lactic acid termed PGL that was developed to be an analog of the ECM and induce skin regeneration following injury. The PGL fibers, approximately 100 μm in thickness, were knitted into a mesh that exhibits a pore microstructure with a characteristic dimension of 280–400 μm. This mesh structure presented a large-weave structure, relative to the characteristic cell dimension of approximately 10 μm that allowed rapid cell incorporation and ample nutrient diffusion. Prior to implantation, the acellular PGL mesh was cultured *in vitro* with fibroblasts until the cells were observed to synthesize several important ECM components such as collagen and elastin *in vitro*. Immediately prior to implantation, the upper surface of the PGL scaffold was seeded with keratinocytes to produce a bilayer graft. Once the keratinocytes reached confluence on the surface of the PGL mesh, the entire structure was grafted into the wound site [106]. A thin, fragile epidermal layer typically developed by 10 days post-grafting and became cornified as early as 20 days post-grafting. Additionally, by 20 days post-grafting the LDR scaffold had degraded completely with minimal inflammatory response. While the interface between the graft and the wound bed stained positive for laminin, consistent with the synthesis of a lamina lucida layer, no other component of the basement membrane was synthesized. In addition, rete ridges were not synthesized and a thick layer of fibrotic tissue with a large fibroblast population and vascular in-growth, characteristic of scar tissue formation, was observed below the newly synthesized epidermal layer. In summary, while the LDR showed the ability to induce regeneration of a neo-epidermis, it did not exhibit the ability to induce regeneration of a complete basement membrane or dermal layer [106].

The LSE was formed by populating a collagen lattice with dermal fibroblasts that *in vitro* contracted the lattice and synthesized additional ECM proteins, forming a neo-dermal layer [107]. Similar to the LDR, after the initial culture period, the upper surface of the neo-dermal layer was seeded with a suspension of keratinocytes. Once seeded, the keratinocytes attached to the collagen construct, proliferated, and differentiated to form a multilayered, epidermal structure within one to two weeks, all *in vitro*. Although short segments of the lamina densa were observed along the dermo-epidermal convergence *in vitro*, the cultured LSE did not exhibit a complete basement membrane layer, Rete ridges, or any other skin appendages at the end of the *in vitro* culture period [108]. At this point, the construct was then implanted into the wound site. Continued structural and biological changes were observed in the LSE following grafting, indicating that remodeling was taking place. A functional, fully differentiated epidermis was observed as early as seven days following grafting, and by fourteen days a vascularized subepidermal layer with many of the structural characteristics of normal dermis, such as a 'basketweave' collagen fiber pattern, was present. However, continued maturation into a formalized dermal region was not observed. Experimental results across multiple animal models and experimental trials were consistent in indicating that the LSE was able to induce regeneration of a mature epidermis and basement membrane, but a mature dermal layer was not observed and the dermo-epidermal junction remained flat.

Instead of relying on technologies to fabricate a three-dimensional scaffold structure from either synthetic or natural materials (i.e., LSE, LDR, DRT), the NDCM technology uses decellularized dermal tissue as a scaffold to induce skin regeneration. Decellularized matrices were explored as platforms that possess favorable mechanical properties for surgical handling, and which contained a surface chemistry in many ways similar to that of the native ECM [109]. The main advantage of the NDCM over a homograft (cellularized dermal tissue from a human donor) and xenograft (cellularized dermal tissue from an animal donor) is that owing to decellularization, the antigenicity of the scaffold is significantly reduced, thereby eliminating the incidence of rejection. The NDCM has been used primarily to treat full-thickness burns and burns to areas of the skin where contraction and scar formation would inhibit functionality (i.e., feet and hands). The NDCM is typically implanted into full-thickness skin wounds and is often covered by a thin autograft of the patient's own epidermis to speed the healing process. This treatment, similar to that observed with the use of the DRT, results in a high percentage of graft take; additionally, the thin autograft of epidermal tissue significantly decreases the time for complete graft re-epithelialization. Patients showed normal range of motion, grip strength, motor control, and functionality along with formation of appropriate anatomical structures (i.e., rete ridges) following treatment [110,111], demonstrating the utility of a naturally derived material in tissue-engineering applications.

Peripheral nerve regeneration

The mammalian peripheral nervous system also consists of a distinct tissue triad. Thousands of axons make up a nerve trunk where each axon is surrounded sequentially by an epithelial, basement membrane, and stromal layer. Schwann cells wrap around the individual axon, forming the myelin sheath that constitutes the cell-continuous, epithelia [112]. Surrounding the epithelia is the acellular ECM layer (basement membrane) connecting the myelin sheath and the stromal layer (Fig. 18.1). The endoneurium is the stromal layer of cells, blood vessels, and ECM that surrounds, insulates, and protects all nerve fibers [1]. Similar to that observed with skin, the myelin sheath (epithelia) and basement membrane regenerate spontaneously following injury (Fig. 18.6) while the endoneurium (stromal layer) does not [1,91].

GENERAL FINDINGS

While there have been a large number of studies investigating many different device designs, few have been shown to perform as well as the peripheral nerve autograft (typically the sural

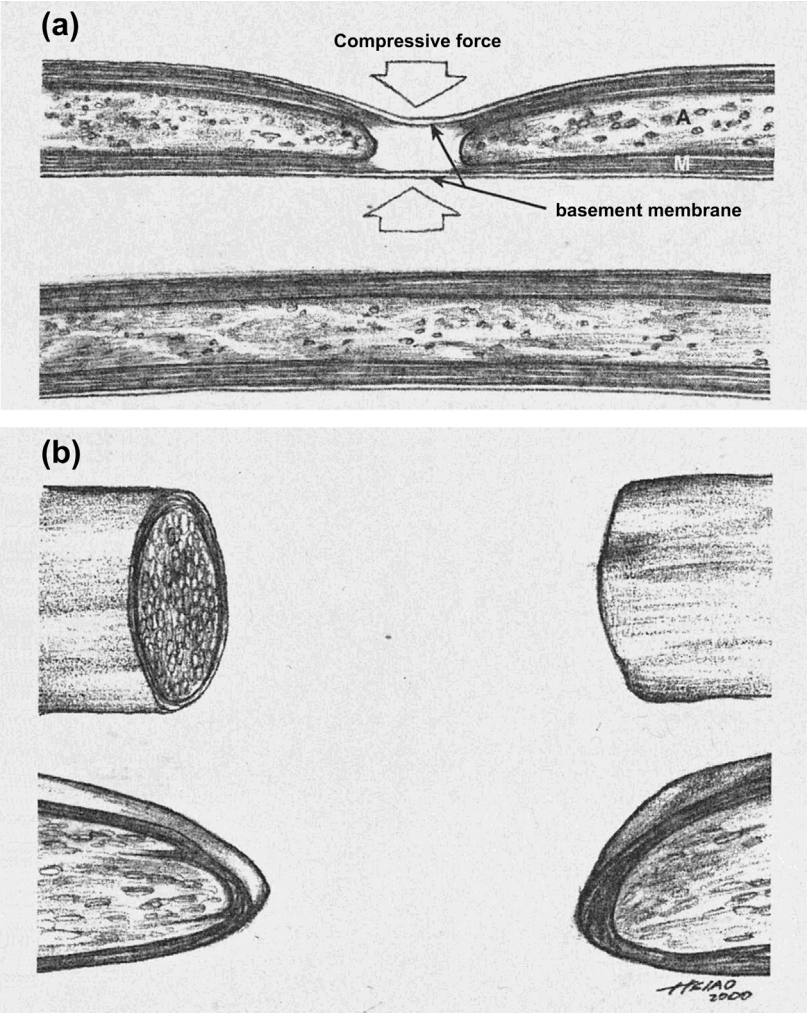

FIGURE 18.6
Axons and myelin sheath inside a nerve fiber are regenerative. Following mild crush injuries, the axoplasm (A) and myelin sheath (M) degenerates along the length of the crushed nerve fiber, but the basement membrane remains intact throughout. Spontaneous regeneration of the nerve fiber occurs within a few weeks of injury (a). Most of the supporting tissues (stroma) surrounding nerve fibers are not regenerative. Although axons are regenerative following transection, the remainder of the nerve trunk is not. Following transection, each stump is closed via cell-mediated contraction and neuroma synthesis (b) [1].

nerve from the leg), and none appear to have been able to improve over the autograft for gaps larger than 10 mm [113]. For studies of peripheral nerve regeneration, complete axotomy (total severance of the nerve trunk) was utilized as the standardized wound model. However, the large number of assays and experimental arrangements employed by independent investigators has made it difficult to effectively compare experimental outcomes. A new methodology has recently been formalized to standardize this comparison, allowing for effective assessment of the quality of peripheral nerve regeneration [114]. This technique compares the frequency of reinnervation (%N) across the experimental gap. The frequency of reinnervation across a gap is reported as percent of nerve trunks fitted with an experimental chamber where axons are observed to bridge the gap between the transected stumps; %N has been used by a large number of investigators as a dimensionless measure of nerve regeneration and, when not reported directly, can often be calculated from data presented by individual investigators.

The investigator reported %N can be normalized to correct for the effect of changes in gap length, animal species, and experimental chamber; the normalization process calculates the critical axon elongation length (L_c) [114], and is based on analysis of data from an early study which showed that a relatively small increase in the gap length bridged by a silicone tube is followed by a sharp drop in %N 115. Data from this and several other reports have been used to construct a sigmoidal curve, termed a 'characteristic curve', for %N versus gap length (L) for the silicone tube configuration. The critical length (L_c) is a single quantitative

parameter that describes the effectiveness of a given nerve repair device in promoting nerve regeneration, and is calculated as the length where the particular device results in 50% reinnervation (and 50% formation of neuroma). Reinnervation at the midpoint is used in this calculation rather than reinnervation at the distal stump because elongating axons reach the gap midpoint faster than the distal stump so midpoint data reach a time-independent state earlier than distal stump data. Given the widespread use of the unfilled silicone tube in studies of rat sciatic nerve defects, its characteristic curve ($L_c = 9.7 \pm 1.8$ mm) is often used to define a laboratory standard for all comparisons, allowing comparison of other experimental devices to the silicone standard by comparing their respective characteristic curves and L_c. Using the silicone tube or any other well-defined construct as a control, it is possible to estimate the length shift, $\triangle L$, the difference between values of L_c for the experimental chamber and that of the control. $\triangle L$ can be used as a simple measurement of relative regenerative activity exhibited by any device. Data obtained from different animal models can also be compared by normalizing each species result to the silicone tube standard tested in most animal models [114].

EFFECT OF TUBULATION

Peripheral nerve regeneration is not observed in the absence of a tubular device connecting the two ends of a transected nerve stump if the gap between the two transected stumps is greater than a few millimeters [1]. It has also been observed that insertion of the distal stump into the tube is required for successful regeneration [1,113]; this result indicates that even though axon elongation takes place from the proximal to the distal stump, the distal stump appears to provide a critical cytokine field responsible for guiding axon elongation (neurotrophic effect). However, while it has been observed that a tube is all that is required to induce regeneration across a gap of modest length following complete transection, the physical parameters of the tube and any material in the tube lumen significantly affect the kinetics and quality of regeneration; these effects will be discussed in the remaining sections of this review.

EFFECT OF TUBE CHEMICAL COMPOSITION

A variety of natural and synthetic polymers have been used in peripheral nerve regeneration conduits, and the choice of tube (conduit) composition has been found to significantly affect the quality of regeneration; for a complete review of the literature, please consult previous publications by the authors [1,116]. Early conduits were produced from naturally occurring materials that were easily harvested and implanted such as bone, dura, perineurium, and blood vessels [1]. More recent devices have utilized non-degradable, synthetic materials such as stainless steel, rayon, and silicone as well as degradable, synthetic polymers such as polyester, polyglactin, and polylactate, and natural polymers such as collagen, laminin, and fibronectin [1,117].

Non-degradable tubes have typically resulted in a poor quality of regeneration marked by significant formation of a neural scar (neuroma) [1,118]. Conversely, conduits fabricated from ECM components, specifically collagen, fibronectin, and laminin, have been generally shown to enhance the quality of peripheral nerve regeneration [1,86,91,119–123]. Collagen tubes in particular have been observed to induce the highest quality of regeneration, as measured by both morphological and electrophysiological methods [1,91,124]. It has been hypothesized that this optimal chemical composition was observed because collagen tubes contain inherently bioactive binding sites (ligands) for attachment and migration of various cell types, and can be manufactured in such a way that the tube wall pore structure can display a range of cell and protein permeabilities. Additionally, the degradation rate of the collagen tubes can be tailored to degrade with half-lives that vary over a very wide range to meet the requirements of the isomorphous tissue replacement model [71,86].

EFFECT OF TUBE PERMEABILITY

Conduit permeability significantly affects the mechanism and quality of peripheral nerve regeneration. Tube permeability can be defined by its initial structural features (i.e., scaffold porosity, pore size) or its degradation characteristics (i.e., rapid degradation quickly permeabilizes the tube). Additionally, the conduits may be protein permeable but cell impermeable or cell and protein permeable. No appreciable increase in the quality of peripheral nerve regeneration was observed for the protein permeable and cell impermeable conduit compared to the impermeable conduit [125–127]. However, cell (and therefore protein) permeable tubes exhibited significantly superior regenerative capacity compared to impermeable as well as protein permeable and cell impermeable conduits [1,127–132]. Device permeability also significantly influences the contractile response following peripheral nerve injury; permeable collagen tubes display a significantly thinner contractile capsule surrounding the regenerating nerve trunk than biodurable silicone tubes [118]. It is hypothesized that device permeability reduces myofibroblast-mediated contraction of the wound site by permitting migration of the contractile cells away from the wound site through the tube wall [1] and by allowing connective tissue cells from the surrounding environment access to the tube lumen [125].

EFFECT OF TUBE DEGRADATION RATE

A biodurable conduit, such as a silicone tube, initially induces partial reinnervation between proximal and distal stumps following implantation [118]. However, as the initially regenerated nerve trunk remodels and matures, the silicone tube constricts this process, resulting in pain and eventual degeneration of the regenerated nerve; prevention of the ultimate degeneration of the initially regenerated nerve trunk requires a difficult second surgical procedure that can further harm the nerve trunk. For this reason, one of the historical goals in studies of peripheral nerve regeneration studies is identification of a suitable degradable conduit. Superior performance of biodegradable tubes compared to tubes made from materials that were either biodurable or which had a very low degradation rate has since been reported in studies by several investigators [1,71,130,133–137]. Examples include comparison of tubes made of two biodurable polymers, silicone and poly(tetrafluorethylene), and two degradable polymers, a copolymer of poly(lactic acid) and ε-caprolactone (PLA/PCL) as well as collagen [137]. This study indicated that the degradable tubes induced a higher quality of regeneration compared to the biodurable tubes over a 6 mm gap in the mouse sciatic nerve. Additionally, a number of degradable collagen tubes have been shown to perform as well as the autograft, considered to be clearly superior to regeneration via a silicone tube and commonly thought to fall short functionally only of physiological (pre-injury) nerve [91,124,138–141].

While the regenerative advantage of a degradable tube versus a biodurable tube is supported by the evidence, there have not been many extensive studies on the effect of the magnitude of the degradation rate and whether an optimal degradation rate exists as has been found in the study of ECM analogs (the DRT) to induce skin regeneration [13]. Recently, one of the first comprehensive studies of the effect of the degradation rate of collagen tubes on the quality of peripheral nerve regeneration has been published by these authors; this study utilized a 15 mm gap in the rat sciatic nerve and evaluated the regenerative capacity of a homologous series of porous, collagen tubes, showing a significant effect of degradation rate on the quality of peripheral nerve regeneration [71]. The chemical composition, pore structure, and permeability of the conduits in this study were kept constant while the crosslink density was steadily increased to create a series of five devices with *in vivo* degradation half-lives varying between <1 week to >100 weeks, The quality of peripheral nerve regeneration was observed to vary significantly with tube degradation rate; the highest quality of peripheral nerve regeneration was observed for tubes with a degradation half-life of two to three weeks (Fig. 18.4) [71]. This data suggests that the positive effects of tubulation in treating peripheral nerve injuries are due to the presence of the tube immediately following injury; tubulation appears to significantly affect the early mechanisms of peripheral nerve regeneration. A speculative view

of the maximum regenerative activity observed in the present study can be based on the putative existence of a lower and higher limit in tube degradation rate similar to that observed for the DRT [13]. When the degradation rate of the collagen tubes is excessively slow, the tubes behave as if they were biodurable, remaining intact long enough to interfere with tissue remodeling. In contrast, tubes that degrade very rapidly fail to maintain a protected environment for regeneration that is the basis for the use of tubulation to promote nerve regeneration.

EFFECT OF TUBE FILLING

A wide variety of solutions, ECM analogs, and cell suspensions have been introduced into the tube lumen in an effort to improve the quality of peripheral nerve regeneration. Use of ECM macromolecules, such as collagen, laminin and fibronectin, in both solution and gel form, has been observed to have no significant effect on peripheral nerve regeneration; furthermore, when gel concentrations exceeded certain critical levels, a negative effect on the quality of regeneration is observed [121]. However, a laminin-coated collagen-based scaffold has been observed to improve the quality of regeneration, implying the requirement of an insoluble structure within the tube lumen in addition to any soluble regulators to improve the quality of regeneration [123].

Several insoluble substrates, ECM analogs inserted into the empty lumen of the tube prior to implantation, have shown significant regenerative activity. Examples include highly oriented fibrin fibers and axially oriented polyamide filaments that significantly improved the quality of regeneration [142–144]. Specific ECM analogs have been observed to significantly increase the maximal gap length that can be bridged by axonal tissue [1,120,145], the speed of axonal bridging [119,123], and the quality of regeneration [1,86,91].

The ECM analog that has been found to induce the highest quality of regeneration is a collagen-based scaffold termed the NRT [1,146]. The NRT has induced regeneration of a functional peripheral nerve across gaps varying from 10 to 15 mm in the rat sciatic nerve [1,91]. Like the DRT, the microstructural and material properties of the NRT have been optimized. The highly bioactive NRT is characterized by axially (extending between the proximal and distal stumps) elongated pore tracks defined by axially oriented ellipsoidal pores with a mean pore size of approximately 35 μm [86,147]. This pore structure is hypothesized to improve the quality of peripheral nerve regeneration by providing directional guidance to the formation of linear Schwann cell columns which act as tracks for axon elongation (microtube hypothesis) [1,148]. The positive effect of an axially oriented fiber structure has also been observed using a fibrin fiber-based ECM analog in the tube lumen [142]. The degradation rate of the NRT has also been found to significantly affect the quality of regeneration, with an *in vivo* degradation half-life on the order of six weeks found to be optimal; NRT variants that degraded too rapidly or too slowly led to significantly poorer functional recovery [1,146]. The long-term morphological structure and electrophysiological function of nerves regenerated using the NRT has been observed to be at the level of an autografted nerve, the current gold-standard for peripheral nerve injury treatment [91,149].

Soluble regulators and cell suspensions have also been introduced into the tube lumen in an effort to improve the quality of regeneration. Suspensions of Schwann cells showed very significant regenerative activity [150], further supporting the microtube hypothesis which describes nerve regeneration as dependent on early formation of linear columns of Schwann cells extending from the proximal towards the distal nerve trunk. In addition to the use of a cell suspension, solutions of acidic (aFGF) and basic fibroblast growth factor (bFGF) were also observed to improve the quality of regeneration [151,152]. However, the use of nerve growth factor (NGF) was not found to improve the quality of regeneration [153].

A variety of conclusions have been drawn about the relative efficacy of the various biomaterials and devices employed in the study of peripheral nerve regeneration. Nerve chamber configurations that had the highest regenerative activity were those in which the tube wall comprised certain synthetic biodegradable polymers such as collagen, was cell-permeable rather than protein permeable or impermeable, and had an *in vivo* degradative half-life on the order of 2 to 3 weeks. Introduction of an insoluble regulator (ECM analog in the form of a scaffold) into the tube lumen, but not of ECM components in a gel or solution form, also significantly improves the quality of regeneration. The optimal ECM structures were found to be highly porous with controlled degradation rates and an axially aligned microstructure. In addition, suspensions of Schwann cells as well as solutions of either acidic or basic fibroblast growth factor placed within the tube lumen with or without an insoluble ECM analog have been shown to improve the quality of regeneration.

Cartilage and fibrocartilage disk tissue-engineering applications

Articular cartilage contains an avascular, non-neural ECM composed primarily of type II collagen and glycosaminoglycans. Compared to other tissues, cartilage possesses a very low cell density and a very high ECM density; the chondrocytes populating the cartilaginous ECM display low proliferative activity and, due to the high ECM protein density, are unable to migrate through the tissue. Due to the low proliferative activity, avascularity, and high ECM protein density, injuries to cartilage display an injury response distinct from traditional injury responses characterized by inflammatory processes, cell-mediated contraction, and scar synthesis. No repair or regeneration processes are observed *in vivo* following cartilage injuries; instead, the scope of injury increases as the wound edges become increasingly degraded, eventually compromising the joint [95]. Severe cartilage injuries are extremely prevalent in today's active society, resulting in pain, decreased patient activity, and eventually disability, profoundly impacting quality of life. The current methods for treating such focal cartilage defects include microfracture, autologous chondrocyte implantation, and osteochondral autografting [95]. In the case of microfracture and autologous chondrocyte implantation, a flap of periosteum is sewn over the cartilage defect area and is sealed with fibrin glue. With microfracture, immediately prior to application of the periosteal flap, microfractures in the subchondral bone are created to allow bone marrow cells access to the then-sealed cartilage defect in an attempt to utilize the stem cell population in the bone marrow to regenerate the damaged cartilage. With autologous chondrocytes implantation, a biopsy of cartilage tissue is removed from the patient prior to surgery and cultured *in vitro* to obtain a large chondrocyte population that is then injected back into the periosteal flap sealed cartilage defect. In the case of osteochondral autografting, a series of osteochondral plugs consisting of cartilage, the underlying subchondral bone, and the tidemark region separating them are removed from a non-loading region at the edge of the damage joint. These plugs are then implanted into the primary cartilage defect using an approach termed mosaicplasty, named after the mosaic pattern of the implanted osteochondral plugs [95]. These procedures, however, exhibit limited long-term success in treating the cartilage injury. Preclinical studies implementing tissue-engineering approaches have yielded results that represent improvements over the currently employed cartilage repair procedures.

Various synthetic and natural materials have been employed to fabricate porous, bioresorbable scaffolds for articular cartilage tissue engineering. Among the list of bioresorbable or partially resorbable materials used for cartilage repair are collagen, hyaluronan, fibrin, polylactic acid (PLA) and polyglycolic acid (PGA), and chitosan scaffolds and gels, devitalized cartilage, hydroxyapatite, demineralized bone matrix, and bioactive glass [95]. Natural polymers such as collagen provide a more native surface to cells, and have been the primary focus of tissue-engineering studies due to previous successes in peripheral nerve and skin regeneration. Studies have confirmed that the addition of cells seeded within these 3D scaffolds enhance matrix synthesis and increase type II collagen production *in vivo* and *in vitro*

[95,154,155]. There are various cell types that may be used to enhance cartilage synthesis when seeded into matrices, including articular chondrocytes and chondroprogenitor cells derived from bone marrow, periosteum, or perichondrium [95,154].

Preliminary investigations have been focused on the bioactivity of chondrocytes in a series of ECM analogs. Promising results have been observed for adult articular chondrocytes cultured *in vitro* in type II CG scaffolds, where the chondrocytes have retained high biosynthetic capacity for producing type II collagen, the predominant ECM component of cartilage [156]. Type II CG scaffolds seeded with autologous, articular chondrocytes have also been evaluated for their *in vivo* regenerative capacity in a full-thickness cartilage injury model; the full-thickness cartilage injury, with cartilage removed down to the subchondral bone, is the standardized, anatomically well-defined wound utilized for *in vivo* studies of cartilage regeneration. The greatest total amount of reparative tissue was found in the cell-seeded type II CG scaffolds as opposed to unseeded type II CG scaffolds and seeded or unseeded type I CG scaffolds. Moreover, examination of the reparative tissue formed in the subchondral region of defects treated with the chondrocyte-seeded type II collagen scaffolds indicated that the majority of the tissue was positive for type II collagen and that good integration was observed between the implant and the surrounding cartilage. These results indicate an influence of the exogenous chondrocytes on the process of chondrogenesis [157]. Such studies of the healing of chondral defects in animal models have revealed that there is some potential for regeneration of this connective tissue. The introduction of certain biomaterial scaffolds along with selected surgical procedures and cell therapies has been demonstrated in animal studies to facilitate the cartilage reparative process and now offers the promise of extending the longevity of clinical treatments of cartilage defects [154,155,158]. Recently, CG scaffolds populated with TGF-β1 transfected meniscus cells were used to successfully fill avascular zone meniscus lesions with repair tissue [159]. Other work has compared type I and type II CG scaffolds for intervertebral disk tissue-engineering applications, finding that type II CG scaffolds were preferential to type I on the basis of cell number as well as protein and GAG synthesis [160].

In a separate series of studies, the regenerative potential of chondrocytes encapsulated in photopolymerized poly(ethylene oxide) hydrogels and self-assembling peptide hydrogels gels have been tested using an *in vitro* culture model followed by *in vivo* implantation. Preliminary results suggest that photocrosslinked [161] and self-assembling hydrogels [162,163] are promising scaffolds for tissue-engineering cartilage as cell viability was maintained, uniform cell seeding was achieved, the biochemical content of the ECM proteins synthesized within the construct were similar to those found in native cartilage [161,162]. Additionally, the importance of the biomechanical environment during *in vitro* culture of chondrocytes within a three-dimensional construct has been observed; improved biosynthesis of ECM components, notably native proteoglycans, is observed when a cyclic loading environment is applied to *in vitro* chondrocytes cultures within a series of hydrogel and scaffold constructs [164]. Collectively these findings provide the basis for the rational development of approaches for the more complete regeneration of articular cartilage, and demonstrate that meaningful clinical outcomes can be achieved even if complete regeneration is not achieved [154,155,158].

Bone, osteochondral regeneration applications

In addition to solidification-induced structural modifications, the CG construct can be chemically modified to create collagen composites. Mineralized CG scaffolds are of particular interest for orthopedic applications due to their potential to mimic the native biochemistry of bone: interpenetrating collagenous matrix (organic) and CaP (mineral) content [165,166]. The addition of a mineral phase to the classic CG scaffold archetype allows for the development of materials with the requisite biochemical and biomechanical properties for bone and osteochondral tissue engineering. Mineralized CG scaffolds have been created via two distinct mechanisms: coating a fully formed CG scaffold, or synthesizing mineralized CG chemistries

prior to scaffold fabrication. Mineralized collagen-GAG have been fabricated via a surface coating process [167]. Here, CG scaffolds were initially synthesized using conventional freeze-drying and then sequentially soaked in phosphate ($NaNH_4HPO_4$) and calcium ($CaCl_2$) solutions. This treatment improved construct compressive modulus nearly 70-fold, from 0.3 kPa to 31 kPa, while maintaining high porosity (95%) [167]. Alternatively, CGCaP scaffolds have been fabricated from a triple co-precipitate suspension of collagen, GAG, and calcium phosphate (CaP) [168,169].

The CGCaP scaffold technology has been used as the basis for creating multiphase collagen scaffolds for the repair of interfacial tissues, notably osteochondral defects. The multi-compartment scaffold was designed to be a single biomaterial construct containing multiple regions ('compartments'), each with distinct microstructural, chemical, and mechanical properties that are connected via a continuous interface between regions. The initial multi-phase scaffold developed via this approach contains an osseous compartment for subchondral bone regeneration (type I collagen, chondroitin 6-sulfate, and CaP) and a cartilagenous compartment for cartilage regeneration (type II collagen and chondroitin 6-sulfate). The continuous interface can be created by layering the cartilaginous compartment and the osseous compartment suspensions in a conventional freeze-drying mold, but then incorporating a processing step to enable partial diffusive mixing between the two suspensions near their interface. After forming an interdiffusion zone, freeze-drying was used to form the final multicompartment scaffold microstructure (Fig. 18.7) [170]. This approach can reduce some complications observed in layered scaffolds with abrupt interfaces including delamination, foreign body contamination (from glue or other adhesive), and inefficient cellular transport between scaffold phases [170]. The differential chemistry, microstructure, and mechanics of the osseous and cartilaginous compartments enable these layered scaffolds to exhibit compressive deformation behavior that mimics behavior observed in natural articular joints [170]. These layered scaffolds are currently the subject of numerous *in vitro* and *in vivo* experiments for various orthopedic tissue-engineering applications. This scaffold system has shown improved healing of osteochondral defects in a caprine model [171] and is currently undergoing Phase I clinical trials for primary and secondary (backfill of traditional mosaicplasty harvest sites) osteochondral defects in the knee. The developed technologies and techniques may hold promise for the regeneration of not only osteochondral defects, but also other orthopedic interfaces such as the osteotendinous insertion.

CONCLUSIONS

Following severe injury, the typical physiological response is characterized by a complex inflammatory response, cell-mediated wound contraction, and scar tissue synthesis characterized as repair. However, introduction of a suitable analog of the extracellular matrix into the wound site has been observed to block cell-mediated contraction of the wound site and induce regeneration of physiological tissue. Although several ECM analogs have been studied, only those with a narrowly defined structure have been shown to be capable of regeneration. The microstructural, chemical compositional, and biodegradation rate specificity of these templates appears to be related to the requirement for inhibition of wound contraction prior to the incidence of regeneration. A number of appropriate materials have been developed and tested for use in particular *in vivo* wound sites, notably the skin, peripheral nerve, conjunctiva, and cartilage. The results of *in vitro* and *in vivo* investigations of many biomaterial constructs suggest that the properties of an ECM analog must be tailored to the specific wound site and species, requiring further development of appropriate *in vivo* and *in vitro* models to further understand the complexity of cell-scaffold interactions and the process of induced regeneration.

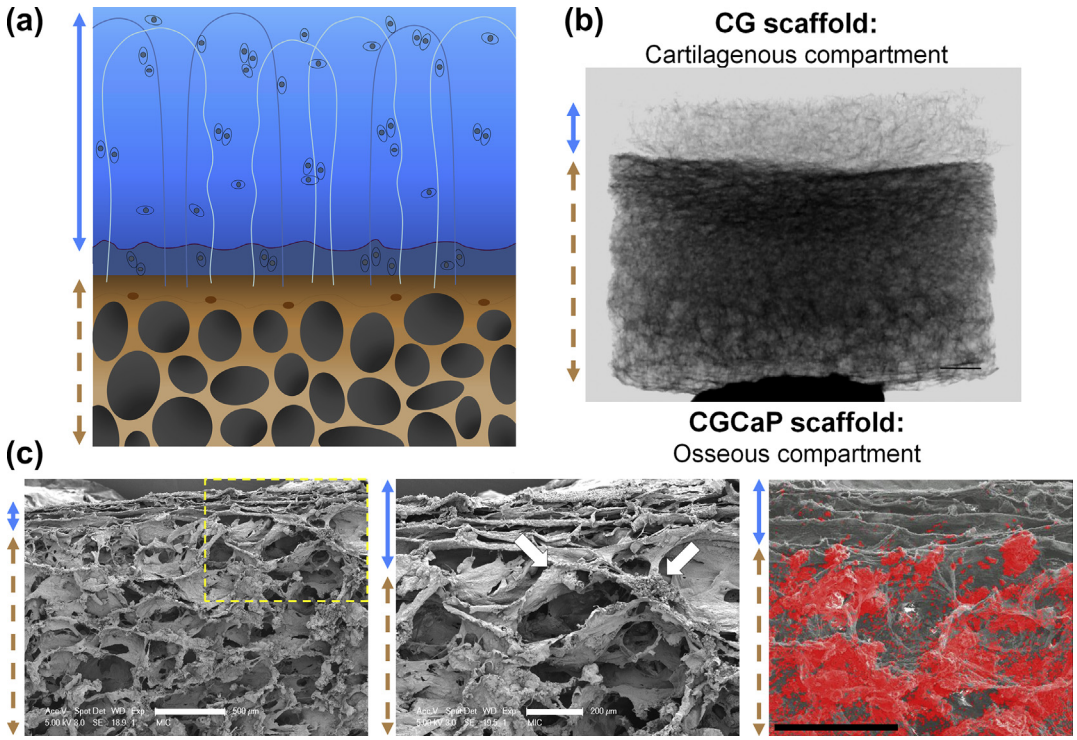

FIGURE 18.7

a) Structure of the natural articular joint showing articular cartilage and subchondral bone joined by a continuous interfacial region. b) X-ray μCT image of the layered osteochondral scaffold showing distinct cartilaginous and osseous compartments (scale bar 1 mm). c) Scanning electron microscope (SEM) images of the osteochondral scaffold showing the complete scaffold microstructure (left; scale bar 500 μm), and the interfacial region (middle; scale bar 200 μm) showing continuity between the osseous (tan dashed arrow) and cartilaginous (blue solid arrow) compartments including collagen struts extending across the transition (white arrows). No regional areas of delamination or debonding are observed between the compartments. Distribution of Ca mineral (P similar but not shown) content (red shading) superimposed over an SEM image of the osteochondral scaffold showed distinct mineralized (high CaP content, tan dashed arrow) and non-mineralized (low/zero CaP content, blue solid arrow) layers (right; black scale bar 400 μm). *Reprinted with permission from [170].*

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PART 4

Biomaterials in Tissue Engineering

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CHAPTER 19

Micro-Scale Patterning of Cells and their Environment

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INTRODUCTION

Control of the cellular environment is crucial for understanding the behavior of cells and for engineering cellular function [1–3]. This chapter describes the use of a set of tools in microfabrication called 'soft lithography' for patterning the substrate to which cells attach, the location and shape of the areas to which cells are confined, and the fluid environment surrounding the cells, all with micrometer precision. We summarize examples where these tools have helped to control the microenvironment of cells, and have been useful in solving problems in fundamental cell biology. The methods described here are experimentally simple, inexpensive, and well suited for patterning biological materials.

How do tissues assemble *in vivo*? How do cells interact with each other in tissues? How do cells respond to stimuli? How do abnormal stimuli give rise to pathological conditions? Answering these fundamental biological questions, and using the information thus obtained for medical applications, requires understanding the behavior of cells in well-controlled micro-environments. Many of the challenges in trying to control the environment experienced by individual cells lie in the relevant scales of size, as well as the character of the stimuli. These scales of size range from angstroms (for molecular detail), through micrometers (for an individual cell), to millimeters and centimeters (for groups of cells); the types of stimuli that must be addressed include the molecular composition of the liquid in which the cell is immersed, the topographical and chemical composition of the surface to which the cells attach, the nature of neighboring cells, and the temperature.

Researchers have used a number of techniques to pattern cells and their environment [4]. Before the 1990s, the most commonly used technique was photolithography, used in biological studies with varying degrees of success [4–6]. Examples include topographical

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features that confine the growth of snail neurons to silicon chips, as first demonstrations of interfacing natural computation with artificial ones [7,8]. However, photolithography is not a technique best suited for biological studies. It is an expensive and time-consuming technology; it is poorly suited for patterning non-planar surfaces; it provides too little control over surface chemistry to pattern sufficiently diverse types of biomolecules on surfaces; it is poorly suited for patterning materials such as hydrogels; the equipment required to use it is rarely routinely accessible to biologists; and it is directly applicable to patterning only a limited set of photosensitive materials (e.g., photoresists).

Microfabrication and micropatterning using stamps or molds fabricated from elastomeric polymers ('soft lithography') provide versatile methods for generating patterns of proteins and ligands on surfaces, micro-scale chambers for culturing cells, and laminar flows of media in capillaries, all in the 0.1–100 micrometer size range [1–3,9]. Soft lithographic methods are relatively simple and inexpensive. The elastomeric polymer most often used in these procedures – polydimethylsiloxane (PDMS) – has several characteristics (optical transparency, ease in manipulation, and low cost) that make it attractive for biological applications [10]. As a new technology, soft lithography is being increasingly used in cell biology, due to its biocompatibility, simplicity, and adaptability to biological and biochemical problems. This chapter gives an overview of the application of soft lithography to the patterning of cells and their fluidic environment, using micro-scale features and laminar flows.

SOFT LITHOGRAPHY

Soft lithography solves many of the problems that required the application of microfabrication to biological problems [1,2,11]. Soft lithographic techniques are inexpensive, are relatively procedurally simple, are applicable to the complex and delicate molecules often dealt with in biochemistry and biology, can be used to pattern a variety of different materials, are applicable to both planar and curved substrates [12], and do not require stringent control (such as a clean room environment) over the environment in which they are fabricated beyond that required for routine experiments with cultured cells [2,3]. Access to photolithographic technology is required only to create a master for casting elastomeric stamps or membranes, and even then, the requirement for chrome masks – the preparation of which is one of the slowest and most expensive steps in conventional photolithography – can often be bypassed in favor of high-resolution printing [13,14]. Soft lithography offers special advantages for biological applications, in that the elastomer most often used (PDMS) is compatible with most types of optical microscopy commonly used in cell biology, is permeable to gases such as O_2 and CO_2, is mechanically flexible, seals conformally to a variety of surfaces (including most types of Petri dishes), is generally biocompatible [15], and can be implantable *in vivo*. The soft lithographic techniques that we will discuss include self-assembled monolayers (SAMs), microcontact printing (μCP), microfluidic patterning (μFP), laminar flow patterning (LFP), patterning with micro-topographies, we also introduce application of these tools in dynamic control of surfaces and three-dimensional (3D) patterning.

SELF-ASSEMBLED MONOLAYERS (SAMS)

Introduction to SAMs

Since many of the studies involving the patterning of proteins and cells using soft lithography have been carried out on SAMs of alkanethiolates on gold, we give a brief introduction to SAMs here [16–20]. SAMs are organized organic monolayer films normally formed by exposing a surface of a gold film to a solution containing an alkanethiol (RSH). SAMs allow control at the molecular level by chemical synthesis of derivatized alkanethiol(s); this molecular control, in turn, gives control over the properties of the interface. The properties of surfaces covered with SAMs are often largely or entirely determined by the nature of the terminal groups of these alkanethiols. The ease of formation of SAMs, and their ability to present a range of

chemical functionality at their interface with aqueous solution, make them particularly useful as model surfaces in studies involving biological components. Furthermore, SAMs can be easily patterned by simple methods such as microcontact printing (μCP) with features down to 100 nanometers in size [21,3]. These features of SAMs make them structurally the best-defined substrates for use in patterning proteins and cells. SAMs on gold are used for many experiments requiring the patterning of proteins and cells, because they are biocompatible, easily handled, and chemically stable. SAMs on silver, although better defined structurally than those on gold, cannot be used in most experiments with cultured cells, due to the toxicity of silver [22]. SAMs on palladium and platinum are just starting to be explored [23,24].

The substrates for SAMs are easy to prepare; once formed, SAMs are stable for weeks under conditions typical for culturing cells. Gold substrates are prepared on glass cover slips or silicon wafers by evaporating a thin layer of titanium or chromium (1−5 nm) to promote the adhesion of gold to the support, followed by a thin layer of gold (10−200 nm) [25]. SAMs formed on these gold substrates are stable to the conditions used for cell culture, but care should be taken to avoid strong light and temperatures above ~70°C since both can result in degradation of the SAM [26,21].

Preventing protein adsorption: 'inert surfaces'

Proteins play an integral part in the adhesion of cells to surfaces: cells require adsorbed proteins (or peptides that mimic parts of a protein) to adhere to the surface [27]. Control of the interaction of proteins with a surface, therefore, enables the control of the interactions of cells with that surface. Most solid surfaces − especially hydrophobic surfaces − adsorb proteins. Thus, the main challenge in controlling the interactions of proteins and cells with surfaces lies in finding surfaces that resist non-specific adsorption of proteins (surfaces that we call 'inert' for brevity). Inert surfaces provide the background necessary for spatially restricting protein adsorption or for preparing surfaces that only bind specific proteins, and are used in patterning proteins and cells, as biomaterials [28,29], and in the construction of biosensors [30].

SAMs terminated in oligo (ethylene glycol) (EG_n, $n > 2$), resist the adsorption from solution of all known proteins and their mixtures [2,19,31]. We know that EG_n groups are not unique in their ability to make inert SAMs: for example, several polar functional groups that do not contain H-bond donors often make good components of inert surfaces [32,33]. The combination of inert and adsorptive surfaces with soft lithographic techniques enables the facile patterning of proteins and cells. Patterns of hydrophobic regions (for example, SAMs terminated in methyl groups) and regions that are 'inert' provides the basis for most work using patterned cells.

A number of other substances also make the surface more or less inert. Many of them are used in connection with soft lithography, for example, bovine serum albumin (BSA) and related proteins [34], man-made polymeric materials (e.g., polyethyleneglycol, or PEG) [35], and dextran [36].

MICROCONTACT PRINTING (μCP)

Patterning ligands, proteins, and cells on SAMs

Microcontact printing (μCP) is a technique that uses topographic patterns on the surface of an elastomeric PDMS stamp to form patterns on the surfaces of various substrates (Fig. 19.1) [3]. The stamp is first 'inked' with a solution containing the patterning component, the solvent is allowed to evaporate under a stream of air, and the stamp is brought into conformal contact with the surface of the gold film for intervals ranging from a few seconds to minutes. The thiol transfers to the gold film in the regions of contact. Other components used as ink for μCP include activated silanes that react with the SiOH groups ($RSiCl_3$ or $RSi(OCH_3)_3$) present on

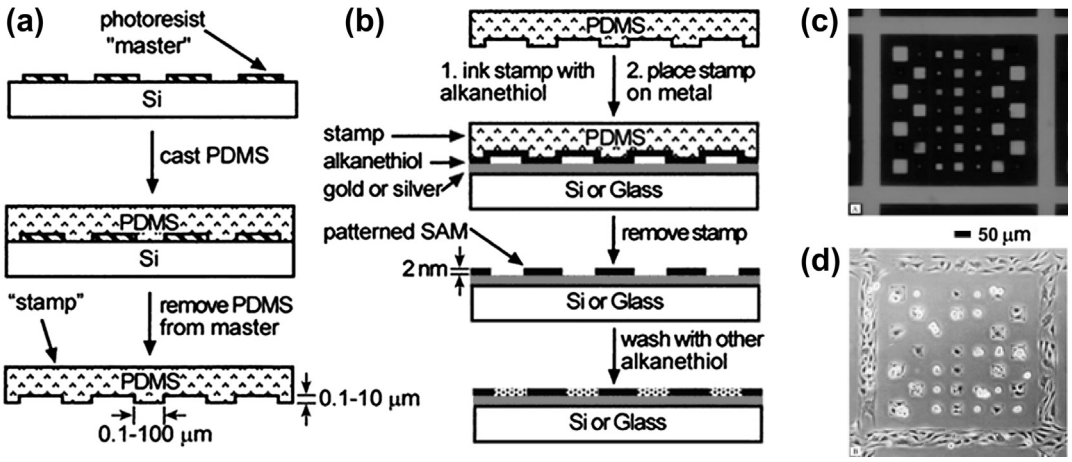

FIGURE 19.1

Molding from a master, microcontact printing (μCP), and patterning of proteins and cells. (a) A method for generating stamps (also applicable for channels and other molds) of PDMS for μCP. A PDMS stamp is prepared by pouring PDMS liquid prepolymer on a 'master' (generally generated by photolithography), followed by curing the PDMS and removing it (as an elastomeric solid) from the master. (b) A typical procedure used for μCP. A solution containing the patterning component of interest ('ink') is applied to this stamp and the solution allowed to dry. This inked stamp is placed on a substrate to allow the ink to transfer to the substrate. A substrate patterned with SAMs remains after removal of the stamp. (c) Selective adsorption of fibronectin onto a surface patterned by SAMs into areas that either promote or resist the adsorption of proteins, by μCP, visualized by immunostaining. (d) Patterned attachment and spreading of cells on the protein patterned substrate in c.

the surface of silicon (with a native film of SiO_2); and various ligands (such as amine-containing compounds) that react with activated SAMs (usually resulting in the formation of a peptide bond that tethers ligands with surfaces) [37,38].

The most general and reliable method for patterning proteins is accomplished by preparing areas of SAMs that promote the adsorption of proteins, surrounded by regions that resist adsorption of proteins (regions that we call 'inert background'), and allowing proteins to adsorb onto the adsorbing regions from solutions. For example, we used μCP to pattern gold surfaces into regions terminating in methyl groups, then filled the rest of the gold surface with an oligo (ethylene glycol)-terminated thiol to form inert regions [25]. Immersion of the patterned SAMs in solutions of proteins such as fibronectin, fibrinogen, pyruvate kinase, streptavidin, and immunoglobulins resulted in adsorption of the proteins exclusively on the methyl-terminated regions [25]. Characterization of the patterns of adsorbed proteins by electron and optical microscopy showed that the layers of adsorbed protein appeared to be homogeneous. Alternatively, proteins can be anchored to ligands patterned onto surfaces by μCP, for example, μCP of biotin onto activated SAMs allows the biospecific immobilization of avidin on the surface [37].

The ability of μCP to create patterns of ligands and proteins allows the patterning of many anchorage-dependent cells (most normal cells in multicellular organisms are anchorage-dependent) [39]; this patterning confines them to specific regions of a substrate, and allows the precise control of the size and shape of the cells (Fig. 19.1). For example, μCP allows the partition of the surface of gold into regions presenting EG_n groups and methyl groups [40]. After coating the substrates with fibronectin, bovine capillary endothelial cells attached only to the methyl-terminated, fibronectin-coated regions of the patterned SAMs. The cells remained attached in the patterns defined by the underlying SAMs for 5–7 days. We have also used SAMs on palladium for the confinement of mammalian cells [23]. EG-terminated SAMs on palladium allow the patterning of individual cells, groups of cells, as well as focal adhesions (subcellular complexes that enable cell-substrate attachment, FAs) for over four weeks; similar SAMs on gold confined cells to patterns for one to two weeks.

The application of μCP in fundamental cell biology

The ability to pattern proteins, groups of cells, single cells and their FAs has led to new studies on the effect of patterned surface environments and cell shape on cell behavior.

Our first attempt in this area was to prepare (via μCP) substrates consisting of square and rectangular islands of laminin surrounded by inert regions, and to study the behavior of rat hepatocytes on them [41]. The cells conformed to the shape of the laminin patterns; the patterning allowed the control of cell shape independently of the density of ligands in the extracellular matrix (ECM). We observed that cell size, regardless of ECM ligand density, was the major determinant of cell growth and differentiation. We then used μCP to prepare substrates that presented circular cell-adhesive islands of various diameters and inter-island spacings [42,43]. Such patterns allowed the control of the extent of cell spreading without varying the total cell-matrix contact area. We found that the extent of spreading (the projected surface area of the cell), rather than the area of the adhesive contact controlled whether the cell divided, remained in stationary phase or entered apoptosis. Dike et al. used μCP to prepare substrates with cell-adhesive lines of varying widths [43]. They found that bovine capillary endothelial cells (BCE) cultured on 10 μm wide lines underwent differentiation to capillary tube-like structures containing a central lumen. Cells cultured on wider (30 μm) lines formed cell-cell contacts, but these cells continued to proliferate and did not form tubes.

Recent progress in understanding how cell adhesion regulates cell physiology has used methods related to μCP. In several model types of cells, the strength of cell-substrate adhesion increased as the allowed area of cell adhesion increased, for small areas (typically less than 300 μm [2]); but the strength of adhesion remained constant for larger areas [44,45]. These results empirically related cell spreading to the strength of cell attachment. Within the FAs, integrin receptors need to aggregate in order to activate the appropriate biochemical pathways for the adhesion of cells [46–48]. It has not been clear, however, what maximum separation is at which integrin receptors can still perform their normal functions. Bastmeyer has used different combinations of micropatterns to determine that cells could adhere to surfaces with arrays of circles with area of 0.1 μm^2, when the spacing between these circles are less than 5 μm; but when the separation between these circles is larger than 30 μm (and when the circles are larger than 1 μm [2]), cells fail to adhere and spread on these surfaces [49]. This result gives a semi-quantitative description of the geometrical requirements for clustered integrins.

Using a combination of self-assembly of nanoparticles and micropatterns, Spatz and colleagues definitively determined the maximum distances (73 nm) between individual integrin receptors at which normal cell adhesion could occur [50]. Another report shows that when FAs mature into larger than normal sizes, they appear to exert four times as much stress on the surface as do normal FAs; this result might be important for myogenesis, the process of the formation of muscle fibers [51].

Micro-scale features enable the studies of the movement of mammalian cells. The extension of lamellipodia is an important process in the movement of cells. Bailly and coworkers used micropatterned substrates to study the regulation of lamellipodia during chemotactic responses of mammalian carcinoma cells to growth factors [52]. On stimulation with epidermal growth factor, the cells extended their lamellipodia laterally out of their patterns of confinement, over the inert part of the substrate. This result showed that the extension of lamellipodia could occur independently of any contact with the substrate. Contact formation was, however, necessary for stabilizing the protrusion. We further observed that when endothelial cells were confined to patterns with corners (such as triangles and squares), their lamellipodia tended to spread most actively from the corners of these shapes [53,54].

Most moving mammalian cells adopt asymmetric shapes. We have examined whether the asymmetry in the shape of a cell is connected to the direction of its movement [55] (Fig. 19.2).

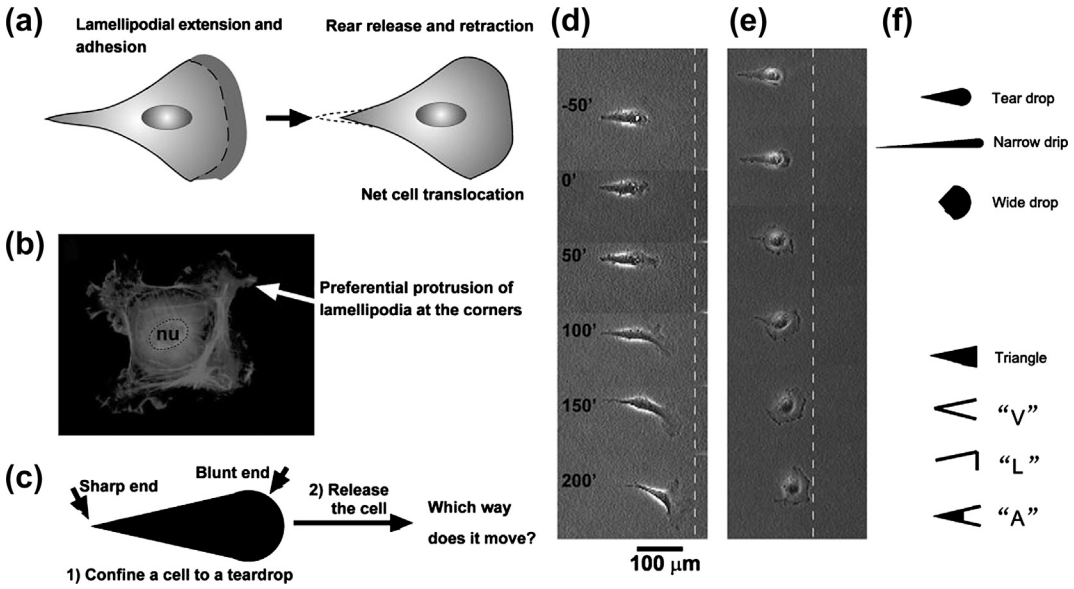

FIGURE 19.2

Understanding the relationship between cell shape and the direction of cell migration. (a) A typical migrating cell has an asymmetric, teardrop-like shape, and moves toward the blunt end. (b) When confined to shapes that have sharp corners, lamellipodia tend to extend out of the sharp corners. (c) A cell initially confined to a teardrop shape then subsequently released may choose which way it moves: toward its blunt or sharp end. (d) (e) Individual 3T3 cells and COS cells, respectively, were initially confined to teardrop shapes, and then released to move freely across the surface. The movement was predominantly toward the blunt end. Numbers indicate time past after the application of electrochemical potential (minutes). (f) Different initial patterns used to confine cells.

Since moving cells often appear to have a teardrop shape, we confined cells to teardrop-shaped patterns, and then used electrochemistry to allow free cell movement (Fig. 19.2a). It is tempting to assume that the released cells would move toward the sharp end of the teardrop pattern, considering that lamellipodia tend to extend from sharp corners (Fig. 19.2b); but when we released teardrop-shaped cells electrochemically, the pre-shaped cells appeared to prefer moving toward their blunt ends (Fig. 19.2c–e). To understand the issue in detail, we varied the initial shapes. It appeared that 'narrow drops' (regardless of details of the shape) had a similar capability to direct cell migration, while 'wide drops' failed to do so. Triangular patterns (having a similar aspect ratio to the teardrop) also direct cell migration, to the same extent as the teardrop, further confirming that the asymmetry of the initial shape alone could direct cell movement. In contrast, symmetric patterns such as rectangles, squares and circles failed to direct cell migration. To determine whether it was the shape of the spread cell or the uneven distribution of FAs underneath the cell that was responsible for directed cell motion, we started cells on 'L', 'V', and 'A'- shaped patterns (Fig. 19.2f). Because individual cells could span these patterns, the overall geometry of immobilized cells resembled each other and was similar to that of cells confined to triangular patterns. However, these patterns allowed different distributions of FAs. Triangles and 'L'-shaped cells allowed more FAs in the blunt end, 'V'-shaped patterns allowed the same amount of the FAs in the blunt end as in the sharp end, while 'A'-shaped patterned allowed more FAs in the sharp end. All these patterns appeared to direct cell migration to the same extent, thus confirming that the overall shape of cells was the determining factor in directing cell motion.

μCP offers opportunities to study not just single cells, but groups of cells. For example, Chen and coworkers devised experiments to control the size and the contact between a pair of cells, thus definitively proving that cell-cell contact, not soluble factors alone, enables cell-contact-mediated proliferation of cells in culture [56]. Toner and colleagues showed, by patterning of hepatocytes and non-parenchymal cells with precise geometrical parameters,

that the interface between the two types of cells is critical for the function of hepatocytes [57]. Ingber and his coworkers discovered, using patterned groups of two or more endothelial cells, that spontaneous ordering arises, and patterns that resemble the Chinese Yin-Yang ideograph would emerge while endothelial cells migrate on the patterns [58,59]. Studying groups of tens to hundreds of cells patterned into defined geometries, Chen and coworkers realized that the shapes of sheets of cells influence the mechanical forces that each cell within the shape experiences, and that these forces affect the physiology of individual cells differently, depending on the location of the cell in the sheet [60].

Local physical interactions between cells and extracellular matrix (ECM) influence directional cell motility, which is critical for tissue development, wound repair, and cancer metastasis. Using μCP, we tested the possibility that the precise spatial positioning of FAs governs the direction in which cells spread and move. We cultured NIH 3T3 cells on circular or linear ECM islands created by μCP. Cells could be driven proactively to spread and move in particular directions by altering either the inter-island spacing or the shape of similar-sized ECM islands. Immunofluorescence microscopy confirmed that FAs assembled preferentially above the ECM islands, with the greatest staining intensity being observed at adhesion sites along the cell periphery. Rac became activated within 2 min after peripheral membrane extensions adhered to new ECM islands, and this activation wave propagated outward in an oriented manner as the cells spread from island to island. The results suggest that physical properties of the ECM may influence directional cell movement by dictating where cells will form new FAs and activate Rac and, hence, govern where new membrane protrusions will form [61].

μCP is also a useful tool for studying dynamic development process of neurons [62]. ECM proteins and cell—cell adhesion molecules play important roles in the development and differentiation of neurons. Recently, we fabricated laminin stripes on a background of poly-L-lysine as substrates for the growth of rat hippocampal neurons, and found that a sharp change of the concentration of laminin guides the growth of neurites by leading the growth cones in a time- and space-dependent manner. The percentage of neurites that grow along the edge of laminin stripes (where there is a sharp change of concentration) decreases as a function of the concentration of laminin under a threshold value. The actin cytoskeleton plays an important role in the process of growth cone's response to the sharp change of concentration of laminin on micropatterns [63].

μCP has also been used in studies of cell division. Even though most mammalian cells become round and almost completely detach from the substrate when they divide, Bornens' group has used μCP to show that the shape of the ECM to which cells initially attach determines the direction of cell division [64].

μCP could also be used to investigate the behavior of stem cells. Micropatterns were reported to bias the differentiation of human mesenchymal stem cells: when allowed to adhere and spread, the stems cells became osteoblasts; when spreading is prohibited by small patterns of confinement, stem cells became adipocytes [65]. We reported a microarray-based approach for the high throughput screening of gene function in stem cells and demonstrated the potential of this method by growing and isolating clonal populations of both adult and embryonic neural stem cells. Clonal microarrays are constructed by seeding a population of cells at clonal density on micropatterned surfaces generated using μCP. Clones of interest can be isolated after assaying in parallel for various cellular processes and functions, including proliferation, signal transduction, and differentiation. We demonstrated the compatibility of the technique with both gain- and loss-of-function studies using cell populations infected with cDNA libraries or DNA constructs that induce RNA interference. The infection of cells with a library prior to seeding and the compact but isolated growth of clonal cell populations will facilitate the screening of large libraries in a wide variety of mammalian cells, including those that are difficult to transfect by conventional methods [66].

Other types of μCP

It is possible to pattern certain proteins (ones that can withstand drying onto the surface of the stamp, and stamping) directly onto surfaces [67–71]. Direct patterning, however, is typically applicable only to structurally stable proteins and is usually more demanding experimentally than patterning via SAMs [77]. The surface of the PDMS stamp used in this type of procedure must be rendered hydrophilic by exposure to a plasma before use [69].

Patterning cells directly with μCP was not thought to be feasible, because most cells are too delicate to be dried or stamped. We have demonstrated the stamping of proteins and cells directly with a soft hydrogel stamp (agarose) that contains large amounts of water [70,72]. The resolution of this technique (tens of micrometers) is not comparable to μCP with PDMS and SAMs, but it makes patterning cells at this large size range easier than patterning with SAMs.

Other researchers have used μCP for different types of cells on other types of surfaces. Craighead and coworkers patterned polylysine on the surfaces of electrodes to confine the growth of neurons [73]. We have synthesized oligopeptides containing a cell adhesion motif at the N-terminus connected by an oligo (alanine) linker to a cysteine residue at the C-terminus [74]. The thiol group of cysteine allowed the oligopeptides to form monolayers on gold-coated surfaces. We used a combination of μCP and these self-assembling oligopeptide monolayers to pattern gold surfaces into regions presenting cell adhesion motifs nd oligo(ethylene glycol) groups that resist protein adsorption. Wheeler et al. created patterns of covalently bound ligands and proteins on glass coverslips and used these patterns to control nerve cell growth [75]. In addition, polymers of EG and supported phospholipids have been used on a series of different substrates for pattering cells [76–79].

Recently, inspired by the composition of adhesive proteins in mussels, Lee et al. used dopamine self-polymerization to form thin, surface-adherent polydopamine films onto a wide range of inorganic and organic materials [80]. Secondary reactions can be used to create a variety of layers, including SAMs through deposition of long-chain molecular building blocks, metal films by electroless metallization, and bioinert and bioactive surfaces via grafting of macromolecules. In a recent study, we studied if polydopamine film can form on OEG SAMs in the same condition as reported for other types of surfaces. The result indicates that polydopamine can transfer onto the SAMs with high fidelity and can form flattened sheets on OEG SAMs, suggesting that polydopamine is a soft and sticky material which can be reshaped under compression. The stable adhesion of polydopamine on antifouling surfaces made it suitable for cell patterning. We confined NIH 3T3 fibroblast cells to the polydopamine patterns which were achieved by either μCP or μFP. Our studies demonstrate that polydopamine is a versatile non-specific adhesive material for cell adhesion [81].

MICROFLUIDIC PATTERNING (μFP)

The use of microfluidic channels allows patterning surfaces by restricting the flow of fluids to desired regions of a substrate. The patterning components such as ligands, proteins, and cells are deposited from the solution to create a pattern on the substrate.

Delamarche and colleagues used μFP to pattern immunogloblins with submicron resolution on a variety of substrates including gold, glass, and polystyrene [82]. Only microliters of reagent were required to cover square millimeter-sized areas. Patel and coworkers developed a method to generate micron-scale patterns of any biotinylated ligand on the surface of a biodegradable polymer [83]. These investigators prepared biotin-presenting polymer films, and patterned the films by allowing solutions of avidin to flow over them through 50 μm channels fabricated in PDMS. The avidin moieties bound to the biotin groups on the surface, and served as a bridge between the biotinylated polymer and biotinylated ligands. Patterns created with biotinylated ligands containing the RGD or IKVAV oligopeptide sequences determined the adhesion and spreading of bovine aortic endothelial cells and PC12 nerve

cells. Both our group and Toner's group used μFP to produce patterns of adsorbed proteins and adherent cells on biocompatible substrates [84–87]. We formed micropatterns of proteins deposited from fluids in separately addressable capillaries. By allowing different cell suspensions to flow through different channels, we could pattern two types of cell on surfaces with high spatial precision. After the adhesion of the two types of cells in different areas on the surface, we could remove the elastomeric stamps to allow movement of both types to be studied. By using μFP, we established an *in vitro* model on glass surfaces for patterning multiple types of cells, to simulate cell-cell interactions *in vivo*. The model employs a microfluidic system and poly(ethylene glycol)-terminated oxysilane (PEG-oxysilane) to modify glass surfaces in order to resist cell adhesion. The system allows the selective confinement of different types of cells to realize complete confinement, partial confinement, and/or no confinement of three types of cells on glass surfaces. The model was used to study intercellular interactions among human umbilical vein endothelial cells (HUVEC), PLA 801 C and PLA801 D cells [84]. By filling individual channels with different fluids, multiple components could be patterned at the same time without the need for multiple steps or the accompanying technical concerns of registration (although registration was required in the fabrication of the stamp itself). By using μFP, we patterned multiple types of cells on a same surface in one step and selectively make a 'wound' by filling low osmotic fluid into a specific channel. Without mechanical tension on adjacent normal cells, the low osmotic fluid leads to 'lysis' of the cells which would release and form gradients of soluble cytoplasmic components to initiate a collective migration of adjacent cells to perform a repair process. We observed the phenomenon of contact inhibition of locomotion, where a cell ceases to continue moving in the same direction after contacting with another cell [88].

Furthermore, by mimicking a 'wound' *in vitro*, we developed a cell-on-a-chip model that can be used to screen wound dressing candidates, thus minimizing the use of animals in the development of better methods for wound care. We simulated a cutaneous wound and screened the performances of several electrospun fibrous wound dressings in enhancing wound healing. For comparison, the performances of the wound dressings were also evaluated in a rat model. It was found that the results acquired by microchip model corroborated well with the animal experiments [89].

Adhesion on a substrate is essential for most types of cells; however, cell-cell interaction dominates certain biological processes under some situations, such as embryonic development. We developed a device consisting of two microchannels separated by a semi-porous polycarbonate membrane which had been treated to make it resistant to cell adhesion. Embryonic stem (ES) cells introduced into the upper channel self-aggregate to form uniformly-sized embryoid bodies. The size and morphology of these bodies are critical for the sequential developmental stages of naturally conceived embryos. The semi-porous membrane also allows subsequent treatment of the non-attached EBs with different reagents from the lower channel. This technique is superior to existing methods which are tedious or provide heterogeneously-sized EBs [90]. Using a similar device, we mimicked niche the microenvironment in which cancer cells reside. A 3D metastatic prostate cancer model that includes the types of surrounding cells in the bone microenvironment was established. Spheroids of metastatic prostate cancer cells (PC-3 cell line), osteoblasts and endothelial cells were formed to ensure uniform incorporation of all co-culture cell types. The model recapitulated the *in vivo* growth behavior of malignant cancer cells within the bone metastatic prostate cancer microenvironment [91]. Chemokines play a key role in the metastasis of cancer cells, however, the exact mechanism is unknown partially due to the lack of adequate research tools. We presented a microfluidic vasculature system to model interactions between circulating breast cancer cells with microvascular endothelium at potential sites of metastasis. The microfluidic vasculature produces spatially-restricted stimulation from the basal side of the endothelium that models both organ-specific localization and polarization of chemokines and many other signaling molecules under variable flow conditions. We demonstrated that

circulating breast cancer cells preferentially adhere to endothelium stimulated from the basal side with CXCL12. CXCL12 functions through CXCR4 on endothelial cells to significantly enhance adhesion of circulating breast cancer cells, independent of CXCR4 or CXCR7 on cancer cells [92]. We also investigated how cells expressing CXCR7 regulate the chemotaxis of a separate population of CXCR4 cells under physiologic conditions in which cells are exposed to gradients of CXCL12. We recapitulated a cancer-stroma microenvironment by patterning CXCR4-expressing cancer cells in microchannels at spatially defined positions relative to CXCL12-producing cells and CXCR7-expressing cells. We demonstrated that chemotaxis of CXCR4 cells depended critically on the presence and location of CXCR7 cells relative to chemokine secreting cells. This simple and robust method should be generally useful for engineering multiple microenvironments to investigate intercellular communication [93].

LAMINAR FLOW PATTERNING

Laminar flow patterning (LFP) is a characteristic method of microfluidic systems that allows two or more different solutions to flow side-by-side in a channel without convective mixing [94,95]. This technique utilizes a phenomenon that occurs in microfluidic systems as a result of their small dimensions — that is, low Reynolds number flow [96,97]. The Reynolds number (Re) is a parameter that describes the ratio of inertial to viscous forces in a particular flow configuration; it is a measure of the tendency of a flowing fluid to develop turbulence. The flow of aqueous fluids in capillaries usually has a low Re and is laminar. Laminar flow allows two or more streams of fluid to flow next to each other without any mixing other than by diffusion of their constituent molecules across the boundary between them (which is usually fairly slow). Diffusional motion of particulate components (e.g., cells) is even slower. This fluid behavior can be used to precisely control the nature and location of an interface between two miscible or non-miscible fluids, without requiring a physical divider. This means that LFP can be used to generate chemical gradients, pattern particles and cell suspensions.

In a typical set-up for LFP experiments, a network of capillaries is made by sealing a patterned PDMS slab with a glass slide or the surface of a Petri dish (Fig. 19.3a) [95]. By passing streams of fluid with different compositions from the inlets, patterns of parallel stripes of flowing fluid are created in the main channel. It is possible, therefore, to treat different parts of a single cell with different reagents if it happens to span these stripes. Fig. 19.3b and 19.3c illustrate the painting of a single cell with dyes that stained mitochondria located in different parts of the same cell [98].

Using this technique, the positions and microenvironments of cells can be controlled simultaneously in several stripes in the same channel [99,100]. Using a similar approach, we could pattern the substrate with different proteins and cells [95]. We can pattern the culture media over an individual cell by delivering chemicals selectively to cells. Since no physical barriers are required to separate the different liquid streams, different liquids can flow over different portions of a single cell.

Ismagilov and colleagues have used LFP to generate a step gradient in temperature to study the development of embryos of the fruit fly *Drosophila melanogaster* [101]. They treated the anterior (front) and posterior (back) of the embryo with media at different temperatures, and observed that the fly embryos developed normally under such a condition. They concluded that in complex biochemical systems, there exist mechanisms for compensation. They further showed that if they reverse the temperature gradient within a certain time, embryos failed to develop normally. This observation shows there is a limit in time for the compensation mechanisms.

Another type of LFP generates gradients with parallel streams of flow of increasing or decreasing concentrations. We have generated gradients of biomolecules both in solution and

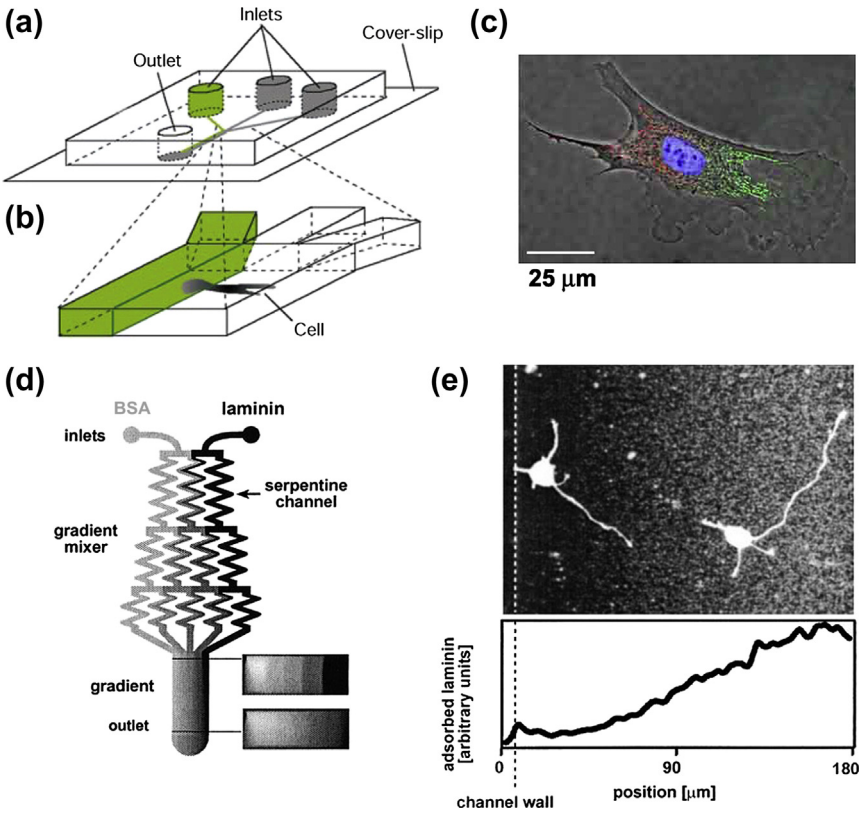

FIGURE 19.3

Manipulation of two regions of a single bovine capillary endothelial cell using multiple laminar flows. (a) Experimental set-up; (b) shows a close-up of the point at which the inlet channels combine into one main channel. (c) Fluorescence images of a single cell after treatment of its right pole with Mitotracker Green FM and its left pole with Mitotracker Red CM-H2XRos. The entire cell is treated with the DNA-binding dye Hoechst 33342. (d—e) Generation of gradients using microfluidic networks, and use of these gradients to study neuronal differentiation. (d) In appropriately designed microfluidic channels, flows generate gradients of BSA and laminin in solution. (e) The gradient in solution became a gradient on the surface when proteins adsorb, and when rat hippocampal neurons grow on the gradient, the neurons extend their longest process (the presumed axon) toward the higher concentrations of laminin in the gradient of proteins on the surface.

on surfaces (Fig. 19.3d) [102,103]. Because we can control the input concentration and the width of the microfluidic channel, it is possible to generate gradients with virtually any characteristics (e.g., the length and the slope) both in solution and on surfaces. We studied the generation of neuronal polarity — the process of the selective formation of one axon and several dendrites from a number of initially equivalent neurites projecting from a single neuron — and found that a surface gradient of laminin was sufficient to guide the orientation of this process (Fig. 19.3e) [104]. We further quantified the slope of the gradient, and determined the minimum slope required for this process to take place. We have also studied the chemotaxis of neutrophils in a solution of gradient of interleukin-8 (IL8) [105]. The neutrophils migrate directionally, toward increasing concentrations of IL8 along linear gradients. They halt abruptly when encountering a sudden drop in the chemoattractant concentration (from maximum directly to zero). When neutrophils encounter a gradual increase or decrease in chemoattractant (from a maximum gradually down to zero), however, they initially cross the crest of maximum concentration, but then they head back towards it. It would be very difficult to carry out experiments to answer questions regarding the detailed responses of cells to gradients over scales of microns without the ability to form precisely controlled gradients.

LFP has some features that make it complementary to other patterning techniques used for biological applications. It takes advantage of easily generated multiphase laminar flows to pattern fluids and to deliver components for patterning. The ability to pattern the growth medium itself is a special feature that cannot be achieved by other processes. This method can pattern even delicate structures such as portions of a mammalian cell. This type of patterning is difficult by other techniques. LFP can also give simultaneous control over surface patterns, cell positioning, and the fluid environment in the same channel. A few recent examples have combined patterned substrates with patterned flows. We have fabricated gradients of proteins on surfaces in microchannels whose floors carry patterns generated by μCP [106]. Jeon and his coworkers have combined substrates patterned in topography and

patterned flows to form a model system that conveniently isolates axons of rat hippocampal neurons from the rest of the cell for studies of their molecular biology [107]. Langer and coworkers have fabricated microchannels that have micropatterns within them to immobilize proteins and cells [108]. Folch and coworkers have used micropatterns to form myotubes from myoblasts, and then used laminar flows to deliver agrin, a proteoglycan found in the neuromuscular junction, precisely to these myotubes. In these experiments, they monitored the clustering of acetylcholine receptor (AChR), and the results corroborated the hypothesis that focalized release of agrin causes the clustering of the AChR [109].

The combination of LFP and hydrogel can accurately model *in vivo* cellular microenvironments. We have demonstrated a flexible method that allows:

1) Controlled spatial distribution (patterning) of multiple types of cells within 3D matrices of a biologically derived, thermally curable hydrogel (Matrigel); and
2) Application of gradients of soluble factors, such as cytokines, across the hydrogel.

The technique uses laminar flow to divide a microchannel into multiple subchannels separated by microslabs of hydrogel. It does not require the use of UV (ultraviolet) light or photoinitiators and is compatible with cell culture in the hydrogel. This technique makes it possible to design model systems to study cellular communication mediated by the diffusion of soluble factors within 3D matrices. This method is particularly useful for studying cells such as those of the immune system, which are often weakly adherent and difficult to position precisely with standard systems of cell culture [110].

Multiplex protein patterns could be created in a single microfluidic channel by combining LFP and μCP. Didar and his colleagues patterned (3-aminopropyl) triethoxysilane (APTES) on a glass surface by using μCP. They then introduced five primary antibodies onto the APTES patterns by LFP to form different protein microarrays in a single channel. The functionality of the microarrays was evaluated through a multiplex immunoassay using secondary antibodies specific to each patterned primary antibody (Fig. 19.4a). Their method proved to be successful for the preparation of highly resistant multiplex functional surfaces for high throughput biological assays [111].

The combination of LFP and μFP can create co-culture patterns of various geometries and compositions for manipulating cell-cell interaction dynamics. We developed a two-layered microfluidic device that sandwiches a semi-porous membrane (Fig. 19.4b). Arbitrary cellular arrangements are enabled by regulating the geometric features of the bottom channel so that as culture media drains, the flow hydrodynamically focuses cells onto the membrane only over specific regions in the bottom channel. Furthermore, when the top channel has multiple inlets, cells can be seeded in adjacent laminar streams, allowing different cell types to be patterned simultaneously in well-defined spatial arrangements. We constructed two juxtaposed non-concentric cell spheroids using mouse embryonic stem (mES) cells and hepatocytes (Fig. 19.4c). The mES cells differentiated in a spatially distinct pattern dictated by the position of the hepatocytes. This contrasts with the uniform mES differentiation observed when co-culture spheroids are formed by the conventional method of randomly mixing the two cell types. This cellular patterning method opens new possibilities for understanding and manipulating interactions between different cell types in 3D [112].

One may ask if the fluid flow required in the generation of laminar flows would cause problems for certain experiments, such as the measurement of chemotaxis, dispersion effects and driving forces. Wikswo and coworkers addressed the chemotaxis problem by measuring the motility of HL60 leukemia cells (which express CXCR2 receptors) in a gradient of CXCL8 [113]. They found that high rates of flow can affect the motility of cells. Reasonably low rates of flow, however, do not affect measurements of motile cells. Dispersion effects resulting from diffusion and convective disturbance are a big problem in LFP. This issue can be partially addressed by using oil-in-water phase systems. However, there are limitations in terms of

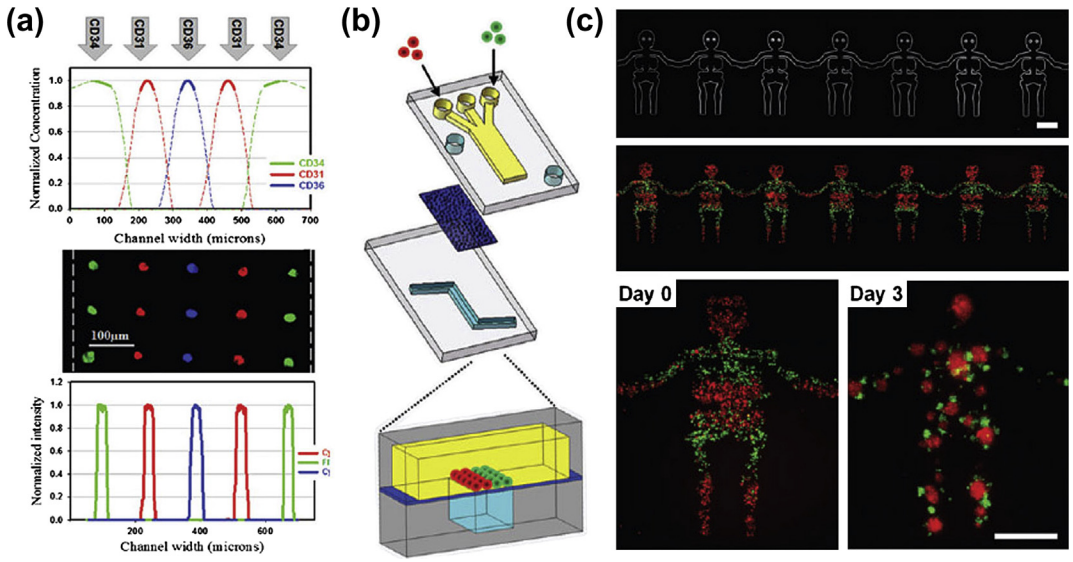

FIGURE 19.4

(a) Patterning multiplex protein microarrays with strong covalent bonds and high throughput biomarker assay. From top to bottom: Schematic representation of laminar flows containing different primary antibody solutions to create the detection interface; Simulation results for concentration distribution inside the flow streams. The areas shown with continuous solid lines correspond to the areas patterned with APTES and thus the antibodies in the flow are expected to attach to the surface at these spots; Superimposed fluorescence microscope images of the surfaces showing the multiplex immunoassay results. The white dashed lines represent the microchannel walls; Fluorescence intensities of fluorescently conjugated secondary antibodies are detected by their specific primary antibodies on the surface. (b) Schematic illustration of the compartmentalized microfluidic system for cellular patterning. Two PDMS channel layers are separated by a semi-porous polycarbonate membrane which is rendered resistant to cell adhesion. The top channel is a straight channel with a dead-end. The bottom channel consists of a straight channel with or without chambers. Cells are introduced into the top channel using multiple laminar flows. (c) Patterning co-culture of multiple cell types. An optical image of the bottom channel and fluorescent images of patterned cells. Two kinds of cells were patterned as 'human' shapes while keeping alternate five equal lines. Scale bars: 500 μm.

the biocompatibility of these systems for adherent cell culture. We presented a fully biocompatible aqueous two-phase flow system (PEG, Dextran) that can be used to pattern cells within simple microfluidic channel designs, as well as to deliver biochemical treatments to cells according to discrete boundaries. We demonstrated that these aqueous, two-phase systems are capable of precisely delivering cells as laminar patterns, or as islands by way of forced droplet formation. The system can also be used to precisely control chemical (trypsin) delivery to preformed monolayers of cells growing within channels [114]. LFP is typically implemented using syringe pumps, which is a relatively complex procedure for a biological lab. Beebe's group presented a passive microfluidic method that enables short-term LFP of multiple fluids using a single pipette and allows each sample to be loaded in any sequence, at any point in time relative to one another. The proposed method is well suited for cell-based assays, reduces the complexity of LFP to a similar level as other cell patterning methods, can be scaled to include more than two streams of fluid, and enables arrays of individually addressable devices for LFP on a single chip [115].

DYNAMIC CONTROL OF SURFACES

Electrochemical desorption of SAMs

It is possible to modulate the ability of surfaces to promote the adhesion of cells by controlling their composition. A relatively simple method to achieve this control is to desorb EG-terminated SAMs electrochemically from a substrate patterned with cells in a buffer containing proteins that promote cell attachment [116]. Electrochemical desorption converts inert areas into regions that can promote the adsorption of proteins and adhesion of cells, and thus allows initially confined cells to move out of their patterns (Fig. 19.5a). By

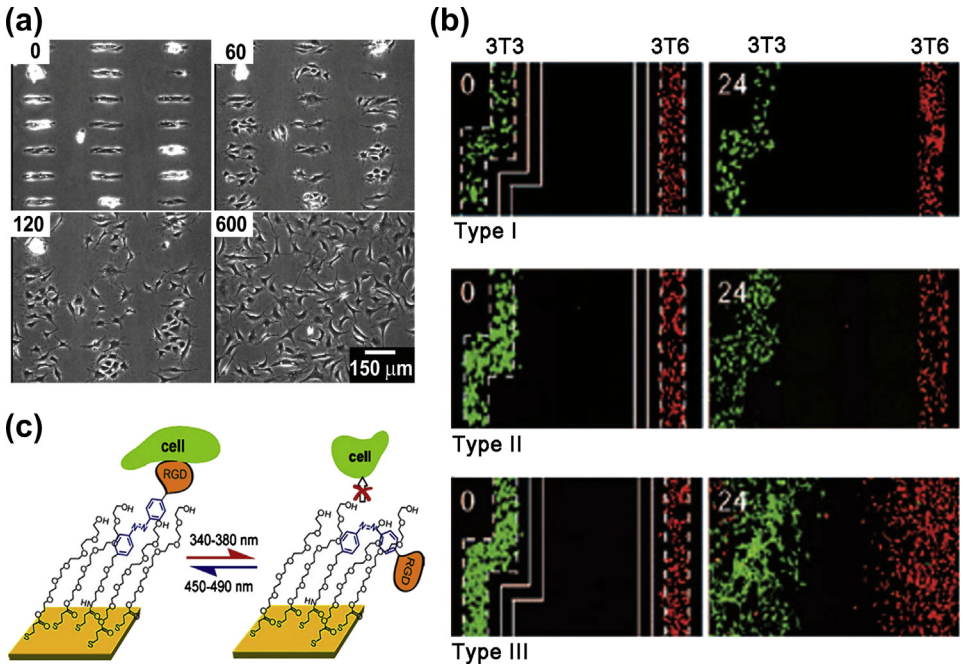

FIGURE 19.5

(a) Using electrochemical desorption of EG-terminated SAMs to release confined BCE cells and allow their free movement. Initially, bovine capillary endothelia cells are confined to a patterned array of hydrophobic regions (SAMs terminated in methyl groups) and surrounded by regions that are 'inert' (SAMs terminated in EG-terminated groups). Application of a cathodic voltage desorbs the EG-terminated SAMs to allow cells initially confined to move freely on the surface. The numbers indicate time after application of electrochemical treatment (in minutes). (b) Time-lapse phase-contrast and fluorescence micrographs for the three types of cell-cell interactions between 3T6 and NIH3T3 cells. (c) The azo moiety can be converted photochemically between the E and Z configurations to either present or mask the RGD ligand and hence modulate biospecific cell adhesion.

electrochemical desorption of SAMs in localized areas defined by a microfluidic system, we patterned multiple types of cells on the same substrate. This technique has the ability to pattern different types of cells with precisely controlled distances while allowing the free exchange of soluble molecules. It also allows these cells to move under each other's influence [117]. Based on this work, we applied selective desorption on complex patterns of cells and simulated three types of cell-cell interactions *in vivo*:

1) Those between two types of cells that are both immobilized and confined to isolated areas, such as epithelial cells and polar cells during ovarian development;
2) Those between one cell type that is immobile and another that moves freely, such as glial cells and neurons in neurodegenerative disorders; and
3) Those between two or more types of cells that are both moving freely, such as hepatocytes and fibroblasts in the liver (Fig. 19.5b).

The system is likely to make co-culturing of different types of cells dramatically more accessible to biologists [118].

Mrksich and coworkers have used the electrochemical conversion of a hydroquinone-terminated SAM into a quinone-terminated SAM to allow the attachment of a cyclopentadiene-tether peptide (which allows the immobilization of cells via the binding of integrin receptors on cell surface) to 'turn on' an otherwise inert SAM for the adhesion of cells [119]. Mrksich has used this technique for the patterning of multiple types of cells on surfaces [120]. Another technique from the same group involves desorption of an immobilized ligand (patterned in certain areas on the surface) and detachment of cells adhered to the surface via this ligand, on application of an electrical reduction, and a subsequent electrical oxidation of the substrate for immobilizing another ligand that allows the migration of

adherent cells (that were initially confined to certain areas on the surface) [121]. Some of the methods for preparing the appropriate thiols employed by Mrksich and colleagues may be too complicated for routine use in a regular cell biology laboratory, but the demonstration of such sophisticated control of the interactions between the cell and solid substrates is unprecedented. Another set of electrochemical methods can be used to activate BSA-rendered inert surfaces for cell adhesion [122,123].

There are a number of other techniques that also allow dynamic patterning of proteins and cells in time and space, such as photochemical methods, surface spatial masking, and thermoresponsive polymers.

Photoresponsive surfaces

Photochemical methods allow the inertness of a SAMs to be tuned. Mrksich and coworkers have devised a SAM that is initially inert, but has in it a nitroveratryloxycarbonyl-protected hydroquinone, which can be oxidized photochemically to generate a benzoquinone group, which, in turn allows the attachment of a ligand for immobilization of cells on surfaces [124]. Another method for photoactivation of the surface utilized a photochemical process that desorbed a ligand from SAMs on surfaces. BSA initially physically adsorbed on the surface via a photocleavable 2-nitrobenzyl group terminated SAM made this surface inert; photochemistry-mediated desorption of the 2-nitrobenzyl group, and therefore BSA, allowed cells to adhere to the surface [125]. Pearson et al. reported a method to incorporate azo group to interacting motifs. The azo capable of binding to chymotrypsin in the Z-form; rendered the system to be photoswitchable. The extent of binding of a-chymotrypsin to the azo-modified surface could be modulated by irradiation with either UV or visible light. Upon UV irradiation, the maximum binding capacity of the surface was activated, after irradiation with visible light, the binding capacity was reduced to approximately 60% of the maximum [126]. We generated a surface that allows the azo unit to reversibly present a ligand that contains RGD peptide on SAMs. The E isomer of azo can present the RGD peptide for cell adhesion, while the Z isomer of azo can mask the RGD peptide in PEG terminated SAMs to prevent cell adhesion. The interconversion between E and Z can be achieved by illuminating with two wavelengths of light (UV light, 340—380 nm, for the E to Z conversion, and visible light, 450—490 nm, for the Z to E conversion) generated by the mercury lamp of a standard fluorescence microscope (Fig. 19.5c). Because the E to Z isomerization is completely reversible, this method provides the only means we know to control cell adhesion reversibly on a molecularly well-defined surface [127].

We fabricated substrates that allow the dynamic control of FAs. We first generated a slab of PDMS with a brittle surface by oxygen plasma treatment, and then made the surface inert by physical adsorption of a polymer containing moieties of PEG [128]. By stretching the slab, we created cracks on the surface, which are not covered with the polymer containing PEG. These cracks could thus promote the formation of FAs and adhesion of cells. Releasing the stress on the slab PDMS closed these cracks, and again prevented the adhesion of cells. This stretch-and-release process could be recycled multiple times.

Thermoresponsive surfaces

Temperature is a very convenient stimulus for the manipulation of cell adhesion by controlling 'on-off' switching of thermoresponsive surfaces prepared using thermoresponsive polymers [129,130]. Thermoresponsive polymers generally exhibit a lower critical solution temperature (LCST), below which they are soluble, and above which they dehydrate and aggregate [129]. Surfaces made from these stimuli-responsive polymers switch from hydrophilic to hydrophobic states in response to changes in temperature. Because of a LCST around 32°C in water, poly (N-isopropylacrylamide) (PIPAAm) has been proven to be an excellent thermosensitive material for controlling cell adhesion [131].

PATTERNING WITH MICRO-TOPOGRAPHIES

In vivo, basement membrane, composed of ECM components, is a complex mesh of pores, fibers, ridges and other features at the nanometer scale [132,133]. Topographical cues generated by the ECM, independent of biochemistry, have direct effects on cell behaviors such as adhesion, migration, cytoskeletal arrangements, and differentiation [134–138]. Cells are inherently sensitive to local micro-scale, mesoscale, and nanoscale topographic and molecular patterns in the ECM environment, a phenomenon called contact guidance [139–141]. The development of microfluidics and micro/nanofabrication methods to control and analyze the cellular response to substrate topography has provided new insights into the interactions of cells with their microenvironments [142–145].

It is possible to confine cells to micropatterns using either elastic membranes that carry holes or microwells [146–148], or allow the initial confinement then release of groups of cells [146,147]. Chen and his coworkers fabricated stamps with multiple levels that allowed the patterning of several different types of proteins and cells at once [149]. They also used bowtie-shaped microwells of agarose gel, both to confine individual cells to particular shapes, and to allow cells to be close to each other without mutual contact [56]. Positioning cells next to each other while preventing their direct contact is difficult to achieve with μCP alone. They also fabricated arrays of micropillars (in sizes much smaller than a single cell) to probe the forces that cells apply to the substrate as they adhere to and migrate on solid surfaces [45]. Tang and coworkers succeeded in molding microstructures in hydrogels of resolution larger than 5 micrometers and used these structures to generate arrays of cells in 3D [150].

We also studied the issue of topographical contact guidance — the way that cells interact with chemically homogeneous surfaces that have topographical features. These studies provide simple methods for further studies of this interesting and complex type of interaction [94,151,152].

Apart from physical and chemical cues, cell-cell interactions also influence cell behaviors. We employed the Madin-Darby canine kidney (MDCK), a cell line with relatively strong intercellular interactions, and NIH 3T3 fibroblast cells, a cell line with relatively weak intercellular interactions, to study the interplay between contact guidance and intercellular connections. The two types of cells were patterned onto PDMS substrates with microgrooves. Although MDCK cells migrate much more slowly than 3T3 cells on flat substrates, the velocity of migration of MDCK cells parallel to the grooves is higher than that of 3T3 cells perpendicular to the grooves and contact and form cell sheet. Because MDCK cells have distinct group behavior, they contact each other very tightly, and the cell sheet acts as a barrier to prevent further migration of 3T3 cells. The 3T3 cell group could migrate only via the space between them. This experiment showed that both cell-cell and cell-substrate interactions simultaneously influence the behaviors of groups of cells [153].

Though current technologies are not developed enough to produce tissue *in vitro* that is identical to that *in vivo*, one important step is to mimic natural tissue architecture. Skeletal muscle is composed of fibrils whose organization defines their functionality. In musculoskeletal myogenesis, the alignment of myoblasts in preparation for myotube formation is a crucial step. The ability to organize myoblasts efficiently, to form aligned myotubes *in vitro* would greatly benefit efforts in muscle tissue engineering. We aligned prefused and differentiated skeletal muscle cells *in vitro* by use of continuous micropatterned wavy silicone surfaces. Alignment was found to be a function of plating density [154]. To simulate the dynamic topographies *in vivo*, by applying and releasing compressive strain, we can introduce and remove the micropatterned wavy of the silicone surfaces respectively. The reversible topography was able to align, unalign, and realign C2C12 myogenic cell line cells repeatedly on the same substrate within 24 hour intervals, and did not inhibit cell differentiation. The method provides a broadly applicable tool for investigating dynamic processes of muscle cells

[155]. Based on this, we presented a method for creating highly ordered tissue-only constructs by using rigid microtopographically patterned surfaces to first guide myoblast alignment, followed by transfer of aligned myotubes into a degradable hydrogel and self-organization of the ordered cells into a functional, 3D, free-standing construct independent of the initial template substrate. Histology revealed an intracellular organization resembling that of native muscle [156]. Furthermore, using the similar device, we developed a 3D physiological model of longitudinal smooth muscle tissue. Acetylcholine (ACh) induced a dose-dependent, rapid, and sustained force generation, vasoactive intestinal peptide and absence of extracellular calcium attenuated the magnitude and sustainability of ACh-induced force, these data were similar to force generated by longitudinal tissue [157].

THREE-DIMENSIONAL PATTERNING

Different types of cells make up tissues and organs hierarchically, and communicate within a complex, three-dimensional environment. It has become evident that 3D cell culture provides more physiological cellular environments compared to conventional 2D cell cultures. Culturing cells in hydrogel has been a widely used method, since the architecture and chemical composition of hydrogels can be easily engineered. However, previous hydrogel cultures lack precise cell positioning and reversibility, which are important characteristics in engineering cellular microenvironments. We have reported a simple reversible hydrogel patterning method for 3D cell culture. In a pre-gelled alginate solution, calcium is chelated by DM-nitrophen (DM-n) to prevent crosslinking. After sufficient UV exposure, the caged calcium is released from DM-n, causing the alginate to cross-link. By exposing a pre-gelled solution to UV selectively, we formed an alginate gel in specific regions of a microfluidic device through the light-triggered release of caged calcium (Fig. 19.6a). Since the amount of crosslinking is based on the calcium concentration, the cross-linked alginate can easily be dissolved by EDTA, thus releasing the patterned cells for further analysis [158].

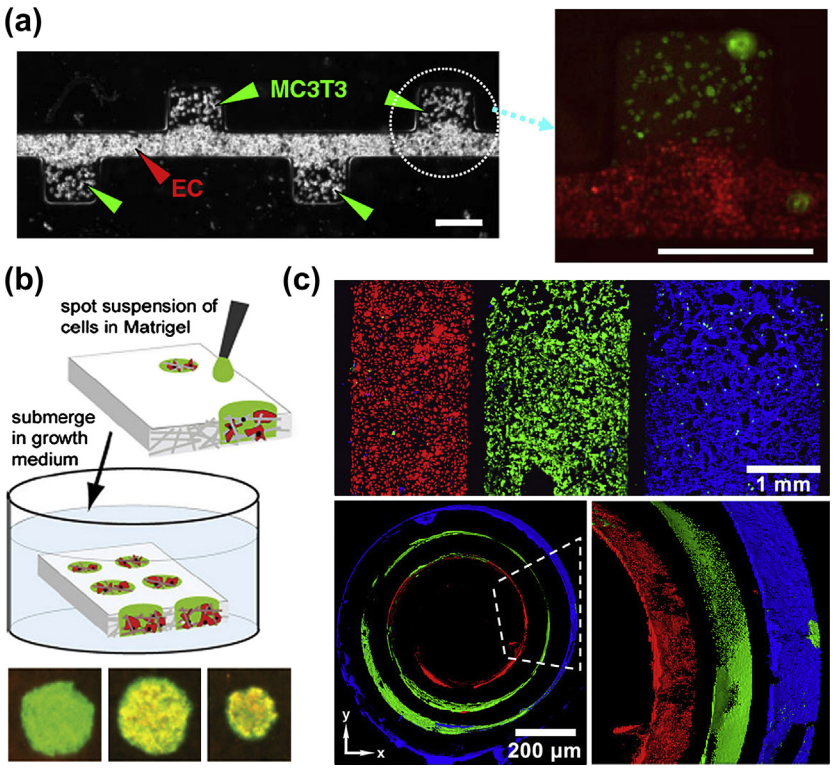

FIGURE 19.6
(a) Patterning cells in alginate hydrogels using light-directed release of caged calcium in a microfluidic device. The image shows patterned co-culture of endothelial cells and MC3T3 cells in a straight microchannel with side chambers. The endothelial cells (red fluorescence) stayed in the straight channel, while the MC3T3 cells (green fluorescence) remained in the side chamber where the alginate maintains the cells in position and in a non-spread rounded state. Scale bars shown in (a) are 200 μm. (b) Generation of 3D cultures of defined physical dimensions in the paper-supported hydrogels. (c) Three types of cells on a SIRM before and after rolling. Each color indicates a different type of cell. Red are endothelial cells; green: smooth muscle cells; blue: fibroblasts.

The density of cells in real tissues is about 10^8–10^9 cells cm^{-3} which is difficult to recapitulate in most present *in vitro* models, due to the difficulty of replicating the spatial distributions of oxygen, metabolites, and signaling molecules in tissues. To tackle this problem, we developed a device for the 3D culture of mammalian cells in microchannels by combining microfluidics and soft lithographic molding of gels containing mammalian cells. Collagen or Matrigel™ made up the matrix of each module of cell-containing gel. Each module had at least one dimension below ~300 μm. The flux of oxygen, nutrients, and metabolic products into and out of the modules was sufficient to allow cells to proliferate to densities comparable to those of native tissue. Multiple cell types with high survival ratios (99%) could be spatially organized in the microfluidic channels [159]. We also stacked and destacked layers of paper impregnated with suspensions of cells in ECM hydrogel ('cells-in-gels-in-paper' or CiGiP) to make it possible to control oxygen and nutrient gradients in 3D and to analyze molecular and genetic responses. Stacking assembles the 'tissue', whereas destacking disassembles it, and allows its analysis (Fig. 19.6b). The study offers a uniquely flexible approach to study cell responses to 3D molecular gradients and to mimic tissue- and organ-level functions [160]. *In vivo*, cells in different locations of 3D tissues are physiologically different, because they are exposed to different concentrations of oxygen, nutrients, and signaling molecules, and to other environmental factors (temperature, mechanical stress, etc). The majority of high throughput assays based on 3D cultures can only detect the average behavior of cells in the whole 3D construct. Isolation of cells from specific regions of 3D cultures is possible, but relies on low-throughput techniques such as tissue sectioning and micromanipulation. To tackle this problem, we developed a simple method for culturing arrays of thin planar sections of tissues, either alone or stacked to create more complex 3D tissue structures based on the 'cells-in-gels-in-paper' approach. The procedure starts with sheets of paper patterned with hydrophobic regions that form 96 hydrophilic zones. Serial spotting of cells suspended in ECM gel onto the patterned paper creates an array of 200 micron-thick slabs of ECM gel containing cells. Stacking the sheets with zones aligned on top of one another assembles 96 3D multilayer constructs. Destacking the layers of the 3D culture, by peeling apart the sheets of paper, 'sections' all 96 cultures at once. Because the 3D cultures are assembled from multiple layers, the number of cells plated initially in each layer determines the spatial distribution of cells in the stacked 3D cultures. This capability made it possible to compare the growth of 3D tumor models of different spatial composition, and to examine the migration of cells in these structures [161].

At the core of tissue engineering is the construction of 3D scaffolds out of biomaterials to provide mechanical support and guide cell growth into forming new tissues or organs. Recently, we reported a new fabrication strategy that results in stable tubular tissue with a high structural similarity to many biological tubular tissues. Using a stress-induced rolling membrane (SIRM) technique, we used two fabrication steps for their tubular structures. First, different types of cells were delivered and patterned on a two dimensional SIRM using microfluidic channels. Then the SIRM was released to roll up into a 3D tube. The tubes have different types of cells at specific locations, i.e., different parts of the tube wall are made up of different cells (Fig. 19.6c). Mimicking structural and functional features is a prerequisite for fabricating functional tubular tissues *in vitro*, and the realization of structural-tissue mimicry may have wide applications in simulation of many tubular tissues and enriches the toolbox for 3D micro/nanofabrication by initially patterning in 2D and transforming it into 3D [162].

CONCLUSION AND FUTURE PROSPECTS

Soft lithography brings to microfabrication low cost, simple procedures, rapid prototyping of custom-designed devices, three-dimensional capability, easy integration with existing instruments such as optical microscopy, molecular level control of surfaces, and biocompatibility [1,2]. These techniques allow patterning of cells and their environments with

convenience and flexibility at dimensions smaller than micrometers. We have described several complementary soft lithographic techniques — μCP, μFP, and LFP — which are useful in their ability to pattern the microenvironment of cultured cells.

μCP is perhaps the simplest method for patterning surfaces. It also provides the highest resolution in patterns with the greatest flexibility in the shape and size of the patterns generated. It provides the best control when one needs to pattern only two types of ligands or proteins. Microfluidic channels are well suited for patterning surfaces using delicate objects such as proteins and cells. They are also useful when multiple ligands, proteins or cells need to be patterned. LFP is similar to patterning with individual microfluidic channels except that the individual flows are kept from mixing with each other by laminar flow, not by physical walls. The ability to pattern the fluid environment is the distinguishing feature of this method, and this enables laminar flow to be used to pattern the distribution of different fluids over the surface of a single mammalian cell. This capability allows patterning of portions of a single cell, and remodeling of the cell culture environment, both in the presence of living cells. Micro-topographies can be useful for certain experiments where micropatterning alone is not sufficient. The combination of two or more of these techniques is starting to become useful for more sophisticated experiments, such as 3D pattering.

Soft lithography is still practiced by only a relatively small number of biologists, but its use is growing rapidly. There are many cell culture environments and related technologies that we have not discussed in this chapter; many of them relate to soft lithographic methods. For example, there are a number of methods of manipulation of chemistry on SAMs, and tools of micropatterns, to allow for molecular level control at the cell-substrate interface [32,163,164].

Although we are starting to have more techniques for fabrication in three dimensions, patterning of cells in three dimensions is still difficult [9,165,166]. We are making rapid progress, however, in the 'fourth dimension'; i.e., time. Since we can turn the surface 'on' and 'off' for adhesion of cells, and change the media at will in laminar flows, real-time monitoring of temporal changes in individual cells is possible [95,116,119]. The optical transparency of PDMS makes it straightforward to pattern the intensity of light in cell cultures [2]. The gas permeability of PDMS may be useful in patterning the gas surrounding cells. PDMS is electrically insulating, and molding or fabricating electrically conducting wires in it should allow patterning of electric fields around cells [95,167]. Gravitational fields can also be affected: microfluidic culture chambers with adherent cells can be turned upside down without loss of the culture media. Temperature, fluid shear, and other factors may also be accurately patterned.

The functional potential of a cell is determined by its genetics. Realization of that potential depends, *inter alia*, on whether the cell is exposed to the appropriate environment for expression of particular sets of genes. Soft lithography provides tools for patterning cells and their environment with precise spatial control. This capability aids efforts in understanding fundamental cell biology, and advances our ability to engineer cells and tissues. The ease with which electronic components or other 'non-biological' components can be fabricated with soft lithography also paves the way for the engineering of cells and tissues for use in biosensors and hybrid systems (e.g., interfaces between semiconductor-based computation and biological computation) that combine living and non-living components.

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CHAPTER 20

Cell Interactions with Polymers

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Scaffolds composed of synthetic and natural polymers have been essential components of tissue engineering since its inception [1]. Polymers are currently used in a wide range of biomedical applications, including applications in which the polymer remains in intimate contact with cells and tissues for prolonged periods (Table 20.1). Many of these polymer materials have been tested for tissue-engineering applications as well. To select appropriate polymers for tissue engineering, it is helpful to understand the influence of these polymeric materials on viability, growth, and function of attached or adjacent cells. In addition, it is now possible to synthesize polymers that interact in predictable ways with cells and tissues; understanding the nature of interactions between cells and polymers provides the foundation for this approach.

This chapter reviews previous work on the interactions of tissue-derived cells with polymers, particularly the types of synthetic polymers that have been employed as biomaterials. The focus of the chapter is on interactions of cells with polymers that might be used in tissue engineering; therefore, both *in vitro* and *in vivo* methods for measuring cell-polymer interactions are described. The interactions of cells in flowing blood, particularly platelets, with synthetic polymer surfaces is also an important aspect of biomaterial design, but is not considered here.

METHODS FOR CHARACTERIZING CELL INTERACTIONS WITH POLYMERS

In vitro cell culture methods

Cell interactions with polymers are often studied *in vitro*, using cell culture techniques. While *in vitro* studies do not reproduce the wide range of cellular responses observed after implantation of materials, the culture environment provides a level of control and quantification that cannot be obtained *in vivo*. Cells in culture are generally plated over a polymer surface and interaction is allowed to proceed for several hours; the extent of cell adhesion and spreading on the surface is then measured. By maintaining the culture for longer periods, perhaps for many days, the influence of the substrate on cell viability, function, and motility can also be determined. Since investigators use different techniques to assess cell interactions with polymers, and the differences between techniques are critically important for interpretation of interactions, some of the most frequently used *in vitro* methods are reviewed in this section.

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TABLE 20.1 Some of the polymers that might be useful in tissue engineering, based on past use in biomedical devices

Polymer	Medical applications
Polydimethylsiloxane, Silicone elastomers (PDMS)	Breast, penile, and testicular prostheses Catheters Drug delivery devices Heart valves Hydrocephalus shunts Membrane oxygenators
Polyurethanes (PEU)	Artificial hearts and ventricular assist devices Catheters Pacemaker leads
Poly(tetrafluoroethylene) (PTFE)	Heart valves Vascular grafts Facial prostheses Hydrocephalus shunts Membrane oxygenators Catheters and sutures
Polyethylene (PE)	Hip prostheses Catheters
Polysulphone (PSu)	Heart valves Penile prostheses
Poly(ethylene terephthalate) (PET)	Vascular grafts Surgical grafts and sutures
Poly(methyl methacrylate) (pMMA)	Fracture fixation Intraocular lenses Dentures
Poly(2-hydroxyethylmethacrylate) (pHEMA)	Contact lenses Catheters
Polyacrylonitrile (PAN)	Dialysis membranes
Polyamides	Dialysis membranes Sutures
Polypropylene (PP)	Plasmapheresis membranes Sutures
Poly(vinyl chloride) (PVC)	Plasmapheresis membranes Blood bags
Poly(ethylene-co-vinyl acetate)	Drug delivery devices
Poly(L-lactic acid), Poly(glycolic acid), and Poly(lactide-co-glycolide) (PLA, PGA, and PLGA)	Drug delivery devices Sutures
Polystyrene (PS)	Tissue culture flasks
Poly(vinyl pyrrolidone) (PVP)	Blood substitutes
Hydroxyapatite	Bone repair
Collagen	Artificial skin
Poly(*p*-dioxanone) and poly(*omega*-pentadecalactone-*co*-*p*-dioxanone) [121]	Sutures

To perform any measurement of cell interaction with a polymer substrate, the polymeric material and the cells must come into contact. Preferably, the experimenter should control (or at least understand) the nature of the contact; this is a critical, and often overlooked, aspect of all of these measurements. Some materials are easily fabricated in a format suitable for study; polystyrene films, for example, are transparent, durable, and strong. Other materials must be coated onto a rigid substrate (such as a glass coverslip) prior to study. But since cell function is sensitive to chemical, morphological, and mechanical properties of the surface,

almost every aspect of material preparation can introduce variables that are known to influence cell interactions.

ADHESION AND SPREADING

Most tissue-derived cells require attachment to a solid surface for viability and growth. For this reason, the initial events that occur when a cell approaches a surface are of fundamental interest. In tissue engineering, cell adhesion to a surface is critical because adhesion precedes other cell behaviors such as cell spreading, cell migration and, often, differentiated cell function.

A number of techniques for quantifying the extent and strength of cell adhesion have been developed: a more complete description of these techniques with early references is available (see Chapter 6 of reference [2]). In fact, so many different techniques are used that it is usually difficult to compare studies performed by different investigators. This situation is further complicated by the fact that cell adhesion depends on a large number of experimental parameters, many of which are difficult to control. The simplest methods for quantifying the extent of cell adhesion to a surface involve three steps:

1) Suspension of cells over a surface,
2) Incubation of the sedimented cells in culture medium for some period of time, and
3) Detachment of loosely adherent cells under controlled conditions.

The extent of cell adhesion, which is a function of the conditions of the experiment, is determined by quantifying either the number of cells that remain associated with the surface (the 'adherent' cells) or the number of cells that were extracted with the washes. Radiolabeled or fluorescently-labeled cells can be used to permit measurement of the number of attached cells. Alternatively, the number of attached cells can be determined by direct visualization, by measurement of concentration of an intracellular enzyme or by binding of a dye to an intracellular component such as DNA. In many cases, the 'adherent' cells are further categorized based on morphologically differences (e.g., extent of spreading, formation of actin filament bundles, presence of focal contacts). This technique is simple, rapid, and, since it requires simple equipment, common. Unfortunately, the force that is provided to dislodge the non-adherent cells is often not controlled, making it difficult to compare results obtained from different laboratories, even when they are using the same technique.

This disadvantage can be overcome by using a centrifuge or a flowing fluid to provide a controllable and reproducible detachment force. In centrifugal detachment assays, the technique described above is modified: after the incubation period, the plate is inverted and subjected to a controlled detachment force by centrifugation. In most flow chambers, the fluid is forced between two parallel plates. Prior to applying the flow field, a cell suspension is injected into the chamber, and the cells are permitted to settle onto the surface of interest and adhere. After some period of incubation, flow is initiated between the plates. These chambers can be used to measure the kinetics of cell attachment, detachment, and rolling on surfaces under conditions of flow. Usually, the overall flow rate is adjusted so that the flow is laminar, and the shear stresses at the wall approximate those found in the circulatory system; however, these chambers can be used to characterize cell detachment under a wide range of conditions.

Radial flow detachment chambers have also been used to measure forces of cell detachment. Because of the geometry of the radial flow chamber, where cells are attached uniformly to a circular plate and fluid is circulated from the center to the periphery of the chamber along radial paths, the fluid shear force experienced by the attached cells decreases with radial position from the center to the periphery. Therefore, in a single experiment, the influence of a range of forces on cell adhesion can be determined. A spinning disk apparatus can be used in a

similar fashion [3], including use with micro- or nano-patterned substrates [4]. Finally, micropipette techniques can be used to measure cell membrane deformability or forces of cell-cell or cell-surface adhesion [5].

MIGRATION

The migration of individual cells within a tissue is a critical element in the formation of the architecture of organs and organisms. Similarly, cell migration is likely to be an important phenomenon in tissue engineering, since the ability of cells to move, either in association with the surface of a material or through an ensemble of other cells, will be an essential part of new tissue formation or regeneration. Cell migration is also difficult to measure, particularly in complex environments. Fortunately, a number of useful techniques for quantifying cell migration in certain situations have been developed. As in cell adhesion, however, no technique has gained general acceptance, so it is difficult to correlate results obtained by different techniques or different investigators.

Experimental methods for characterizing cell motility can be divided into visual assays and population assays: a more complete description with early references is available in Chapter 7 of reference [2]. In visual assays, the movements of a small number (usually ~100) of cells are observed individually. Population techniques, on the other hand, allow the observation of the collective movements of larger numbers of cells: in filter chamber assays the number of cells migrating through a membrane or filter is measured, while in under-agarose assays the leading front of cell movement on a surface under a block of agarose is monitored. Both visual and population assays can be quantitatively analyzed, enabling the estimation of intrinsic cell motility parameters, such as the random motility coefficient and the persistence time: these methods are described in reference [2].

AGGREGATION

Cell aggregates are important tools in the study of tissue development, permitting correlation of cell-cell interactions with cell differentiation, viability and migration, as well as subsequent tissue formation. The aggregate morphology permits re-establishment of the cell-cell contacts normally present in tissues; therefore, cell function and survival are often enhanced in aggregate culture. Because of this, cell aggregates may also be useful in tissue engineering, enhancing the function of cell-based hybrid artificial organs or reconstituted tissue transplants [6].

Gentle rotational stirring of suspensions of dispersed cells is the most common method for making cell aggregates [7]. While this method is suitable for aggregation of many cells, serum or serum proteins must be added to promote cell aggregation in many cases, thus making it difficult to characterize the aggregation process and to control the size and composition of the aggregate. Specialized techniques can be used to produce aggregates in certain cases, principally by controlling cell detachment from a solid substratum. For example, stationary culture of hepatocytes above a non-adherent surface [8] or attached to a temperature sensitive polymer substratum [9] have been used to form aggregates. Synthetic polymers produced by linking cell binding peptides (such as RGD and YIGSR) to both ends of poly(ethylene glycol) (PEG) have been used to promote aggregation of cells in suspension [10].

The kinetics and extent of aggregation can be measured by a variety of techniques. Often, direct visualization of aggregate size is used to determine the extent of aggregation, following the pioneering work of Moscona [7]. The kinetics of aggregation can be monitored in this manner as well, by measuring aggregate size distributions over time. This procedure is facilitated by the use of computer image analysis techniques or electronic particle counters, where sometimes the disappearance of single cells (instead of the growth of aggregates) is followed. Specialized aggregometers can provide reproducible and rapid measurements of

the rate of aggregation; in one such device, small angle light scattering through rotating sample cuvettes is used to produce continuous records of aggregate growth [11].

CELL PHENOTYPE

In tissue-engineering applications, particularly those in which cell-polymer hybrid materials are prepared, one is usually interested in promotion of some cell-specific function. For example, protein secretion and detoxification are essential functions for hepatocytes used for transplantation or liver support devices; therefore, measurements of protein secretion and intracellular enzyme activity (particularly the hepatic P_{450} enzyme system) are frequently used to assess hepatocyte function. Similarly, the expression and activity of enzymes involved in neurotransmitter metabolism (such as choline acetyltransferase or tyrosine hydroxylase) are often used to assess the function of neurons. Production of extracellular matrix (ECM) proteins is important in the physiology of many tissues, particularly connective tissues and skin; collagen and glycosaminoglycan production has been used as an indicator of cell function in chondrocytes, osteoblasts, and fibroblasts. In some cases, the important cell function involves the coordinated activity of groups of cells, such as the formation of myotubules in embryonic muscle cell cultures, the contraction of the matrix surrounding the cells, or the coordinated contraction of cardiac muscle cells. In these cases, cell function is measured by observation of changes in morphology of cultured cells or cell communities.

HIGH-THROUGHPUT METHODS FOR CHARACTERIZATION OF CELL-POLYMER INTERACTIONS

Cell-polymer interactions can be analyzed using methods that allow for many parallel measurements. For example, small spots of ECM proteins can be deposited onto glass slides — or other substrates — to allow high-throughput screening of cell interactions with ECM proteins and protein combinations [12]. This microprinting method can also be applied to three-dimensional materials appropriate for tissue engineering [13]. Microarray printing of polymer and polymer blends allows for screening of interactions of cells with synthetic polymer surfaces as well [14]. Although these methods allow the parallel testing of many different cell/polymer combinations, the measurements of cell interaction are usually limited to light or fluorescence microscopy.

In vivo methods

The context of cell-polymer interactions *in vivo* is inherently complex due to the presence of blood, interstitial fluids, and the presence of multiple cell types in various activation states. Proteins from the blood and interstitial fluid adsorb to polymer surfaces in a non-specific manner and provide a substrate for cell adhesion [15]. Thus, it is believed that cells interact directly with this proteinaceous layer instead of the polymer surface, an assumption that explains the similar *in vivo* reactions elicited by a diverse array of polymers. Almost all implanted polymers induce a unique inflammatory response termed the foreign body response (FBR) [16].

The FBR can be divided in several overlapping phases that include non-specific protein adsorption, inflammatory cell recruitment — predominantly of neutrophils and macrophages, macrophage fusion to form foreign body giant cells (FBGC), and involvement of fibroblasts and endothelial cells (Fig. 20.1). The end result of the FBR is the formation of FBGCs directly on the polymer surface, and the subsequent encapsulation of the implant by a fibrous capsule that is largely avascular. To date, the molecular and cellular determinants of the FBR remain largely unknown. However, recent studies have suggested specific roles for several proteins and a key role for macrophages.

A number of implantation techniques in rodents and larger animals (typically rabbits, pigs, or sheep) have been adopted for the investigation of cell-polymer interactions. Most notably,

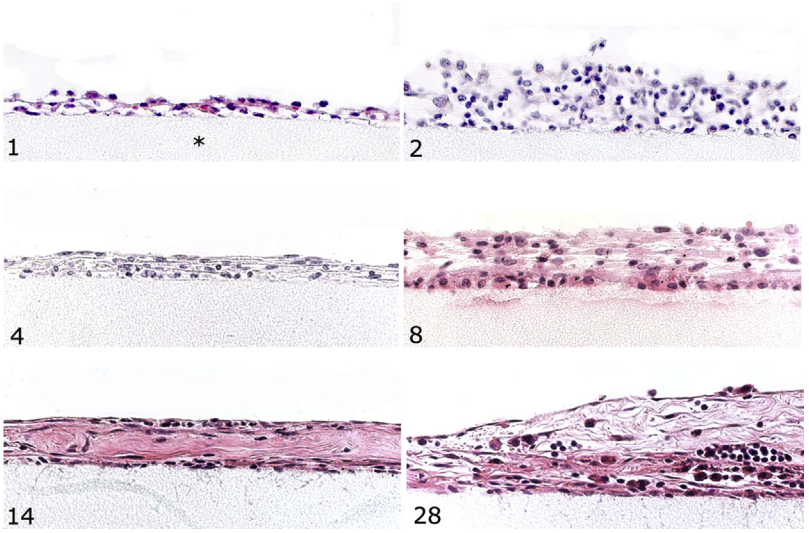


FIGURE 20.1
Development of the foreign body response. Representative images of hematoxylin and eosin-stained Millipore filter disks (mixed cellulose ester) implanted in the peritoneal cavity of wild type mice. The number on each panel indicates the number of days after implantation. In the first two days following implantation, the disks display loose and expanding layers of attached inflammatory cells. Between four and eight days, the cell layers show increased organization that appears to coincide with the deposition of collagen fibers. Continuous deposition of collagenous matrix and an overall reduction in cellular content are characteristic of the two and four week time points. * indicates the disk. Original magnification 400X.

short-term studies for the analysis of protein adsorption, inflammatory cell recruitment and adhesion, and macrophage fusion most often employ either intra-peritoneal (IP) implantation or the subcutaneous (SC) cage-implant, also known as the wound chamber model. In the IP implant model, a sterilized piece of polymer is placed in the peritoneal cavity through a surgical incision [17]. This model has been used extensively in the investigation of protein adsorption onto polymer surfaces and its impact during the early inflammatory response (0–72 hr). In general, due to the short duration, it is difficult to extrapolate the information obtained from such studies to the fate of the FBR. Implant studies of intermediate duration (days to weeks) have been executed using an SC cage-implant; in this system, a polymer is placed within a cage made of stainless steel wire mesh and then implanted through a surgical incision [18]. The SC cage-implant is useful because biologically active soluble agents can be injected through the wire mesh in the immediate vicinity of the polymer.

Both IP and SC implantations allow for the analysis of recruited, non-adherent cell populations, which are collected by lavage of the peritoneal cavity or aspiration of host-derived exudates from the cage. In addition, implant materials can be recovered and analyzed for the presence of adherent cells by various techniques, immunohistochemistry being the most reliable. Thus, both implantation models can provide information regarding the migration of cells to the implantation site and the interaction of cells (adhesion, survival, fusion) with the polymer surface. Other commonly used *in vivo* models for the analysis of cell-polymer interactions involve the implantation of test polymers directly into host tissues such as dermis (subcutaneous), muscle, bone, and brain. Generally, such studies have been carried for longer incubation periods (weeks to months). Furthermore, polymer-based constructs have been surgically implanted at sites where they are expected to perform biological functions, for example vascular grafts [19,20], heart valves [21], and cardiac patches [22]. Thus, in addition to biocompatibility and toxicity, polymers can be evaluated for their role in function of a tissue or organ. The direct implant model can be considered the most representative for most

polymer applications and can provide information regarding the FBR, such as inflammation (macrophage accumulation and polarization, FBGC formation), fibrosis (collagen content), encapsulation (capsule thickness), and angiogenesis (vascular density). Finally, in cases where the polymer is susceptible to degradation, additional analyses can be performed to evaluate the role of chemical and physical changes of the material in interactions with the tissue.

CELL INTERACTIONS WITH POLYMERS

Protein adsorption to polymers

A polymeric material that is placed in solution or implanted in the body becomes coated with proteins quickly, usually within minutes. Many of the subsequent interactions of cells with the material depend on, or derive from, the composition of the protein layer that forms on the surface. Polymers have been shown to adsorb a large number of proteins *in vitro* [23]. For example, proteomic analysis of PP, PET, and PDMS incubated with serum identified immunoglobulins, transferrin, albumin, serum amyloid P, and complement C4, among other proteins, on the polymer surface [24]. The C3 component of complement has also been shown to adsorb to medical grade PEU and adhesion of inflammatory cells to the polymer was reduced in the presence of C3-depleted serum but not fibronectin-depleted serum [25]. In contrast, *in vivo* studies employing the IP implantation method suggest that adsorption of fibrinogen is the critical determinant of the FBR [26]. Specifically, adsorption and denaturation of fibrinogen on polymer surfaces might lead to the exposure of cryptic cell adhesion motifs (integrin binding sites) which influence subsequent cellular interactions. Despite the recognized importance of adsorbed proteins, it is possible that direct contact between polymers and cells can have significant impact on cell function. Supporting evidence for such interactions was shown in study where large PMMA beads (150 μm in diameter) induced the production of a pro-inflammatory cytokine by macrophages in the absence of serum proteins [27].

Effect of polymer chemistry on cell behavior

SYNTHETIC POLYMERS

For cells attached to a solid substrate, cell behavior and function depend on the characteristics of the substrate. Consider, for example, experiments described by Folkman and Moscona, in which cells were allowed to settle onto surfaces formed by coating conventional tissue culture polystyrene (TCPS) with various dilutions of pHEMA [28]. As the amount of pHEMA added to the surface was increased, cell spreading decreased as reflected by the average cell height on the surface. The degree of spreading, or average height, correlated with the rate of cell growth (Fig. 20.2), suggesting that cell shape, which was determined by the adhesiveness

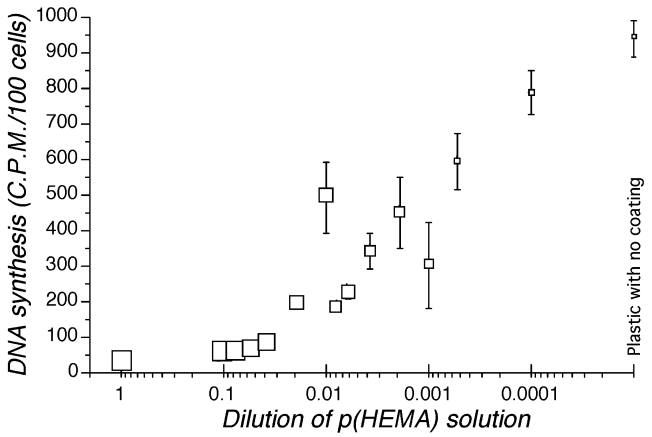


FIGURE 20.2

Cell shape and growth are modulated by properties of a polymer surface. Cell culture surfaces were produced by evaporating diluted solutions of pHEMA onto TCPS. The uptake of [^{3}H]thymidine was used as a measure of proliferation. The size of the symbol represents the relative cell height; small symbols represent cells with small heights, and therefore significant spreading, large symbols represent cells with large heights, and therefore negligible spreading. *Data replotted from Folkman and Moscona [28].*

of the surface, modulated cell proliferation. In these experiments, two well-known polymers (TCPS and pHEMA) were used to produce a series of surfaces with graded adhesivity, permitting the identification of an important aspect of cell physiology. These experiments clearly demonstrate that the nature of a polymer surface will have important consequences for cell function, an observation of considerable significance with regard to the use of polymers in tissue engineering.

Following an experimental design similar to that employed by Folkman and Moscona, a number of groups have examined the relationship between chemical or physical characteristics of the substrate and behavior or function of attached cells. For example, in a study of cell adhesion, growth, and collagen synthesis on synthetic polymers, fetal fibroblasts from rat skin were seeded onto surfaces of 13 different polymeric materials [29]. The polymer surfaces had a range of surface energies, as determined by static water contact angles, from very hydrophilic to very hydrophobic. On a few of the surfaces (PVA and cellulose), little cell adhesion and no cell growth was observed. On most of the remaining surfaces, however, a moderate fraction of the cells adhered to the surface and proliferated. The rate of proliferation was relatively insensitive to surface chemistry: the cell doubling time is ~24 hr, with slightly slower growth observed for the two very hydrophobic surfaces (PTFE and PP). Collagen biosynthesis was also correlated with contact angle, with higher rates of collagen synthesis per cell for the most hydrophobic surfaces.

Results from a number of similar studies have been summarized previously: see Table 12.1 and 12.2 in reference [2], for example. Some general conclusions emerge from this past work. For example, in a number of studies, cell adhesion appears to be maximized on surfaces with intermediate wettability (Fig. 20.3). For most surfaces, adhesion requires the presence of serum and, therefore, this optimum is likely related to the ability of proteins to adsorb to the surface. (In the absence of serum, however, adhesion is enhanced on positively charged surfaces, due to the net negative charge of most cell membranes.) Cell spreading on copolymers of HEMA (hydrophilic) and EMA (hydrophobic) was highest at an intermediate HEMA content, again corresponding to intermediate wettability [30]; in this case, spreading correlated with fibronectin adsorption [30]. The rate of fibroblast growth on polymer surfaces appears to be relatively independent of surface chemistry. Cell viability may also be related to interactions with the surface, although this is not yet predictable. The migration of surface-attached fibroblasts, endothelial cells, and corneal epithelial cells is also a function of polymer surface chemistry: see reference [2] for more details.

SURFACE MODIFICATION

Polymers can frequently be made more suitable for cell attachment and growth by surface modification. In fact, TCPS substrates used for tissue culture are obtained by surface treatment

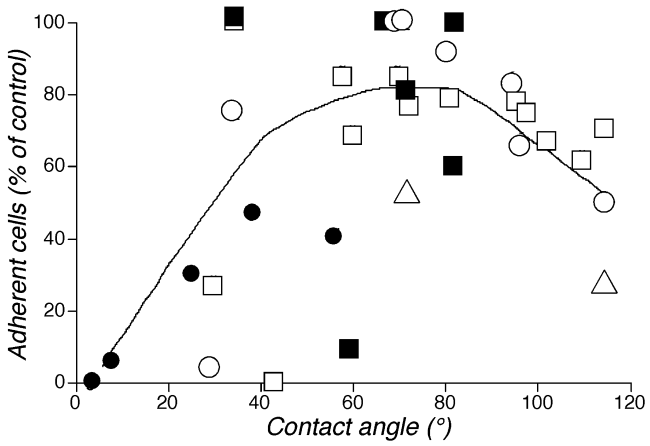


FIGURE 20.3
The relationship between cell adhesion and water-in-air contact angle. *Data replotted from Tamada and Ikada for fibroblasts (open squares) [29], Ikada for L cells (open circles) [54], Hasson et al. for endothelial cells (open triangles) [118], van Wachem et al. for endothelial cells (filled circles) [119].*

of polystyrene (PS) by glow discharge or exposure to chemicals, such as sulfuric acid, to increase the number of charged groups at the surface, which improves attachment and growth of many types of cells. Other polymers can also be modified in this manner. Treatment of pHEMA with sulfuric acid, for example, improves the adhesion of endothelial cells and permits cell proliferation on the surface [31]. Modification of PS or PET by radio frequency plasma deposition enhances attachment and spreading of fibroblasts and myoblasts [32]. The effects of these surface modifications appear to be secondary to increased adsorption of cell attachment proteins, such as fibronectin and vitronectin, to the surface. On the other hand, some reports have identified specific chemical groups at the polymer surface — such as hydroxyl (-OH) [33] or surface C-O functionalities [32] — as important factors in modulating the fate of surface-attached cells.

So far, no general principles that would allow prediction of the extent of attachment, spreading, or growth of cultured cells on different polymer surfaces have been identified. Correlations have been made with parameters such as the density of surface hydroxyl groups [33], density of surface sulfonic groups [34], surface free energy [35,36], fibronectin adsorption [32], and equilibrium water content [37] for specific cells, but exceptions to these correlations are always found. Perhaps general predictive correlations will emerge, as more complete characterization of polymer systems — including bulk properties, surface chemical properties, and nanoscale topography (as described below) — are collected. But many commonly used polymer materials are complicated mixtures, containing components that are added to enhance polymerization or to impart desired physical properties, often in trace quantities. Lot-to-lot variations in the properties of commercially available polymers can be significant.

The surface chemistry of polymers appears to influence cell interactions *in vivo*. For example, the ability of macrophages to form multinucleated giant cells at the material surface correlates with the presence of certain chemical groups at the surface of hydrogels: macrophage fusion decreases in the order $(CH_3)_2N$- > -OH = -CO-NH- > $-SO_3H$ > -COOH (-COONa) [38]. A similar hierarchy has been observed for CHO (Chinese Hamster Ovary) cell adhesion and growth on surfaces with grafted functional groups: CHO cell attachment and growth decreased in the order $-CH_2NH_2$ > $-CH_2OH$ > $-CONH_2$ > -COOH [39].

Layer-by-layer (LbL) assembly of nanofilms is a versatile approach for the control of surface properties to modulate cell attachment and function. In the most general technique, alternating layers of two polymers — one polycationic and the other polyanionic — are deposited onto a substrate to create a film of controlled thickness and chemical properties. By using a cell attachment protein, such as fibronectin, as one of the polyelectrolytes, thin films that control cell adhesion to the substrate can be created [40]. Control of conditions during LbL assembly — such as solution pH — can create films with different physical structures, which further modulate cell interactions [41]. Covalent reactions between the polymers — produced either by addition of crosslinking agents after LbL film formation or during the assembly of the film using reactive polymers — allows for the creation of more stable films [42].

BIODEGRADABLE POLYMERS

Biodegradable polymers slowly degrade and then dissolve following implantation. This feature may be important for many tissue-engineering applications, since the polymer will disappear as functional tissue regenerates. For this reason, interactions of cells with a variety of biodegradable polymers have been studied. Biodegradable polymers may provide an additional level of control over cell interactions: during polymer degradation, the surface of the polymer is constantly renewed, providing a dynamic substrate for cell attachment and growth.

Homopolymers and copolymers of lactic and glycolic acid (PLA, PGA, PLGA) have been frequently examined as cell culture substrates, since they have been used as implanted sutures

for several decades. Many types of cells will attach and grow on these materials. For example, chondrocytes proliferate and secrete glycosaminoglycans within porous meshes of PGA and foams of PLA [43]. Similarly, rat hepatocytes attach to blends of biodegradable PLGA polymers and secrete albumin when maintained in culture [44]. Neonatal rat osteoblasts also attach to PLA, PGA, and PLGA substrates and synthesize collagen in culture [45].

Cell adhesion and function has been examined on materials made from other biodegradable polymers. As an example, cells from an osteogenic cell line attach onto polyphosphazenes produced with a variety of side groups; the rate of cell growth as well as the rate of polymer degradation depends on side group chemistry [46].

Use of degradable polymers increases the complexity of analysis in the *in vivo* setting because their degradation products can cause excessive and prolonged biological responses. Depending on the material used, degradation products can be released by hydrolysis, enzymatic digestion, degradative activity of macrophages and FBGC, or combinations of these mechanisms. The effects of polymer-derived products that are released by hydrolysis or enzymatic digestion depend on the chemical properties of the polymer and are not difficult to determine. On the other hand, the effects of products released due to cellular activities are difficult to predict, because the chemical and biological mechanisms responsible for their generation and release have not been established. Furthermore, the activity of cells, and thus the rate of release, can be influenced by several parameters such as local concentrations of growth factors. It is expected that biodegradable polymers should allow for the favorable resolution of the FBR after their complete disappearance from the host tissue. The disappearance of FBGC from implantation sites after degradation of polymers to microparticles smaller than 10 μm in size has been observed (Kyriakides et al., unpublished data). Furthermore, macrophages were observed to persist until the degradation of the polymer was complete. In SC implantation sites the formation of a vascularized collagenous work as a replacement for the polymer was observed. Presumably, the physiology of the implantation site, the acuteness of the inflammatory response, and the rate of polymer degradation all influence the nature of the FBR. Favorable *in vivo* results have been obtained with biodegradable elastomeric polyesters composed of poly(diol citrates) [47]. However, the duration of the FBR was shown to exceed 12 months in implanted compressed naltrexone-poly[trans-3,6-dimethyl-1,4-dioxane-2,5-dione] (DL-lactide) loaded microspheres [48].

SYNTHETIC POLYMERS WITH ADSORBED PROTEINS

As mentioned above, cell interactions with polymer surfaces appear to be mediated by proteins, adsorbed from the local environment. Since it is difficult to study these effects *in situ* during cell culture, often the polymer surfaces are pretreated with purified protein solutions. In this way, the investigators hope that subsequent cell behavior on the surface will represent cell behavior in the presence of a stable layer of surface-bound protein. A major problem with this approach is the difficulty in determining whether surface conditions, i.e., the density of protein on the surface, change during the period of the experiment.

As described earlier, cell spreading, but not attachment, correlates with fibronectin adsorption to a variety of surfaces. Rates of cell migration on a polymer surface are usually sensitive to the concentration of pre-adsorbed adhesive proteins [49], and migration can be modified by addition of soluble inhibitors to cell adhesion [50]. It appears that the rate of migration is optimal at intermediate substrate adhesiveness, as one would expect from mathematical models of cell migration [51]. In fact, a recent study shows a clear correlation between adhesiveness and migration for CHO cells [52].

The outgrowth of corneal epithelial cells from explanted rabbit corneal tissue has been used as an indicator of cell attachment and migration on biomaterial surfaces [53]. When corneal cell outgrowth was measured on ten different materials that were pre-adsorbed with

fibronectin, outgrowth generally increased with the ability of fibronectin to adsorb to the material. Exceptions to this general trend could be found, suggesting that other factors (perhaps stability of the adsorbed protein layer) are also important.

HYBRID POLYMERS WITH IMMOBILIZED FUNCTIONAL GROUPS

Surface modification techniques have been used to produce polymers with surface properties that are more suitable for cell attachment [54]. For example, chemical groups can be added to change the wettability of the surface, which often influences cell adhesion (Fig. 20.3), as described above. Alternatively, whole proteins such as collagen can be immobilized to the surface, providing the cell with a substrate that more closely resembles the ECM found in tissues. Collagen and other ECM molecules have also been incorporated into hydrogels by either adding the protein to a reaction mixture containing monomers and initiating the radical polymerization, or mixing the protein with polymerized polymer, such as pHEMA, in appropriate solvents. To isolate certain features of ECM molecules, and to produce surfaces that are simpler and easier to characterize, smaller biologically active functional groups have been used to modify surfaces. These biologically active groups can be oligopeptides, saccharides, or glycolipids.

Certain short amino acid sequences, identified by analysis of active fragments of ECM molecules, appear to bind to receptors on cell surfaces and mediate cell adhesion. For example, the cell-binding domain of fibronectin contains the tripeptide RGD (Arg-Gly-Asp) [55].[1] Cells attach to surfaces containing adsorbed oligopeptides with the RGD sequence and soluble, synthetic peptides containing the RGD sequence reduce the cell binding activity of fibronectin, demonstrating the importance of this sequence in adhesion of cultured cells. A large number of ECM proteins (fibronectin, collagen, vitronectin, thrombospondin, tenascin, laminin, and entactin) contain the RGD sequence. The sequences YIGSR and IKVAV on the A chain of laminin also have cell binding activity, and appear to mediate adhesion in certain cells.

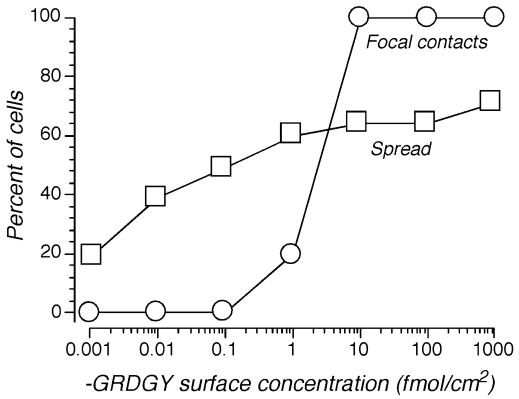
Because RGD appears to be critical in cell adhesion to ECM, many investigators have examined the addition of this sequence to synthetic polymer substrates. The addition of cell binding peptides to a polymer can induce cell adhesion to otherwise non-adhesive or weakly-adhesive surfaces. Cell spreading and focal contact formation are also modulated by the addition of peptide (Fig. 20.4). Since cells contain cell adhesion receptors that recognize only certain ECM molecules, use of an appropriate cell binding sequence can lead to cell-selective surfaces, where the population of the cells that adhere to the polymer is determined by the peptide [56].

The presence of serum proteins attenuates the adhesion activity of peptide-grafted PEU surfaces [57], highlighting a difficulty in using these peptide-grafted materials *in vivo*. This problem may be overcome, however, through the development of base materials that are biocompatible yet resistant to protein adsorption. One of the most successful approaches for reducing protein adsorption or cell adhesion is to produce a surface rich in PEG. A variety of techniques have been used including surface grafting of PEG, adsorption of PEG-containing copolymers, semi-interpenetrating networks, or immobilization of PEG-star polymers to increase the density of PEG chains at the surface.

As an alternate approach, matrices can be formed directly from synthetic polypeptides. For example, genes coding the β-sheet of silkworm silk have been combined with genes coding fragments of fibronectin to produce proteins that form very stable matrices with cell adhesion domains (Pronectin F®). Synthetic proteins based on peptide sequences from elastin have been used as cell culture substrates: in the presence of serum, fibroblasts and endothelial cells

[1] Amino acids are identified by their one letter abbreviations: A=alanine, R=arginine, N=asparagine, D=aspartic acid, B=asparagine or aspartic acid, C=cysteine, Q=glutamine, E=glutamic acid, Z=glutamine or glutamic acid, G=glycine, H=histidine, I=isoleucine, L=leucine, K=lysine, M=methionine, F=phenylalanine, P=proline, S=serine, T=threonine, W=trypotophan, Y=tyrosine, V=valine.

(a)



(b)

Peptide	Human foreskin fibroblasts	Human vascular smooth muscle cells	Platelets	Human umbilical vein endothelial cells
-GRGDY	85	93	0	90
-GYIGSRY	79	88	0	84
-GPDSGRY	56	62	0	66
-GREDVY	9	7	0	89
-None	9	10	0	8

FIGURE 20.4
Cell adhesion to surfaces with immobilized peptides: data from [56,120]. a) Fibroblast spreading on surfaces with immobilized -GRGDY. b) Cell-selective surfaces: fraction of cells spread for several immobilized peptides.

adhered to the surfaces of matrices formed by γ-irradiation crosslinking of polypeptides containing repeated sequences GGAP, GGVP, GGIP, and GVGVP [58].

Surface adsorption of homopolymers of basic amino acids, such as polylysine and polyornithine, are frequently used to enhance cell adhesion and growth on polymer surfaces. Similarly, covalently bound amine groups can also influence cell attachment and growth. Polymerization of styrene with monamine- or diamine-containing monomers produced copolymers with ~8% mono- or diamine side chains, which enhanced spreading and growth: diamine-PS > monoamine-PS > PS [59].

The immobilization of saccharide units to polymers can also influence cell attachment and function. As an example, N-p-vinylbenzyl-o-β-D-galactopyranosyl-(1−4)-D-gluconamide has been polymerized to form a polymer with a polystyrene backbone and pendant lactose functionalities [60]. Rat hepatocytes adhere to surfaces formed from this polymer, via asialoglycoprotein receptors on the cell surface, and remain in a rounded morphology consistent with enhanced function in culture. In the absence of serum, rat heptaocytes will adhere to similar polymers with pendant glucose, maltose, or maltotriose. Similar results have been obtained with polymer surfaces derivatized with N-acetyl glucosamine, which is recognized by a surface lectin on chicken hepatocytes [61].

Films produced by LbL techniques can also be surface modified to modulate cell interactions. For example, for films that are assembled using covalent reactions, residual reactive groups can be used to attach small molecules that control the interaction of the film with cells. In one study, PEI films produced by covalent LbL assembly were further modified by coupling of small molecules. Coupling of hydrophobic molecules produced films that permitted cell attachment and growth, whereas covalent coupling of hydrophilic sugar groups reduced cell attachment, even in the presence of serum proteins [62].

Electrically charged or electrically conducting polymers

A few studies have examined cell growth and function on polymers that are electrically charged. Piezoelectric polymer films, which were produced by high intensity corona poling of poly(vinylidene fluoride) or poly(vinylidene fluoride-*co*-trifluoroethylene) and should generate transient surface charge in response to mechanical forces, enhanced the attachment and differentiation of mouse neuroblastoma cells (Nb2a), as determined by neurite number and mean neurite length [63]. These observations may be important *in vivo*, as well. For example, positively-poled poly(vinylidene fluoride-*co*-trifluoroethylene) nerve guidance

channels produced greater numbers of myelinated axons than either negatively-poled or unpoled channels [64]. Electrically conducting polymers might be useful for tissue-engineering applications, because their surface properties can be changed by application of an applied potential. For example, endothelial cells attached and spread on fibronectin-coated polypyrrole films in the oxidized state, but became rounded and ceased DNA synthesis when the surface was electrically reduced [65].

Influence of surface morphology on cell behavior

The microscale texture of an implanted material can have a significant effect on the behavior of cells in the region of the implant. This has long been observed *in vivo*. For example, fibrosarcomas developed with high frequency, approaching 50% in certain situations, around implanted Millipore filters; the tumor incidence increased with decreasing pore size in the range of 450 to 50 μm [66].

The behavior of cultured cells on surfaces with edges and grooves is different than behavior on smooth surfaces. In many cases, cells oriented and migrated along fibers or ridges in the surface, a phenomenon called contact guidance from early studies on neuronal cell cultures [67]. Fibroblasts orient on grooved surfaces [68], with the degree of cell orientation depending on both the depth and pitch of the grooves. Not all cells exhibit the same degree of contact guidance when cultured on identical surfaces: a comparison of cell responses to surfaces with grooves and edges, collected from early studies, is provided in Table 12.3 of reference [2]. These measurements are relevant to design of materials for tissue engineering: adhesion and initial migration of an osteoblast cell line was examined on materials with grooves ranging in pitch from 150 to 1000 nm and found to be optimal on grooves of intermediate spacing [69]. Cultured stem cells behave differently on grooved surfaces, when compared to smooth surfaces, which appears to be secondary to changes in integrin assembly, focal contact formation, and cytoskeletal organization [70].

Other textures also influence cell behavior. For example, substrates with peaks and valleys influence the function of attached cells [71]. PDMS surfaces with 2 to 5 μm texture maximized macrophage spreading. Similarly, PDMS surfaces with 4 or 25 μm^2 peaks uniformly distributed on the surface provided better fibroblast growth than 100 μm^2 peaks or 4, 25 or 100 μm^2 valleys.

The microscale structure of a surface has a significant effect on cell migration, at least for the migration of human neutrophils. In one study, microfabrication technology was used to create regular arrays of micron-size holes (2 μm × 2 μm × 210 nm) on fused quartz and photosensitive polyimide surfaces [72]. The patterned surfaces, which mimicked a structural element of 3D network (i.e., spatially separated mechanical edges), were used to study the effect of substrate microgeometry on neutrophil migration. The edge-to-edge spacing between features was systematically varied from 6 μm to 14 μm with an increment of 2 μm. The presence of evenly distributed holes at the optimal spacing of 10 μm enhanced migration by a factor of 2 on polyimide, a factor of 2.5 on collagen-coated quartz and a factor of 10 on uncoated quartz. The biphasic dependence on the mechanical edges of neutrophil migration on 2D patterned substrate was strikingly similar to that previously observed during neutrophil migration within 3D networks, suggesting that microfabricated materials provide relevant models of 3D structures with precisely defined physical characteristics. Perhaps more importantly, these results illustrate that the microgeometry of a substrate, when considered separately from adhesion, can play a significant role in cell migration.

Use of patterned surfaces to control cell behavior

A variety of techniques have been used to create patterned surfaces containing cell adhesive and non-adhesive regions. Patterned surfaces are useful for examining fundamental determinants of cell adhesion, growth, and function. For example, individual fibroblasts were

attached to adhesive micro-islands of palladium that were patterned onto a non-adhesive pHEMA substrate using microlithographic techniques [73]. By varying the size of the micro-island, the extent of spreading and hence the surface area of the cell was controlled. On small islands (~500 μm^2) cells attached, but did not spread. On larger islands (4,000 μm^2), cells spread to the same extent as in unconfined monolayer culture. Cells on large islands proliferate at the same rate as cells in conventional culture, and most cells attached to small islands proliferate at the same rate as suspended cells. For 3T3 cells, however, contact with the surface enhanced proliferation, suggesting that anchorage can stimulate cell division by simple contact with the substrate as well as by increases in spreading.

A number of other studies have employed patterned surfaces in cell culture. Micrometer-scale adhesive islands of self-assembled alkanethiols were created on gold surfaces using a simple stamping procedure [74], which served to confine cell spreading islands. When hepatocytes were attached to these surfaces, larger islands (10,000 μm^2) promoted growth, while smaller islands (1,600 μm^2) promoted albumin secretion. Stripes of a monoamine derivatized surface were produced on fluorinated ethylene propylene films by radio frequency glow discharge [75]. Since proteins adsorbed differently to the monamine-derivatized and the untreated stripes, striped patterns of cell attachment were produced. A similar approach, using photolithography to produce hydrophilic patterns on a hydrophobic surface, produced complex patterns of neuroblastoma attachment and neurite extension [76]. A variety of substrate microgeometries were created by photochemical fixation of hydrophilic polymers onto TCPS or hydrophobic polymers onto PVA through patterned photomasks: bovine endothelial cells attached and proliferated preferentially on either the TCPS surface (on TCPS/hydrophilic patterns) or the hydrophobic surface (on PVA/hydrophobic patterns) [77]. When chemically patterned substrates were produced on self-assembled monolayer films using microlithographic techniques, neuroblastoma cells attached to and remained confined within amine-rich patterns on these substrates [78].

CELL INTERACTIONS WITH POLYMERS IN SUSPENSION

Most of the studies reviewed in the preceding section concerned the growth, migration, and function of cells attached to a solid polymer surface. This is a relevant paradigm for a variety of tissue-engineering applications, where polymers will be used as substrates for the transplantation of cells or as scaffolds to guide tissue regeneration *in situ*. Polymers may be important in other aspects of tissue engineering, as well. For example, polymer microcarriers can serve as substrates for the suspension culture of anchorage dependent cells, and therefore might be valuable for the *in vitro* expansion of cells or cell transplantation [79]. In addition, immunoprotection of cells suspended within semi-permeable polymer membranes is another important approach in tissue engineering, since these encapsulated cells may secrete locally active proteins or function as small endocrine organs within the body.

The idea of using polymer microspheres as particulate carriers for the suspension culture of anchorage dependent cells was introduced by van Wezel [80]. As described above for planar polymer surfaces, the surface characteristics of microcarriers influence cell attachment, growth, and function. In the earliest studies, microspheres composed of diethylaminoethyl (DEAE)-dextran were used; these spheres have a positively charged surface and are routinely used as anion-exchange resins. DEAE-dextran microcarriers support the attachment and growth of both primary cells and cell lines, particularly when the surface charge is optimized. In addition to dextran-based microcarriers, microspheres that support cell attachment can be produced from PS, gelatin, and many of the synthetic and naturally-occurring polymers described in the preceding sections. The surface of the microcarrier can be modified chemically, or by immobilization of proteins, peptides, or carbohydrates.

Suspension culture techniques can also be used to permit cell interactions with complex three-dimensional polymer formulations. For example, cells seeding onto polymer fiber

meshes during suspension culture often results in more uniform cell distribution within the mesh than can be obtained by inoculation in static culture [81].

In cell encapsulation techniques, cells are suspended within thin-walled capsules or solid matrices of polymer. Alginate forms a gel with the addition of divalent cations under very gentle conditions and, therefore, has been frequently used for cell encapsulation. Certain synthetic polymers, such as polyphosphazenes, can also be used to encapsulate cells by cation-induced gelation. Low-melting point agarose has also been studied extensively for cell encapsulation. Methods for the microencapsulation of cells within hydrophilic or hydrophobic polyacrylates by interfacial precipitation have been described [82], although the thickness of the capsule can limit the permeation of compounds, including oxygen, through the semi-permeable membrane shell. Interfacial polymerization can be used to produce conformal membranes on cells or cell clusters [83], thereby providing immunoprotection while reducing diffusional distances.

Hollow fibers are frequently used for macroencapulsation; cells and cell aggregates are suspended within thin fibers composed of a porous, semi-permeable polymer. Chromaffin cells suspended within hollow fibers formed from copolymer of vinyl chloride and acrylonitrile, which are commonly used as ultrafiltration membranes, have been studied as potential treatments for cancer patients with pain [84], Alzheimer's disease [85], and retinitis pigmentosa [86]. Other polymer materials — such as chitosan, alginate, and agar — have been added to the interior of the hollow fibers to provide an internal matrix that enhances cell function or growth.

Polymer nanoparticles are rapidly becoming important materials for drug delivery and tissue engineering, but little is known about how particles with diameters in the range of 50 to 200 nm interact with cells. When PLGA nanoparticles (~100 nm diameter) were suspended over monolayers of airway, intestinal, or renal tubular epithelial cells, particles were internalized as soon as 30 min after first exposure, becoming widely distributed through the intracellular space over a 24 hour period [87], but the extent of nanoparticle internalization varied among the epithelial cell types. PS nanoparticle interactions with model cell membranes depend on particle size, shape, and surface chemistry, as well as membrane composition, which may be a factor in the variable interactions of particles with different cells [88]. Although multiple mechanisms of nanoparticle entry into cells have been suggested, endocytosis is important in most cells, although it is not the only mechanism. For example, PS particles (20 nm diameter) translocated — in and apical to the basolateral direction — through monolayers of cultured alveolar cells, with particles carrying a positive surface charge moving more effectively than particles with a negative surface charge, apparently by transcellular, non-endocytic mechanisms [89].

Proteins absorb readily to the surface of polymer nanoparticles, as they do to all polymer surfaces. The presence of surface-bound proteins influences the interaction of nanoparticles with cells [90]. Particle shape may also have an influence on polymer particle interactions with cells [91], although there are few studies that have examined the effect of shape, particularly for particles with diameters less than 1 μm. As in other systems, tethering of ligands to the polymer surface can influence nanoparticle interactions with cells, including enhancing association and internalization [92].

CELL INTERACTIONS WITH THREE-DIMENSIONAL POLYMER SCAFFOLDS AND GELS

Cells within tissues encounter a complex chemical and physical environment that is quite different from commonly used cell culture conditions. Three-dimensional cell culture methods are frequently used to simulate the chemical and physical environment of tissues. Often, tissue-derived cells cultured in ECM gels will reform multicellular structures that are reminiscent of tissue architecture.

Gels of agarose have also been used for three-dimensional cell culture. Chondrocytes dedifferentiate when cultured as monolayers, but re-express a differentiated phenotype when cultured in agarose gels [93]. When fetal striatal cells are suspended in three-dimensional gels of hydroxylated agarose, ~50% of the cells extended neurites in gels containing between 0.5 and 1.25% agarose, but no cells extended neurites at concentrations above 1.5%. This inhibition of neurite outgrowth correlates with an average pore radius of greater than 150 nm [94]. Neurites produced by PC12 cells within agarose gels, even under optimal conditions, are much shorter and fewer in number than neurites produced in gels composed of ECM molecules [95].

Macroporous hydrogels can also be produced from pHEMA-based materials, using either freeze-thaw or porosigen techniques. These materials, when seeded with chondrocytes, may be useful for cartilage replacement [96]. Similar structures may be produced from PVA by freeze-thaw crosslinking. Recently, a PEG-based macroporous gel was used as a scaffold for endothelial cells to form microvessel networks *in vivo* [97]. Although cells adhere poorly to pHEMA, PVA, and PEG materials, adhesion proteins or charged polymers can be added during the formation to encourage cell attachment and growth. Alternatively, water-soluble, non-adhesive polymers containing adhesive peptides, such as RGDS, can be photopolymerized to form a gel matrix around cells (see [98], for example).

Fiber meshes and foams of PLGA, PLA, and PGA have been used to create three-dimensional environments for cell proliferation and function, and to provide structural scaffolds for tissue regeneration. When cultured on three-dimensional PGA fiber meshes, chondrocytes proliferate, produce both glycosaminoglycans and collagen, and form structures that are histologically similar to cartilage [99]. The internal structure of the material, as well as the physical dimensions of the polymer fiber mesh, influence cell growth rate, with slower growth being observed in thicker meshes. Changing the fluid mechanical forces on the cells during the tissue formation also appears to influence the development of tissue structure.

In addition to fiber meshes, porosity can be introduced into polymer films by phase separation, freeze drying, salt leaching and a variety of other methods (reviewed in reference [100]). It is now possible to make porous, degradable scaffolds with controlled pore architectures and oriented pores [101,102]. Fabrication methods that provide control over the structure at different length scales may be useful in the production of three-dimensional tissue-like structures.

Most methods for producing fiber meshes are limited to producing fibers ~10 microns in diameter, which is much larger than the diameter of natural fibers that occur in the extracellular matrix, and also larger than many of the features that are known to be important in orienting or guiding cell activity. Electrospinning techniques can be used to make small diameter fibers and non-woven meshes of a variety of materials including poly(caprolactone), PLA, collagen, and elastin mimetic polymers.

CELL INTERACTIONS UNIQUE TO THE *IN VIVO* SETTING

While cell interactions with polymers *in vitro* can be described by examination of cell behaviors — such as adhesion, migration, or gene expression — or the coordinated behavior of cell groups — such as aggregation, cell interactions with polymers *in vivo* can lead to other responses, involving cells that are recruited to the implantation site and remodeling of the tissue space surrounding, or even within, the polymeric material. Inflammation, the FBR, and angiogenesis are three examples of these more global responses to an implanted material.

There is much still to learn in this area, but it is clear that both the implant material and the physiology of the implant site are important variables. A study describing a relatively simple experiment, in which ePTFE implants were placed in adipose tissue, in subcutaneous tissue, or epicardially illustrates the variability of these responses [103]. Moreover, studies

in MCP1-null mice have shown that the extent of the FBR was dependant on the implantation site [104,105]. This short section introduces these physiological responses to implanted materials.

Inflammation

The implantation of polymers through surgical incisions means that an initial component of the FBR involves a wound healing-like response and it is reasonable to assume that the early inflammatory response is mediated, at least in part, by wound-derived factors. Analysis of inflammatory cells has been pursued in several implantation models and was shown to involve predominantly neutrophils (early) and monocyte/macrophages (late). Subsequent to their recruitment, these cells are believed to utilize adhesion receptors to interact with adsorbed proteins. Studies in mice that lack specific integrins or fibrinogen have provided supporting evidence for this hypothesis [106,107]. Specifically, short-term (18 hr) IP implantation of polyethylene terephthalate (PET) disks in mice that lack fibrinogen indicated normal recruitment but reduced adhesion of macrophages and neutrophils to the polymer. In the same study, an analysis of the response in mice that lack plasminogen, indicated no changes in cell adhesion to the polymer, despite a reduction in the recruitment of both cell types in the peritoneal cavity. Thus, in addition to fibrinogen for adhesion, inflammatory cells can utilize plasminogen for migration/recruitment. Moreover, studies in mice lacking plasma fibronectin displayed altered foreign body response [108]. More recently, it was shown that polymer-macrophage interactions can lead to activation of the inflammasome leading to the secretion of the pro-inflammatory cytokine IL-1β [27]. In the same study, it was shown that mice that lack components of the inflammasome mount reduced FBR suggesting that initial interactions between cells and polymers are critical determinants of the FBR. Surprisingly, the chemokine CCL2 (also known as monocyte chemoattractant protein or MCP-1), was shown not to be important in monocyte/macrophage recruitment in long-term implants in the subcutis [104]. However, CCL2 was shown to be important for macrophage fusion leading to FBGC formation. Subsequently, it was shown that CCL2 was critical for the recruitment of inflammatory cells in the FBR when implants were placed in the peritoneal cavity of mice [105]. Consistent with the notion that macrophage responses are critical in the FBR, IP implants in CCL2-null mice displayed minimal encapsulation.

FBGC can cause damage to polymer surfaces through their degradative and phagocytic activities, and thus pose a significant obstacle to the successful application of polymer-based biomaterials and devices. *In vivo* studies have identified a critical role for interleukin (IL)-4 in the formation of FBGC [18], but the regulation of macrophage fusion is not fully understood [109]. On the other hand, several studies have focused on the role of polymer surface chemistry on macrophage function and FBGC formation. For example, analysis of macrophage adhesion, apoptosis, and fusion on hydrophobic (PET and BDEDTC-coated), hydrophilic (PAAm), anionic (PAANa), and cationic (DMAPAAmMel) surfaces implanted in the rat cage-implant model revealed that PAAm and PAANa induced more apoptosis and reduced adhesion and fusion [110].

Fibrosis and angiogenesis

Unlike wound healing, the resolution of the polymer-associated inflammatory response is characterized by the excessive deposition of a highly organized collagenous matrix and a striking paucity of blood vessels [111,112]. The collagenous capsule can vary in thickness but usually exceeds 100 μm, presumably to limit diffusion of small molecules to and from the polymer. The dense and organized nature of the collagen fibers in the capsule could play a role in limiting blood vessel formation. Implantation studies in mice that lack the angiogenesis inhibitor TSP2 indicated that an increase in vascular density in capsules surrounding PDMS disks was associated with significant loosening of the collagenous matrix [113]. However, a direct link between the arrangement of collagen fibers in the capsule and blood vessel

formation has not been established. Interestingly, the modification of the PDMS surface from a hydrophobic to a hydrophilic state altered its cell adhesive properties *in vitro* but did not cause a change in the FBR *in vivo* [113]. Such observations underscore the significance of *in vivo* evaluation of cell- and tissue-polymer interactions. Reduced encapsulation of polydimethylsiloxane (silicone rubber) disks and cellulose Millipore filters implanted SC has been reported in mice that lack SPARC (secreted protein, acidic and rich in cysteine), a matricellular glycoprotein that modulates the interactions of cells with the extracellular matrix. Interestingly, mice that lack SPARC and its close homolog, hevin, display diminished vascular density in encapsulated Millipore filters (type HA, mixed cellulose ester) [114]. Taken together, implantation studies in genetically-modified mice suggest that members of the matricellular protein group play critical roles in the FBR [112]. The process however, can also be influenced by parameters such as polymer special geometry and porosity. Comparison of the FBR elicited by expanded and condensed PTFE showed similar encapsulation but more mature fibrous capsule formation in the latter [115]. In addition, the effect of polymer porosity in the FBR was examined in SC-implanted PTFE membranes in rats where it was shown that the vascular density could be increased in capsules surrounding polymers with a pore size in the range of 5 μm [116]. However, it is unclear whether the same porosity would enhance the vascular density of capsules surrounding other polymers. Finally, an additional concern with polymer encapsulation is the presence of contractile cells, myofibroblasts, which can cause contraction of the capsule and misshape or damage polymer implants. For example, silicone-based breast implants have been shown to be susceptible to this phenomenon [117].

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CHAPTER 21

Matrix Effects

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INTRODUCTION

The extracellular matrix serves at least three functions in its role controlling cell behavior: it provides adhesion signals, it provides growth factor binding sites, and it provides degradation sites to give way to the enzymatic activity of cells as they migrate. An understanding of these interactions is important in tissue engineering, where one may desire to mimic the biological recognition molecules that control the relationships between cells and their natural biomaterial interface, namely the extracellular matrix. The components of the extracellular matrix are on one level immobilized, but not necessarily irreversibly. Cell-derived enzymes, such as tissue transglutaminase and lysyl oxidase, serve to chemically cross-link certain components of the extracellular matrix, such as fibronectin chains to other fibronectin chains and to fibrillar collagen chains. Other components are more transiently immobilized, such as growth factors that are sequestered within the extracellular matrix proteoglycan and protein network. This network can be partially degraded, and the growth factors themselves can be proteolytically cleaved, to mobilize the growth factors under cellular control. Not all of the signals of the extracellular matrix are biochemical in nature. A biomechanical interplay between cells and their extracellular matrix also plays an important role in the functional regulation in many tissues, particular in load-bearing tissues. This chapter will consider only the biochemical aspects of biological recognition; the reader is referred elsewhere for treatments of the role of the extracellular matrix as a biomechanical regulator of cell behavior [1–5].

EXTRACELLULAR MATRIX PROTEINS AND THEIR RECEPTORS

Interactions between cells and the extracellular matrix are mediated by cell-surface glycoprotein and proteoglycan receptors interacting with proteins bound within the extracellular matrix. This section will begin with an introduction of the glycoprotein receptors on cell surfaces involved in cell adhesion. The topic will then turn to the proteins in the extracellular matrix to which those receptors bind, with a discussion of the active domains of those proteins that bind to the cell-surface receptors. Finally, the roles of cell-surface associated enzymes in the processing and remodeling of the extracellular matrix will be addressed.

There are four major classes of glycoprotein adhesion receptors that are present on cell surfaces, three of which are involved primarily in cell-cell adhesion and one of which is involved in both cell adhesion to other cells and cell adhesion to the extracellular matrix. The first three are introduced briefly, and the fourth more extensively.

The cadherins are a family of cell-surface receptors that participate in homophilic binding (i.e., the binding of a cadherin on one cell with an identical cadherin on another cell) [6–10]. These molecules allow a cell of one type (e.g., endothelial cells) to recognize other cells of same type

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and are important in the early stages of organogenesis. These interactions depend upon the presence of extracellular Ca^{2+} and may be dissociated by calcium ion chelation. Since all cadherins are present on cell surfaces, cadherins are not involved directly in cell interactions with the extracellular matrix. They may be involved indirectly, in that they may organize cell-cell contacts in concert with another receptor system that is involved in regulation of cell-extracellular matrix binding.

A second class of receptors is the selectin family [11–14]. These membrane-bound proteins are involved in heterophilic binding between cells, such as blood cells and endothelial cells, in a manner that depends, as the cadherins, on extracellular Ca^{2+}. These proteins contain lectin-like features and recognize branched oligosaccharide structures in their ligands, namely the sialyl Lewis X and the sialyl Lewis A structures. As with the cadherins, these receptor-ligand interactions are important primarily in cell-cell interactions, and they are particularly important in the context of inflammation.

A third class of receptors represents members of the immunoglobulin superfamily, cell adhesion molecule proteins that are denoted as Ig-CAMs or simply CAMs [15]. These proteins bind their protein ligands in a manner that is independent of extracellular Ca^{2+} and they participate in both homophilic and heterophilic interactions. As for the cadherins and the selectins, they bind to other cell-surface proteins and are thus primarily involved in cell-cell interactions. One class of ligand for these receptors includes selected members of the integrin class of adhesion receptors, discussed below.

The fourth class of adhesion receptors is the integrin family [16–22]. While the other three classes of receptors described briefly above are involved primarily in cell-cell recognition, the integrins are involved in both cell-cell and cell-extracellular matrix binding. The integrins are dimeric proteins, consisting of an α and a β subunit assembled non-covalently into an active dimer. There are many known α and β subunits, with at least 18 such α subunits and eight such β subunits that are capable of assembly into at least 24 αβ combinations. Some of the αβ combinations present in the β1, β2, β3 and β4 subclasses are shown in Table 21.1; these

TABLE 21.1 Selected members of the integrin receptor class and their ligands [20]

Integrin heterodimer	Ligands
α1β1	Collagen, laminin
α2β1	Collagen, laminin
α3β1	Collagen, fibronectin, laminin, thrombospondin-1
α4β1	Fibronectin, osteopontin, vascular cell adhesion molecule-1
α5β1	RGD, fibronectin, L1
α6β1	Laminin
α7β1	Laminin
α8β1	RGD, fibronectin, tenascin
α9β1	Collagen, laminin, osteopontin, tenascin, vascular cell adhesion molecule-1
α10β1	Collagen
α11β1	Collagen
αvβ1	RGD, collagen, fibrinogen, fibronectin, vitronectin, von Willebrand factor
αXβ2	Complement protein C3bi, fibrinogen
αMβ2	Complement protein C3bi, fibrinogen, intercellular adhesion molecule-1, vascular cell adhesion molecule-1
αLβ2	Intercellular adhesion molecule-1 - intercellular adhesion molecule-5
αDβ2	Intercellular adhesion molecule-3, vascular cell adhesion molecule-1
αvβ3	RGD, bone sialoprotein, fibrinogen, fibronectin, thrombospondin, vitronectin, von Willebrand factor
αIIbβ3	Fibrinogen, fibronectin, thrombospondin, vitronectin, von Willebrand factor
α6β4	Laminin, hemidesmosomes

are the most commonly expressed integrins and are thus arguably the most generally important. The β2 integrins are involved primarily in cell-cell recognition; for example, the integrin αLβ2 binds to ICAM-1 and ICAM-2, both members of the immunoglobulin superfamily subclass of cell adhesion molecules described in the preceding paragraph. By contrast, the β1, β3 and β4 integrins are primarily involved in cell-extracellular matrix interactions.

The β1 and β3 integrins bind to numerous proteins present in the extracellular matrix, as illustrated in Table 21.1. These proteins include collagen, fibronectin, vitronectin, von Willebrand factor and laminin.

Collagen is the primary structural protein of the tissues; the reader is referred elsewhere for a focused review on this extensive topic [23,24]. Many forms of collagen exist, several of which are multimeric and fibrillar. To these collagens many other adhesion proteins bind, thus putting collagen in the role of organizing many other proteins that interact with and organize cells. Collagen also interacts directly with integrins, primarily α1β1 and α2β1.

Fibronectin is a globular protein that is present in nearly all tissues; fibronectin has been extensively reviewed elsewhere [25,26]. Fibronectin also exists in many forms, depending upon the site in the tissues and the regulatory state of the cell that synthesized the fibronectin. Almost all cells interact with fibronectin, primarily through the so-called fibronectin receptor α5β1, and to a lesser extent through the β3 integrin αvβ3 as well as other integrins as will be described farther below.

Vitronectin is a multifunctional adhesion protein found in the circulation and in many tissues [27–29]. The protein is active in promoting the adhesion of numerous cell types and binds primarily to the so-called vitronectin receptor, αvβ3, as well as αvβ1 and to the platelet receptor αIIbβ3.

Von Willebrand factor is an adhesion protein that is primarily involved in the adhesion of vascular cells; the reader is referred elsewhere for a detailed review [30–32]. It is synthesized by the megakaryocyte, the platelet-generating cells of the bone marrow, and is stored in the α-granules of circulating platelets. Activation of platelets leads to the release of the granule contents, including von Willebrand factor. Von Willebrand factor is also synthesized by and stored within the endothelial cell. A multimeric form of the protein, where tens of copies of the protein may be linked together into insoluble form, is found in the subendothelium and is involved in blood platelet adhesion to the subendothelial tissues upon vascular injury.

Laminin is a very complex adhesion protein that is generally present in the basement membrane, the proteins immediately beneath the epithelia and endothelia, as well as in many other tissues, as reviewed in detail elsewhere [33–35]. It is present as a family of forms [36]. The classic form was purified from the extracellular matrix of Engelbreth-Holm-Swarm tumor cells and consists of a disulfide crosslinked trimer of one α1 (400,000 Da), one β1 (210,000 Da) and one γ1 (200,000 Da) polypeptide chains. This form binds to the β1 integrins α1β1, α2β1, α3β1, α6β1 and α7β1, and to the β3 integrins αvβ3 and αIIbβ3, as well as to other integrins. A number of other laminin forms exist, composed of αβγ combinations of α1, α2, α3, α4 or α5, β1, β2 or β3, and γ1 or γ2 chains. The details of the differences in function of all of the various laminin forms remain only partially elucidated, but it is clear that several of them do stimulate very different behaviors in a variety of cell types. Laminin is a particularly important component of the basal lamina, i.e., the extracellular matrix beneath monolayer structures such as epithelia, mesothelia and endothelia [37]. For example, laminin contains numerous domains that bind to endothelial cells [38], and these are undoubtedly important in regulating a variety of cell-type specific functions, as discussed below.

There are several cell morphological hallmarks of integrin binding to adhesion proteins in the extracellular matrix. These include spreading of the cells, extension of cellular membrane processes called focal contacts to within approximately 20 nm of the extracellular matrix

surface [39], clustering of integrin receptors at the sites of focal contacts and assembly of intracellular accessory proteins at the site of clustered integrins to assist in the attachment of the integrin complex to the f-actin cytoskeleton [40]. These sites of clustered receptors and interaction of the transmembrane receptors with the intracellular cytoskeleton carry most of the stress between the cells and the extracellular matrix or artificial surfaces; indeed, both theoretical analysis and experimental results demonstrate that without the formation of focal contacts and without the connection of numerous transmembrane integrin αβ heterodimer complexes into much larger multi-integrin complexes by intracellular proteins such as talin, vinculin and α-actinin, cell adhesion would be very much weaker than it is in reality [40]. The focal contact serves as an important center for regulation of cell signaling, both mechanically and chemically, as discussed below.

The extracellular matrix proteins described above are very complex. They contain sites responsible for binding to collagen, for binding to glycosaminoglycans (as described below), for crosslinking to other extracellular matrix proteins via transglutaminase activity, for degradation by proteases (as described briefly below) and for binding to integrin and other adhesion receptors (as described in detail immediately below). Since the proteins must be so multifunctional, the sites that serve the singular function of binding to integrins comprise a small fraction of the protein mass. In most cases, the receptor-binding domain can be localized to an oligopeptide sequence less than ten amino acid residues in length, and this site can be mimicked by linear or cyclic oligopeptides of identical or similar sequence as that found in the protein [41]. The first such minimal sequence to be identified was the tripeptide RGD [42] (using the single letter amino acid code, shown in a footnote to Table 21.2). Synthetic RGD-containing peptide, when appropriately coupled to a surface or a carrier molecule (see below), is capable of recapitulating much of the adhesive interactions of the RGD site in the protein fibronectin, including integrin binding. At least for the case of integrin binding via αvβ3, the RGD ligand alone is capable of also inducing integrin clustering and, when the signal is presented at sufficient surface concentration, focal contact formation and cytoskeletal organization [43]. Many receptor-binding sequences other than the RGD tripeptide have been identified by a variety of methods, and a few of these sequences are shown in Table 21.2. In these receptor-binding sequences, the affinity is highly specific to the particular ordering of the amino acids in the peptide; for example, the peptide RDG, containing the same amino acids but in a different sequence, is completely inactive in binding to integrins.

TABLE 21.2 Selected cell binding domain sequences of extracellular matrix proteins (from [117], after [118])

Protein	Sequence[a]	Role
Fibronectin	RGDS	Adhesion of most cells, via α5β1
	LDV	Adhesion
	REDV	Adhesion
Vitronectin	RGDV	Adhesion of most cells, via αvβ3
Laminin A	LRGDN	Adhesion
	SIKVAV	Neurite extension
Laminin B1	YIGSR	Adhesion of many cells, via 67 kD laminin receptor
	PDSGR	Adhesion
Laminin B2	RNIAEIIKDI	Neurite extension
Collagen I	RGDT	Adhesion of most cells
	DGEA	Adhesion of platelets, other cells
Thrombospondin	RGD	Adhesion of most cells
	VTXG	Adhesion of platelets

[a]*Single letter amino acid code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.*

One class of adhesion peptides contains the central RGD sequence. These are modified by their flanking residues, which modify the receptor specificity of the receptor-binding sequence. For example, the sequence found in fibronectin is RGDS, in vitronectin it is RGDV, in laminin it is RGDN and in collagen it is RGDT. Other adhesion peptides maintain the central D residue, such as the REDV and LDV sequences of fibronectin. The REDV and LDV sequences are relatively specific in their binding and interact with the integrin $\alpha 4\beta 1$; RGD peptides also bind to $\alpha 4\beta 1$, but the REDV and LDV sequences bind essentially only to $\alpha 4\beta 1$.

In addition to peptides that bind to the integrin adhesion receptors, there are other peptide sequences that bind to other non-integrin receptors. As an example, laminin bears several such sequences, such as the YIGSR, SIKVAV, and RNIAEIIKDI peptides. The YIGSR sequence binds to a 67 kD monomeric non-integrin laminin receptor [44]. As do the integrin receptors, this laminin receptor also interacts via its cytoplasmic domain with intracellular proteins involved with linkage to the f-actin cytoskeleton. The YIGSR sequence is involved in the adhesion and spreading of numerous cell types (see below). The SIKVAV sequence in laminin binds to neuronal cell-surface receptor and stimulates the extension of neurites [45].

In addition to the highly sequence specific binding of adhesion peptides to cell-surface receptors, most of the adhesion proteins also bind to cell-surface components by less specific mechanisms. These proteins contain a heparin-binding domain (so called because of the use of heparin affinity chromatography in purification of the protein) that binds to cell-surface proteoglycans that contain heparan sulfate or chondroitin sulfate glycosaminoglycans [46]. The peptide sequences that bind to cell-surface proteoglycans are rich in cationic residues, such as arginine (R) and lysine (K) relative to their content in the anionic residues aspartic acid (D) and glutamic acid (E), and they also contain hydrophobic amino acids, such as alanine (A), isoleucine (I), leucine (L), proline (P) and valine (V). Several of these sequences are shown in Table 21.3. For example, the heparin-binding sequence in fibronectin bears a sequence of PRRARV, having a motif of XBBXBX, which is observed in several cell adhesion proteins, X being a hydrophobic residue and B being a basic residue, either K or R. These sites within adhesion proteins such as fibronectin and laminin bind to cell-surface proteoglycan in parallel with interaction by the integrin binding sites to stabilize the adhesion complex [47]. Cell-cell adhesion molecules also employ cell-surface proteoglycan binding affinity, e.g., N-CAM bears the domain KHKGRDVILKKDVR, which binds to heparan sulfate and chondroitin sulfate proteoglycans [48]. The interactions with cell-surface proteoglycans are much less specific than those with integrins; the binding is not as sensitive to the order of the oligopeptide sequence and moreover the effect can be mimicked simply by R or K residues immobilized upon a surface, albeit certainly with a great loss in specificity [49].

The extracellular matrix is subject to dynamic remodeling under the influence of cells in contact with it. Cells seeded *in vitro* upon an extracellular matrix of one composition may adhere, spread, form focal contacts, remove the initial protein, secrete a new extracellular matrix of different protein composition and form new focal contacts. Cell-surface bound and cell-derived free enzymes play an important role in this remodeling of the extracellular matrix [50]. For example, cell-released protein disulfide isomerases are released from cells to

TABLE 21.3 Proteoglycan binding domain sequences of extracellular matrix proteins (after [117], references contained in [49])

Sequence[a]	Protein
XBBXBX	Consensus sequence
PRRARV	Fibronectin
YEKPGSPPREVVPRPRPGV	Fibronectin
RPSLAKKQRFRHRNRKGYRSQRGHSRGR	Vitronectin
RIQNLLKITNLRIKFVK	Laminin

[a]*X indicates a hydrophobic amino acid. Basic amino acids are shown underlined.*

covalently crosslink protein in the extracellular matrix by disulfide bridging. Cell-derived transglutaminases also form an amide linkage between the ε-amino group of lysine and the side group amide of glutamine to chemically cross-link proteins in the extracellular matrix. These processes are responsible, for example, for the assembly of the globular adhesion protein fibronectin into fibrils within the extracellular matrix beneath cells.

Membrane-bound and cell-released enzymes are also involved in degradation of the extracellular matrix to permit matrix remodeling and cell migration [51]. Cell-released matrix metalloproteinases such as collagenase and gelatinase, serine proteases such as urokinase plasminogen activator and plasmin, and cathepsins are each involved in both remodeling and degradation during cell migration. Accordingly, the matrix-cell interaction should be understood to be bi-directional: the cell accepting information from the matrix, and the matrix being tailored by the cell. This phenomenon has been referred to as dynamic reciprocity and has been elucidated elsewhere [52].

One of the important roles of cell-associated enzymatic degradation of the extracellular matrix is in mobilization of growth factor activity. Because growth factors are such powerful regulators of biological function, their activity must be highly spatially regulated. One means by which this occurs in nature is by high-affinity binding interactions between growth factors and the three-dimensional extracellular matrix in which they exist. Many such growth factors bind heparin, meaning that they, like adhesion proteins, bear domains that bind extracellular matrix heparan sulfate and chondroitin sulfate proteoglycans. For example, basic fibroblast growth factor binds heparin with high affinity [53]. Vascular endothelial growth factor is another example of a heparin-binding growth factor [54]. These growth factors are strongly immobilized by binding to extracellular matrix proteoglycans, and they can be mobilized under local cellular activity, e.g., by degradation of these proteoglycans, or, in the case of vascular endothelial cell growth factor, by cleavage of the main chain of the growth factor away from the heparin-binding domain by plasmin activated at the surface of a nearby cell.

Extracellular matrix proteins also play important roles in matrix interactions with growth factors. This has been best studied in the context of fibronectin [55], although other matrix molecules have also been shown to bind growth factors. In the case of fibronectin, biophysical interactions with a domain consisting of the twelfth to fouteenth type III repeats was shown to lead to binding to a host of growth factors with a dissociation constant of order 1–10 nM [55]. In this manner, just as matrix proteoglycans, matrix proteins can sequester growth factors. Matrix proteins can play a more important role though: in some matrix proteins, such as fibronectin, the integrin-binding domain is close to the growth factor-binding domain, so allowing synergistic signaling between the bound integrin and the growth factor receptor that ligates the bound growth factor [56]. In this manner, one observes that adhesion and growth factor presentation of the matrix are highly interrelated.

Fundamental studies have demonstrated that the interaction between growth factors and the extracellular matrix can dramatically alter their local behavior, where the length scale of the local response is measured in single cell diameters. Specifically, very low interstitial flows can convect cell-derived proteases directionally downstream of a cell, and these proteases can liberate matrix-bound growth factors that were previously homogeneously distributed throughout the matrix. Since the protease activity is preferentially downstream of the cell, growth factor liberation is also preferentially downstream. This can create gradients of growth factor, allowing the cell to sense the directionality of flow, even when the flows are extremely subtle [57,58].

MODEL SYSTEMS FOR STUDY OF MATRIX INTERACTIONS

Since the extracellular matrix adhesion proteins may be mimicked, at least to some degree, by small synthetic peptides, it is possible to investigate cell-substrate interactions with

well-defined systems. Foundational to them all are the interactions of cells with the surface, other than with adhesion peptides intentionally endowed upon the surface, which would produce cell adhesion. These so-called non-specific interactions are between cell-surface receptors and proteins that have adsorbed to the surface. Due to this role played by adsorbing proteins, some introduction to the protein and surface interactions leading to adsorption is warranted. The thermodynamic and kinetic aspects of protein adsorption have been reviewed and the reader is referred elsewhere for a more detailed treatment [59]. The primary driving force for protein adsorption is the hydrophobic effect: water near a hydrophobic material surface fails to hydrogen bond with that surface, and thus assumes a more highly ordered structure in which the water is more thoroughly hydrogen bonded to itself than is the case in water far away from the surface. A protein can adsorb to this surface, acting like a surfactant, and thus replace the hydrophobic material surface with a polar surface capable of hydrogen bonding with water. This releases the order in the water, with a net result of a large entropic gain. Electrostatic interactions, e.g., between charges on D, E, K or R residues on the protein with cationic or anionic functions on the material surface play a lesser but important role as well; since proteins generally bear a net negative charge, anionic surfaces typically adsorb less protein than do cationic surfaces. These observations, overly generalized in the above sentences, guide one to examine model surfaces that are hydrophilic and nonionic, as well as being derivatizable to permit coupling of the adhesion peptide under study.

The tendency for proteins to adsorb to material surfaces has been exploited as a method by which to immobilize peptides onto substrates for study. Pierschbacher et al. have described the peptide Ac-GRGDSPASSKGGGGSRLLLLLLR-NH2 (where the Ac indicates that the N-terminus is acetylated and the -NH2 indicates that the C terminus is amidated to block the terminal charges) for this purpose [60]. The LLLLLL stretch is hydrophobic and so adsorbs avidly to hydrophobic polymer surfaces, effectively immobilizing the cell-binding RGDS sequence from fibronectin. Non-adhesive proteins such as albumin have also been grafted with RGD peptide, e.g., by binding to amine groups on lysine residues on the albumin; adsorption of the albumin conjugate thus immobilizes the attached RGD peptide [61].

Surfaces coated with hydrophilic polymers have also been employed to graft adhesion peptides. One simple system that has been useful is glass modified with a silane, 3-glycidoxypropyl triethoxysilane; once the silane is grafted to the surface, the epoxide group is hydrolyzed to produce -CH2CH(OH)CH2OH groups pendant from the surface (glycophase glass). The hydroxyl groups serve as sites for covalent immobilization of adhesion peptide, e.g., via the N-terminal primary amine [62]. Titration of the surface density of grafted RGD peptides versus cell response using this system revealed quantitative information on the number density of interactions required to establish morphologically complete cell spreading [43]. A surface density of approximately 10 fmol/cm^2 of RGD was required to induce spreading, focal contact formation, integrin $\alpha_v\beta3$ clustering, α-actinin and vinculin co-localization with $\alpha_v\beta3$, and f-actin cytoskeletal assembly in human fibroblasts cultured upon this synthetic extracellular matrix. This surface density corresponds to a spacing of roughly 140 nm between immobilized RGD sites, demonstrating that far less than monolayer coverage is sufficient to promote cell responses. Silane-modified quartz has been employed as a surface to study the role of RGD sites and heparin-binding domains of adhesion proteins in osteoblast adhesion and mineralization, demonstrating a strong benefit for the involvement of both modes of adhesion [63]. The base material, glycophase glass, is only modestly resistant to protein adsorption and thus non-specific cell adhesion; accordingly, the investigation of long-term interactions, during which the adherent cells may be synthesizing and secreting their own extracellular matrix to adsorb to the synthetic one experimentally provided, are difficult to investigate. This has motivated exploration with substrates that are more resistant to protein adsorption.

An enormous amount of research has been expended into grafting material surfaces with water soluble, non-ionic polymers such as polyethylene glycol, HO(CH2CH2O)$_n$H, abbreviated

PEG. This vast body of research has been extensively reviewed elsewhere [64]. Polyethylene glycol has been immobilized upon surfaces by numerous means; three particularly effective means will be addressed in the following paragraphs.

Thiol compounds bind by chemisorption avidly to gold surfaces [65,66]. When those thiols are terminal to an alkane group, R-$(CH2)_n$-SH, the thiol adsorbs in perfect self-assembling monolayers; the thiol-gold interaction contributes about half of the energy of interaction, and the alkane-alkane van der Waals interaction contribute the other half. Accordingly, it is easy to employ alkanethiols to display, in very regular fashion, some functionality R upon a gold-coated substrate (so long as the R group is not large enough to sterically inhibit monolayer packing, in which case it can be diluted with a non-functional alkanethiol. Using this approach, Prime and Whitesides immobilized oligoethylene glycol-containing alkanethiol, HS-(CH2)11(OCH2CH2)nOH, on gold surfaces [67]. Protein adsorption was investigated upon surfaces formed with this alkanethiol and a hydrophobic co-reactant, HS-(CH2)10CH3. Degrees of polymerization (n) as low as 4 were observed to dramatically limit the adsorption of even very large proteins, such as fibronectin. When the oligoethylene glycol monolayer was incomplete, i.e., when the monolayer was mixed with the hydrophobic alkanethiol, longer oligoethylene glycol functions were able to preserve the protein repulsiveness of the surface. Since the background amount of protein adsorption on these materials is so low, one would expect them to be very useful as substrates for peptide attachment for studies with model synthetic extracellular matrices, e.g., with HS-(CH2)11(OCH2CH2)n-NH-RGDS.

Drumheller and Hubbell have developed a polymeric material that was highly resistant to cell adhesion for use in peptide grafting [68,69]. Materials that contain large amounts of polyethylene glycol generally swell extensively, rendering material properties sometimes unsuitable for cell culture or medical devices. To circumvent this, the polyethylene glycol swelling was constrained by distributing it as a network throughout a densely crosslinked network of a hydrophobic monomer, trimethylolpropane triacrylate. This yielded a material with the surface hydrophilicity of a hydrogel but with mechanical and optical properties of a glass. These materials were highly resistant to protein adsorption, even after an adsorptive challenge to the material with a very large adhesion protein, laminin, and even over multiweek durations. When the polymer network was formed with small amounts of acrylic acid as a comonomer, the polymer still remained cell non-adhesive. The carboxyl groups near the polymer surface were useful, however, as sites for derivatization with adhesion peptides such as the RGD and YIGSR sequences. Since the adsorption of proteins to those surfaces was so low, materials endowed with inactive peptides such as the RDG supported no cell adhesion.

Numerous other approaches are possible. One of particular interest, because of its ease of use, is physisorption. Block copolymers, consisting of adsorbing domains and non-adsorbing domains, can be adsorbed to material surfaces and can be used to regulate biological interactions. As an example, when the non-adsorbing domains are polyethylene glycol, surfaces can be generated which display very low levels of non-specific adhesion [70]. One convenient class of polymers are ABA block copolymers of polyethylene glycol (the A blocks) and polypropylene glycol (B), in which the hydrophilic and cell repelling polyethylene glycol domains flank the central hydrophobic and adsorbing polypropylene glycol block. The central hydrophobic block adsorbs well to hydrophobic surfaces, thus immobilizing the hydrophilic polymer and thereby resisting cell adhesion [70]. Cell adhesion peptides can be displayed at the tips of these hydrophilic chain termini, and a very effective and simple model surface can be obtained [71,72]. Similar constructions can be designed for anionic surfaces, e.g., by using a polycationic block as a binding domain, with polyethylene glycol chains attached thereto [73–75]. Adhesion-promoting peptides may be grafted to the termini of the dangling polyethylene glycol chains, to permit cell attraction to these ligands on an otherwise remarkably non-adhesive background [76].

Model systems have also been employed for ligand discovery, i.e., to determine which parts of an adhesion protein are responsible for binding to an adhesion receptor. This has been most

convincingly implemented using peptide arrays, i.e., surfaces in which peptides have been immobilized or even more powerfully synthesized in small domains upon an otherwise passive substrate [77,78]. Arrays of peptides that constitute overlapping sequences order 10 amino acids long spanning the entire length of a candidate adhesion protein can be constructed. If a receptor for the putative binding site is already known, it can be labeled, e.g., with a fluor, and the identity of peptides that bind can be determined by the location of the spots that bind the fluorescent protein. If a candidate receptor is not known, then larger spots can be synthesized (which somewhat limits the size of the peptide library that is formed), and the identity of adhesion-promoting peptides can be determined by the location of spots that promote cell adhesion and spreading, for example. Once the identity of a binding peptide is determined, the binding receptor can be determined by affinity chromatography of cell-derived proteins on columns containing bound peptide, with proteomic analysis of the protein that bind.

The above-mentioned model systems for study of cell-matrix interactions are all two-dimensional systems. Three-dimensional systems have also been developed, in an effort to mimic the spatial complexity of the natural extracellular matrix [3,79,80]. Some of these utilize natural proteins, such as cell-derived extracellular matrix, fibrin and collagen [58,81,82]. Some systems enable the identity and amounts of adhesion ligands, and potentially other ligands, to be precisely controlled. Two approaches are presented in the following paragraphs by way of example.

When fibrin forms spontaneously, non-fibrin proteins such as fibronectin, are grafted into the nascent fibrin network by the enzymatic activity of the coagulation transglutaminase factor XIIIa. This feature of coagulation has been exploited to engineer fibrin matrices, by coagulating fibrinogen in the presence of exogenous and even synthetic factor XIIIa substrates [83]. E.g., if an adhesion ligand is synthesized as a fusion with a factor XIIIa substrate peptide, the adhesion ligand will be immobilized within the fibrin network. This approach has been carried out with small synthetic adhesion peptides [84], with recombinant proteins that are fusions with a factor XIIIa substrate domain [85], and with peptides that bind glycosaminoglycans, which can in turn bind to growth factors [86]. One can incorporate other bioactive molecules such as growth factors directly within the fibrin matrices, also by expressing them as recombinant fusion proteins with factor XIIIa substrate domains [87]. Using these approaches, three-dimensional matrices for cell culture investigations of basic cellular processes can be constructed.

Synthetic three-dimensional matrices that allow precise control of cell adhesion ligand display have also been developed [79]. In one system, reactively-functionalized branched polyethylene glycol is crosslinked by a counter-reactive peptide, the peptide being designed with a sequence that is a substrate for proteases that cells use when they migrate, such as matrix metalloproteinases or plasmin [88]. To the ends of some of the polyethylene glycol arms are also grafted adhesion ligands, such as a reactive RGD peptide. Although the porosity of these hydrogels is very small compared to the length scale of the smallest of processes that cells extend when they migrate, local activation of proteases at the cell membrane surface enables the cells to proteolytically tunnel their way through the materials [89]. Another of other very clever chemical schemes have been developed for crosslinking biomimetic gels, including through chemical [90,91] and enzymatic [92,93] means. Like the fibrin materials described above, these materials are useful both as model systems for study of cell biology, as well as for therapeutic ends.

CELL PATTERN FORMATION BY SUBSTRATE PATTERNING

The ability to control material surface properties precisely enables the formation of designed architectures of multiple cells in culture and potentially *in vivo* as well. Large-scale architectures

have been formed by patterning adhesive surfaces. Four methods for patterning have been particularly powerful, photolithography, mechanical stamping, microfluidics and lift-off.

Photolithographic methods have been employed to impart patterns on cell adhesion surfaces. Alkoxysilanes have been chemisorbed to glass surfaces (using the same grafting chemistry as with the glycophase glass described above), and ultraviolet light was employed to selectively degrade the alkoxy group to yield patterns of surface hydroxyl groups [94]. These hydroxyl groups were used as sites for reaction with a second layer of an amine-containing alkoxysilane. These aminated regions supported cell adhesion and thus formed the cell-binding regions on the patterned substrate [95]. Patterned amines on polymer surfaces have also been employed to induce cell patterning via adhesive domains patterned upon a non-adhesive background [96]. These approaches have been combined with the bioactive peptide technology described above. For example, patterned amines have been used as grafting sites for the adhesive peptide YIGSR to pattern neurite extension upon material surfaces [97]. One of the goals of this work was to create neuronal networks as a simple system in which to study communication among networks of neurons. A powerful system for such work has been provided by using adhesive aminoalkylsilanes patterned upon a non-adhesive perfluoroalkylsilane background [98]. Polymers have been synthesized explicitly for the purpose of attaching adhesive peptide sequences such as RGD, and these will be very useful in future studies of cell-cell interactions in neuronal and other cell systems [99]. Such patterned surfaces have been formed to control cell shape and size, in order to gain deeper insight into the interplay between cell biomechanics and cell function [100]. It is particularly convenient in photopatterning studies to develop photochemistries on materials specifically for the purpose of immobilizing polymers and polymer-peptide adducts [101]; this has been addressed, e.g., with phenylazido-derivatized surfaces [102,103].

Alkanethiols upon gold have also been patterned using simple methods. Contact printing has been employed for this purpose [65,66]. Conventional photolithographic etching of silicon was employed to make a master printing stamp, a negative of which was then formed in silicone rubber. Structures as small as 200 nm were preserved in the silicone rubber stamp. The stamp was then wetted with a cell adhesion promoting alkanethiol HS-(CH2)15-CH3. Stamping a gold substrate resulted in creation of a pattern of the hydrophobic alkane group. The stamped gold substrate was then treated with the cell-resistant alkanethiol HS-(CH2)11(OCH2CH2)6OH. Using this system it was possible to create adhesive patches of defined size upon a very cell non-adhesive substrate [104]. Microcontact printing can also be employed with binding approaches other than alkane thiols binding to gold; e.g., adhesion proteins such as laminin have been stamped onto reactive silane-modified surfaces to produce patterns to guide neurite outgrowth in culture [105]. This very flexible and powerful system will be useful in a wide variety of cell biological and tissue-engineering applications.

A third powerful method is based on microfluidic systems, in which silicone rubber stamps are formed with silicon masters; the stamps are pressed to a surface, and the thin spaces patterned thereby are employed as capillaries to draw up fluid, containing a treatment compound, onto desired regions of the surface. The fluid can contain a soluble, adsorbing polymer with an attached adhesion peptide [71], or it can contain a peptide with some affinity linker for the surface. In the practice of the latter, it is powerful to employ the very high affinity streptavidin-biotin pair, e.g., by biotinylating the polymer at the surface and exposing, with the aid of the microfluidics channels, to peptide conjugated to streptavidin [106].

In a fourth method, also involving silicone layers on material surfaces, silicone layers can be used to pattern directly the locations in which cells adhere to surfaces, and the silicone layers can be lifted off the substrate, if desired, after such cell attachment is desired [107]. Using such approaches, it is possible to pattern 2D surfaces as well as 3D microwells atop such 2D surfaces.

CONCLUSIONS

While it is tempting to think of the matrix to which cells attach as providing primarily a mechanical support, it is clear from the text above that this is only a small part of the picture. Cell interaction with adhesive substrates is known to provide signaling information to the cells via numerous means, both biochemical and biomechanical, and this topic has been extensively reviewed [1,3,80,108–112]. The biochemical mechanisms underlying these interactions are likely numerous and have not yet been fully elucidated. One key mechanism involves the focal contact as a site for catalysis. Integrin clustering induces tyrosine phosphorylation of several proteins, many of which still have unknown function [113]. One of these proteins is a 125 kD tyrosine kinase that localizes, after it is tyrosine phosphorylated, at the sites of focal contacts; this protein has been accordingly named pp125 focal adhesion kinase, or pp125fak. Thus, although the cytoplasmic domain of integrins bears no direct catalytic activity, clustering of integrins is known to stimulate tyrosine phosphorylation and further specific kinases are know to assemble at the sites of clustered integrins. Interestingly, when cells were permitted to spread via a non-integrin mediated mechanism, specifically by interaction of cell-surface proteoglycans with surface-adsorbed polycations, phosphorylation of intracellular proteins did not occur [113]. The phosphorylation of these proteins, associated with focal contact formation, is known to be an important signal for survival of a variety of cell types [114]. Thus, the matrix plays not only a mechanical role as a support for cell adhesion and migration, but also a key signaling role in determining the details of cell behavior, ranging from survival to differentiation.

Engineered biomaterials will play an increasingly important role in deciphering the language of the interaction between cells and their extracellular matrix [79]. Indeed, this represents one of the key challenges for researchers as the field moves forward, to more faithfully represent the complexity of the natural extracellular matrix in synthetic analogs. It is clear that the complex cellular interactions that exist with the 3D milieu *in vivo* cannot be represented well by culture of cells on simple 2D substrates like cell culture flasks [80], and it falls to the tissue engineer to develop more physiologically representative models.

While one goal of biomaterials and tissue-engineering research is certainly to develop systems for the quantitative study of biological interactions, another is to develop practical novel therapeutics. Many of the concepts described above, both in terms of development of model surfaces and especially 3D matrices and with regard to manipulating cellular behavior, are directly transferable, however some cautionary comments should be made. It is not only the chemical identity of an adhesion peptide that determines its biological activity, but also its amount and distribution. This was very clearly demonstrated by Palecek et al. [115], who showed that small amounts of an adhesion molecule could enhance cell migration, whereas larger amounts could inhibit it. They further demonstrated that this effect depended on, among other features, the affinity of the receptor-ligand pair, the number of receptors, and the polarization of receptors from the leading to the trailing edge of the cell. Given that many of these features depend upon on the state of the cell and can be modulated by the cell's biological environment, e.g., by the growth factors to which the cell is exposed [116], many confounding features must be considered in translation from model to practical application [79].

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CHAPTER 22

Polymer Scaffold Fabrication

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INTRODUCTION

The abbreviations used in this chapter are summarized in Table 22.1.

While this summary seeks to review the emerging field of bioprinting as a novel processing technique, an overview of the other main scaffold processing techniques (Table 22.2), including their benefits and limitations as compared to three-dimensional printing (3DP), will be provided. The use of 3DP as a scaffold processing technique seeks to accomplish the goal of developing more biomimetic scaffolds that incorporate the complex hierarchy of natural tissue, providing a better model for understanding tissue function and remodeling [1]. Due to the advances in the specificity and resolution of 3DP, this processing technique has the capacity to print structures with tissue-appropriate heterogeneities in terms of cell distribution and biochemical and biophysical properties [1,2]. While most processing techniques fail to produce scaffolds with microstructures that allow for cell proliferation and migration, bioprinting may be used to tailor the desired porosity and interconnectivity to create an optimal microstructure for cell growth and production of extracellular matrix (ECM) proteins [3]. Furthermore, the capacity of this method to produce scaffolds with complex porous microstructures shows promise in creating scaffolds with an internal vascular network that has not otherwise been achieved with other processing methods.

The overarching goals of printing for tissue engineering include the ability to:

1) Print cells individually or in aggregates,
2) Deposit material in a layer-by-layer approach,
3) Maintain proper structural integrity with layer fusion, and
4) Fabricate a vascular structure, all of which will be elucidated in the following sections of this chapter [4].

To address these goals, a bioprinted scaffold generally contains two important base components, which include a cell carrier material and a cell-free structural material. The cell carrier material, bioink, consists of cellularized materials embedded in hydrogels, microspheres, or as simple cellular aggregates and may include synthetic extracellular matrix (sECM) components [5]. In addition to the cell carrier material, biopaper acts as a cell-free structural material that supports the bioink and provides the foundation for the scaffold [5]. The bioprinting mechanism then uses a three-axis control system to print layer-by-layer structures of bioink and biopaper into a pre-determined three-dimensional (3D) object, such as a scaffold in the shape of a specific organ or tissue [4,6]. The hybrid bioprinting process is illustrated in

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00022-7

TABLE 22.1 List of abbreviations

Name	Abbreviation
Micro-computed tomography	μCT
Three-dimensional fiber deposition	3DF
Three-dimensional printing	3DP
Bone morphogenetic protein-2	BMP-2
Computer-aided design/computer-aided manufacturing	CAD/CAM
Charge coupled device	CCD
Computed tomography	CT
Demineralized bone matrix	DBM
Extracellular matrix	ECM
Environmental scanning electron microscopy	ESEM
Fourier transform infrared spectroscopy	FTIR
Glycosaminoglycan	GAG
High internal phase emulsion	HIPE
Hyaluronic acid	HA
Hydroxyapatite	HAp
Infrared	IR
Laser-assisted bioprinting	LAB
Laser-induced forward transfer	LIFT
Muscle-derived stem cells	MDSCs
Matrix metalloproteinases	MMP
Magnetic resonance imaging	MRI
Mesenchymal stem cells	MSCs
3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium	MTS
National Electrical Manufacturers Association	NEMA
Poly(ε-caprolactone)	PCL
Poly(ester amide)	PEA
Poly(ethylene glycol)	PEG
Poly(ethylene glycol)-diacrylate	PEG-DA
Positron emission tomography/computed tomography	PET/CT
Poly(hydroxymethylglycolyde-co-ε-caprolactone)	PHMGCL
Poly(lactic acid)	PLA
Poly(lactic-*co*-glycolic acid)	PLGA
Poly(N-isopropylacrylamide)	PNiPAAm
Poly(propylene fumarate)	PPF
Poly(vinyl alcohol)	PVA
Arg-Gly-Asp	RGD
Rapid prototyping	RP
Synthetic extracellular matrix	sECM
Scanning electron microscopy	SEM
Stereolithography	STL
Tricalcium phosphate	TCP
Ultraviolet	UV

Fig. 22.1 [7]. It is possible to print biopaper layers that integrate complex patterning of multiple cell types [1,3,6,8] at a high resolution. Moreover, biopaper may be printed with a layer thickness that promotes sufficient cell migration and that is conducive to stacking into a three-dimensional scaffold [9]. Depending on the complexity of the scaffold architecture, a computer-aided design (CAD) program may be used to fabricate organ-specific geometries [5,10]. Initially, the CAD model is created by a computed tomography (CT) or magnetic resonance imaging (MRI) scan from human or animal tissue. Because the scanned biological tissue may be directly implemented as the CAD model, bioprinting is advantageous because it can be tailored to a specific patient. In the case where it would be impossible to obtain CT or MRI results from the patient prior to surgery, it may also be possible to compare archived tissue scan data from patients of similar age groups and organ size [10].

TABLE 22.2 Methods of scaffold fabrication

Technique	Description	Advantages	Disadvantages
Rapid prototyping: 3DP	CAD-modeled deposition of material and/or cells; layer-by-layer stacking to create a 3D scaffold	Wide variety of materials; highly defined porosity and architecture; accurate repeatability; allows incorporation of bioactive molecules and cells	High-throughput manufacturing not available; time consuming process
Fiber bonding	Polymer fibers are heated and fused at points of intersection	Simple procedure; high surface area-to-volume ratio promotes cell attachment	High processing temperatures; limited control over porosity
Electrospinning	Voltage is applied to spin polymer fibers in a non-woven mesh	Very fine fiber thickness may be achieved	Limited control of fiber deposition and porosity; poor mechanical integrity
Solvent casting/ particulate leaching	Porogen incorporated into polymer and solidified; porogen is later dissolved/ leached	Minimal material needed for procedure; high porosity; allows incorporation of composite materials	Pore structures not interconnected; residual solvents or porogens; detrimental to bioactivity
Melt molding	Polymer and porogen heated in mold; solidification and removal from mold; porogen leached out	Control of shape with mold; flexible geometry; allows incorporation of composite materials	High processing temperatures; lack of pore interconnectivity
Membrane lamination	Thin layers of porous polymer are chemically fused	Defined contours and geometries; no visible boundary between layers	Necessary to define shape for each layer; time consuming; cytotoxic solvents
Extrusion	Polymer is heated and forced through a die; forms 3D profile of die's cross-sectional area	Ability to fabricate tubular structures	High temperatures or pressures needed
Freeze-drying	Polymer solution is emulsified; emulsion poured in mold and frozen	Some pore interconnectivity	Lower porosity than most other procedures; scaffold and pore size limitations
Phase separation	Polymer dissolved in solvent; bioactive molecules added to make homogenous mixture; cooling and freezing separates phases; solvent sublimed	Potential for drug delivery applications; bioactive molecules protected from solvents	Residual solvents; limited pore sizes
High-internal-phase emulsion	Water-in-oil emulsion following HIPE standards; monomers from organic phase used to synthesize and cross-link polymer scaffolds with pores	Injectability; good pore morphology; biodegradable polymers may be used	Limited polymer types; high processing temperatures
Gas foaming	Compressed polymer is exposed to high pressure gas; pores form with decreased pressure	Eliminates need for organic solvents; improved cell adhesion	Lack of pore interconnectivity; limited pore sizes
Peptide self-assembly	Designer peptides form into complex geometries (spheres, fibers, sheets) via hydrophobic/hydrophilic interactions	May be designed to promote various cell behaviors; compatible with *in vitro* culture	Expensive materials; design expertise required; limited scaffold size
In situ polymerization	Polymer is injected at site and polymerizes or cross-links after implantation	Immediate implantation possible; minimal processing necessary	Limited mechanical properties; limited porosity; harmful polymerization by-products; limited material choice

Adapted from references [64–66]

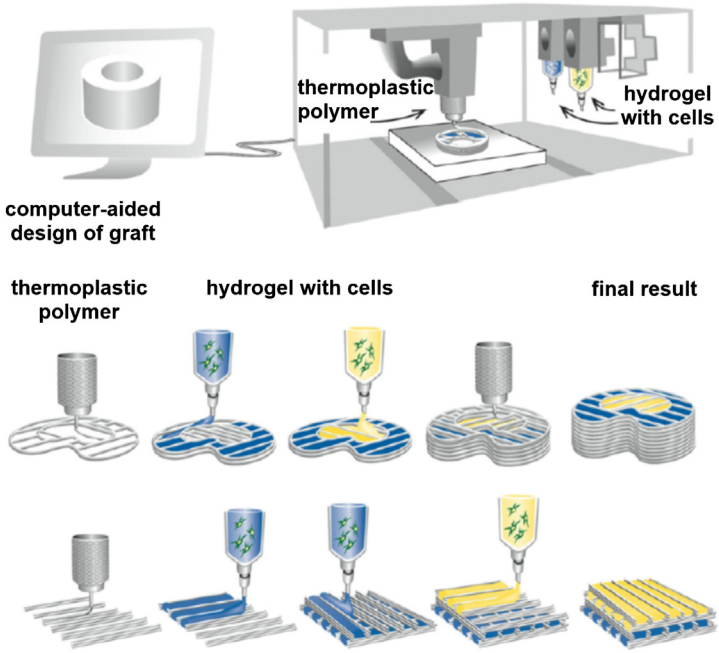

FIGURE 22.1
Step-by-step process of bioprinting, including development of computer model and printing bioink and biopaper. *Reprinted with permission from reference [7].*

After a model has been generated by CT or MRI scan, the file is converted to stereolithography (STL) format for implementation by the CAD software. The software allows further design modification based on geometric scaling, desired porosity, and resolution, to modulate structure and function of the engineered tissue. Standard software must be implemented with the printing system in order to translate the code from the CT scan into a 3D model for printing. Some commercial printers come with CAD software that is specific to the printing instrument; however, open-source printers available allow modification of user-defined software or integration with other commercial programs, such as SolidWorks® [11]. SolidWorks®, among other commercially available computer-aided design/manufacturing (CAD/CAM) software programs, provides a relatively simple interface for designing a 3DP model with parameters that may easily be modified to accommodate sample size, pore size, resolution, and geometry (Dassault Systèmes SolidWorks Corp., Waltham, MA, USA). Implementation of the CAD/CAM software may also be combined with a CT image of a tissue defect to create customized designs that are appropriate for surgical implantation [10]. Images of the tissue taken before and after the defect placement, can be combined to create the relative shape of the defect to be printed *in situ* or implanted post-printing [10]. The geometric fidelity of this shape can be later analyzed by a laser distance sensor, which detects defects in the printing uniformity and compares the resolution of the printed scaffold to that of the computer model. Once the tissue model has been developed and characterized for proper function, the final processing methods depend upon the desired method of implantation. Scaffolds may be cultured in flow perfusion conditions to allow cell-development of ECM and then implanted into the patient after the scaffold meets the structural needs of the specific tissue. On the other hand, the processing parameters of the printing system may be tuned to print *in situ* tissue replacements or repairs, which may have a promising clinical application in surgical procedures.

BIOINK: CELL PRINTING AND CHARACTERIZATION

The first goal of 3DP for tissue engineering is to develop processing methods that allow printing of cells and cellular components. Bioink, in addition to containing cells, may also incorporate bioactive components such as signaling molecules (e.g., cytokines, growth

factors), DNA, ECM molecules, and others, depending on the desired effect on the printed tissue [1]. Since cells and bioactive molecules respond to external environmental cues, it is important to determine whether processing has an effect on the biological components in the scaffold, specifically on their morphology, composition, and function [1,12]. Before printing cell-based solutions, it is important to confirm that the printing material is compatible with the processing conditions of the printer. Consequently, it is important to note that most work has investigated the encapsulation of cells in hydrogels due to their ease of use with printing conditions [5], functionality and biocompatibility [13]. Material choice will be discussed later in this section and in the following section. After testing the compatibility of the polymer solution with the printing process, it is then possible to incorporate cells and biological molecules into the material. A sterile environment for cell printing must first be prepared to minimize contamination and protect cell viability before processing. Polymer solutions must be sterile filtered, and the printer components that come in contact with cells must be sterilized with methods that take into consideration the material composition of the printer. Further cell encapsulation in a material requires consideration of many printing factors. Thus, measures must be taken to ensure proper environmental conditions to minimize the effect of printing on the material properties and biological identity of the cellular components in the scaffold.

External processing conditions that could have an impact on cell fate may include polymerization reactions, temperature, pressure, shear stress, and deposition force, some of which may not otherwise be considered in cases of cell seeding post-printing [9,14,15]. For those materials that require external environmental cues for polymerization, these factors may also threaten viability and proliferation. For instance, crosslinking may be necessary to enhance the mechanical strength of the printed polymer construct and drive cell differentiation [16]. Depending on the rate and completeness of the material's polymerization mechanism, the scaffold may be subject to ultraviolet (UV), infrared (IR), or white light after each successive layer is printed, or after the entire construct has been fabricated. In some studies, a hydrogel solution was partially crosslinked several minutes prior to printing for initial structural integrity and later fully crosslinked to fuse layers for the final structure [5,10]. Due to processing effects of shear stress from the printing nozzle, crosslinking via UV, IR, or white light, and other factors, cell viability in the printed scaffolds should be determined and compared to *in vitro* cultures.

In order to determine whether crosslinking has an effect on the mechanical integrity of the material or the fate of cells encapsulated in the material, several characterization methods may be employed. Mechanical moduli can be measured, for example, for printed scaffolds with varying crosslinking densities to optimize material parameters [5]. A comparison between the viability of printed cells to that of cells in standard culture medium would be necessary to determine if crosslinker concentrations are cytotoxic [2]. Early studies investigating the simultaneous printing of a $CaCl_2$ crosslinker with endothelial cells showed cell attachment and spreading on an alginate hydrogel after printing, offering preliminary evidence that cells can survive the printing and crosslinking process [17]. Some mechanisms have been developed to protect cells from harmful degradation or polymerization by-products until reactions have reached completion. For instance, cells encapsulated in gelatin microspheres have been successfully implemented in poly(propylene fumarate) (PPF) [18]. This temporary encapsulation technique allows cells to remain viable during crosslinking reactions induced in the polymer and will then release the cells upon completion of the crosslinked construct by dissolution of the gelatin, allowing cell migration and proliferation throughout the scaffold [19]. Furthermore, histological assays may be performed after printing to determine preferred cell differentiation pathways among crosslinked scaffolds.

In addition to influences from crosslinking, processing temperatures may restrict the materials used for cell-based printing applications. Fortunately, since 3DP can be performed at

ambient temperature, fabrication in a normal laboratory setting is more feasible [10,20,21]. Although there have been many studies investigating the incorporation of cells in hydrogels for 3DP [2,4,5,7,11,17,22,23], few investigations have been conducted on simultaneous printing of cells and scaffold for rigid polymer constructs. This may be attributed to the fact that many rigid polymers cannot be extruded in liquid form at near-ambient temperatures [24,25]. For some 3DP applications that do not employ cellular components, rigid polymers are often melted in powder form when printed [26,27], but the high melting temperatures required would have an adverse impact on cell viability, and would not be conducive to cell encapsulation. To accommodate printing of scaffolds with encapsulated cells, it is necessary for the polymer to be in its liquid form upon extrusion from the deposition tool. This is because polymers printed in solid powder form, if not melted beforehand, require the addition of solvents to dissolve and fuse into scaffold layers, but common printing deposition tools often degrade as a result of the solvent addition [28]. Moreover, there are some printing nozzles available that are resistant to solvent degradation, but printing using these tools often compromises resolution, and compatibility with powder-based polymers is limited [28]. Another group has looked at printing starch-based polymers with distilled water, but while this approach is less cytotoxic, the porogen size is limited and not amenable to high resolution applications [29]. Printing a polymer in its liquid state is also advantageous because a lower deposition pressure is required for extrusion. At high deposition pressures, shear thinning may occur, involving a sudden drop in polymer viscosity and shear stress at deposition [24]. Moreover, the polymer viscosity has a substantial impact on the injectability of the solution [25,30]. A more viscous solution requires a greater deposition force, resulting in exertion of a greater shear stress on the material and cells [10,20]. Further injectability testing may be needed to determine the optimal viscosity to promote structural integrity and cell viability when subject to shear conditions from the printer head.

While processing techniques have an impact on cell viability and behavior, the cell carrier material may need further modifications to allow proper tissue growth. In some cases, the ability to interact with cells necessitates functionalization of the material via end groups, ECM molecules, or other bioactive elements. Examples of bioactive elements may include Arg-Gly-Asp (RGD) peptides functionalized on poly(ethylene glycol) (PEG) hydrogels for cell attachment, or modification with matrix metalloproteinases (MMP)- or plasmin-sensitive crosslinkers for degradation [13,31]. A hydrophilic material, such as PEG [31–33], is relatively inert to cell interactions and requires addition of bioactive molecules to allow cell adhesion and proliferation throughout the matrix. On the contrary, a highly hydrophobic polyester such as poly(ε-caprolactone) (PCL) has been functionalized as a printable copolymer — poly(hydroxymethylglycolide-co-ε-caprolactone) (PHMGCL) — to allow cell attachment and tunable degradation [34]. There are a few other methods of manipulating cell-material interactions by printing components that allow modification post-printing. For instance, one such printing technique has been investigated using magnetized nanoparticles in alginate and in cells [20]. This technique allows direct migration of bioactive factors and spatial distribution of cells via an external magnetic field, which would modify the organization of matrix post-printing toward different paths of cell differentiation [20]. By selective printing of ECM components and other bioactive molecules throughout the scaffold, it is possible to direct cell patterning and cellular responses in the matrix, such as proliferation, migration, cell death, and differentiation [1,12,24,35].

Cells that have attached and proliferated on the scaffold also have the capacity to modify their matrix [12]. First of all, cell-secreted enzymes may have an effect on the mechanical integrity of the material. For instance, while scaffold degradation may have a negative effect on cell viability [36], the production of ECM and subsequent scaffold degradation by the cells has an effect on mechanical stability of the material [37,38]. This effect may be controlled or enhanced by external mechanical conditions; specifically, pre-loading or other means of mechanical stimulation on the scaffold prior to implantation may provide ways to modulate

cell differentiation toward hard or soft tissue lineages. Sources of mechanical stimulation may have a subsequent effect on scaffold integrity via production of different types and quantities of ECM proteins [37]. The interplay between matrix stiffness and cell migration behavior has a drastic impact on directing cell lineage, as well as the propensity for matrix degradation by proteolytic enzymes or other molecules contained in the scaffold [31]. sECM materials have been developed to allow sensitivity, via cleavage sites or other functionalities, to degradation enzymes secreted by the cells [13,31]. In addition, if the cell density comprises a significant portion of the scaffold, this may have an effect on its crosslinking ability and mechanical properties [5,20,37].

Depending on how the cells are incorporated into the scaffold, whether seeded [28] or encapsulated [13], attachment and proliferation may occur over the course of days to weeks. Immunohistochemical and colorimetric assays, fluorescent imaging, and histological staining are performed to analyze cell distribution and viability in samples after several weeks of culture. For example, a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay may be used to determine cell viability via quantification of mitochondrial activity [5]. Fluorescent tags may also be transfected in cells or encapsulated in cell-containing microspheres in order to visualize proliferation, viability, and distribution throughout the scaffold after printing [5,17,39,40]. Epifluorescence microscopy may be used to visualize the fluorescent molecules in the scaffold [17,40] and detect foreign-body responses *in vivo* [41]. In addition to monitoring cell viability and behavior, histological staining may be utilized to identify the presence of cell-secreted ECM molecules, such as collagen and glycosaminoglycans (GAGs). Some stains used for visualization include Safranin-O for GAG content, hematoxylin and eosin for cell morphology, masson trichrome for collagen presence, toluidine blue for proteoglycan content, and von Kossa's staining for mineralization [38,41,42]. Results from staining determine if the cell carrier material provides an appropriate microenvironment for cell proliferation and remodeling.

BIOPAPER: STRUCTURAL MATERIAL PRINTING AND CHARACTERIZATION

In order to achieve the second goal of printing with a layer-by-layer approach, cell-free structural material, or biopaper, must be printed alongside the cell carrier material to enable proper layer deposition and maintenance of resolution. When considering a structural material for printing scaffolds containing cells, it is important to minimize harmful processing procedures, such as high curing temperature and polymerization solvents, while enabling extrusion and proper layer fusion of the scaffold material. As stated previously, hydrogels make up a substantial percentage of materials used for cell-based printing applications. In order to account for both the biocompatibility and the structural needs for the scaffold, polymer hydrogels are often functionalized via crosslinking or bioactive agents to allow enhanced mechanical strength and cell attachment, respectively [5,13,24,38]. However, hydrogels often have poor mechanical strength even with the addition of functional agents, so researchers are investigating a wider variety of materials that will be amenable to printing in order to cater to the need for load-bearing tissues. For example, Boland et al. found that printed samples of alginate had significantly lower elastic modulus and ultimate tensile strength than alginate samples that were manually crosslinked [17]. This result was attributed to high porosity in the printed sample, but may have also been due to other processing factors [17]. Solving the problem of mechanical integrity would either require replacement of the hydrogel with stronger, biocompatible materials, or designing scaffolds that would allow complete cellular remodeling and degradation of the material to create naturally functioning tissue.

Within the acceptable list of materials that are both biocompatible and amenable to printing with cells, the material of choice is primarily based on the mechanical and chemical properties that most closely match the organ in question. For instance, common materials used for

chondral tissue engineering include alginate [11] and agarose [3]. Both agarose [5] and PEG [32] have been used in combination with hyaluronan as materials to print vascular structures. Manipulating the properties of alginate for its rapid gelation time and collagen for its bioactivity, Pataky et al. were able to print beads of this hydrogel blend for applications in vascular printing as well [2]. 3DP may be implemented for printing hydrogels as well as rigid polymers and ceramics. For instance, some materials employed for bone tissue engineering include PCL [26], hydroxyapatite (HAp) with PPF [43], and demineralized bone matrix (DBM) [10]. Although most major organs have not yet been successfully printed, a list of materials and cells used for printing major tissue types are summarized in Table 22.3.

Material properties are of paramount importance when considering the application and processing method for scaffold engineering, and these may have an effect on the printed resolution of the construct. As emphasized before, it is especially important to consider the viscosity and injectability of the material for a 3DP application due to limitations of the deposition mechanism. For example, the maximum deposition force for certain printer models places restrictions on highly viscous materials [10]. These material parameters have a direct influence on the final printing resolution of the material. When considering inherent injectability properties, the printed resolution should be adequate for millimeter-sized defects, which are commonly seen in most *in vivo* tissue-engineering work in small animal models [10,27,41,42,44,45]. The resolution places restrictions on not only the viscosity of the material and extrusion force, but also the syringe tip size, all of which must be carefully balanced in order to achieve uniform printing of the desired tissue-engineered scaffold [39,46]. The resolution quality may later be evaluated by imaging tools to determine accuracy to design standards.

Besides the effect of viscosity, rigidity, and degree of crosslinking on resolution, other material properties may affect cell fate, including composition, degradation and polymerization by-products, degradation rate, biocompatibility, crystallinity, porosity, and hydrophobicity. Its composition must mimic natural ECM and be amenable to cellular remodeling via production of new ECM components and work synergistically with cell signaling processes to allow proper tissue growth [1]. The degradation timescale must be appropriate to the tissue regeneration rate and the matrix must be amenable to cellular remodeling via production of ECM proteins. Because the degradation mechanics of tissue implants have variable effects on surrounding healthy tissue, including eliciting inflammatory, fibrotic, or hyperplastic responses, materials that have the potential to release cytotoxic degradation and polymerization by-products must be thoroughly investigated before use in a 3DP application to ensure

TABLE 22.3 Materials for 3DP scaffold fabrication

Tissue	Material	Cell-laden applications
Heart valve and heart tissue	PEG-DA, PEA [55]; alginate [55,68]	Human cardiac progenitor cells [68]
Skin	PCL [7], collagen [69]	Fibroblasts and keratinocytes [50,69]
Liver	Gelatin [60,70] and fibrinogen [70]; PCL, PLGA, HA, gelatin, collagen hybrid [59]	Hepatocytes [59, 60, 70], with pre-osteoblasts [59]
Peripheral nerve tissue and neural cell printing	PLA-PEG, PLA, DL-lactide [61]; collagen [71]	Neurons, astrocytes [71]
Bone	HAp and TCP [27]; brushite and monetite [44]; PCL [26,34], HAp with PPF [43], DBM [10], PHMGCL [34]; agarose, collagen, gelatin, Matrigel, PNiPAAm, fibrin, Pluronics [67]	MDSCs with BMP-2 [35]; osteoblasts, MSCs, osteogenic cells [67]
Cartilage	Alginate [11]; agarose [3]; chitosan, gelatin, collagen, PEG, PNiPAAm, PVA, fibrin [67]	Chondrocytes, MSCs [67]

Adapted from reference [67]

biocompatibility *in vitro* and *in vivo* [25]. Moreover, the degree of crystallinity will have a direct impact on scaffold rigidity and mechanical strength, as well as indirect guidance of cell proliferation and ultimate differentiation. The material geometry, in addition to mimicking the desired tissue in function and integrity, must contain interconnected pore structures to allow fluid and mass transport of waste and nutrients, as well as allowing cell attachment, proliferation, and tissue formation. These material properties may be further characterized to determine if the scaffold appropriately models the tissue or organ.

Material properties should be characterized after printing, including surface composition, morphology, porosity, resolution, and interaction with surrounding tissue. To characterize the composition of the surface after printing, Fourier transform infrared spectroscopy (FTIR) may be utilized. The morphology — specifically, the degree of porosity control — may be visualized by gold sputtering of the printed polymer and imaging the sample using scanning electron microscopy (SEM) or environmental SEM (ESEM) for wet, uncoated samples. It is expected that the morphology and composition of the material surface will encourage cell attachment and proliferation once the cells are released from encapsulation [9]. For specific printing applications, some characterization methods may provide helpful information regarding material interaction with surrounding tissue. When characterizing printed implants for calvarial defects, *in vivo* MRI has been performed in mice to characterize the printed scaffold via T1- and T2-weighted images [42]. Clinically, T1- and T2-weighted images show tissues with different gray scales and are both helpful in determining abnormal tissue behavior or morphology in the brain. Signal intensities from these images may be measured to determine the change in microstructure and biochemical composition over time, as well as how these intensities correspond with the intensities of the healthy surrounding tissue [42]. Additionally, the MR images are helpful in measuring inflammation and the progression of wound healing [45]. Comparable imaging techniques for other parts of the body, including positron emission tomography/computed tomography (PET/CT) or X-ray scanning, may provide similar information on implant integration *in vivo*.

PRINTING MECHANISM: LAYER-BY-LAYER APPROACH

For layer-by-layer deposition to be successful, the type of printing system employed is equally as important as the choice of materials and method of cell encapsulation used to print the tissue. The printer choice will determine how the cells and materials will be deposited and organized and ultimately delegate the layered structural make-up of the scaffold. Due to the versatility of printing liquid-based materials, bioprinting has largely moved away from the traditional powder and binder process of early 3DP and more toward hydrogel and soft polymer printing [25,47]. To address further soft polymer processing applications, some of the pioneering models of 3D printers manipulated the skeleton of commercial inkjet printers, such as the HP Deskjet® [17,39,48], and filled recycled ink cartridges with cell solution to print micron-scale cell droplets [4]. This inkjet-based system allows a variety of materials and cells to be printed by simply switching out the cartridge [39]. While X- and Y-axis positioning capabilities were already available within the inherent properties of the printer, Z-axis control was implemented via a metal rod and stepper motor regulation, which is comparatively similar to the basic mechanical principle for most commercial bioprinter models today [17,42,49]. On the other hand, these preliminary models had some limitations. For instance, the scaffold dimensions in a design by Boland et al. were confined to a 50 mL conical tube filled with uncross-linked polymer solution, as pictured in Fig. 22.2 [17]. A crosslinker was deposited via the printer head on a specified area of the polymer surface, which was defined within the dimensions of a glass substrate. Crosslinking of the polymer allowed formation of the first hydrogel layer, which was then lowered by the rod to allow more uncrosslinked polymer to cover the top of the first layer and allow printing of the subsequent layer [17].

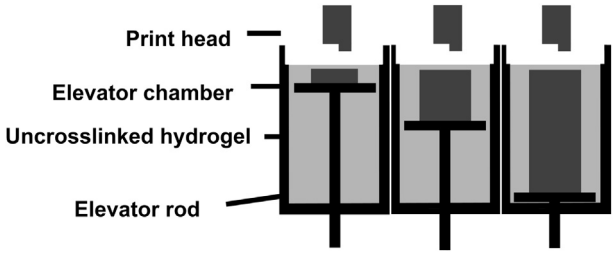

FIGURE 22.2
Model of elevator chamber for inkjet printing.
Reprinted with permission from reference [17].

Since then, there have been improvements in the field with new printers in the past few years. Most bioprinting techniques under current investigation utilize 3D Plotting – also known as 3D fiber deposition (3DF) – to print layers from polymer melts instead of powders [24,25,38]. 3DF allows much easier fiber deposition and layer fusion and does not require the addition of harmful organic solvents, unlike the powder-binder method employed with previous inkjet-based models [25]. Some commercial systems (3D-Bioplotter™ Fourth Generation, Envisiontec GmbH, Germany; Fab@Home V 2.0, Cornell University, Ithaca, NY) use this method of fiber deposition. Furthermore, for inkjet-based printers, some attempts have been made to improve vascular printing [2,39]. Specifically, printing vessel-like structures that support physiological flow conditions can be achieved by modifying settings to allow printing over void space [2]. Other current models have expanded the area of the printing stage for overall structures of greater than 20 cm [49]. Additionally, improved models have eliminated the need for an elevator chamber by use of multiple deposition tools with the capacity to combine the crosslinker and uncrosslinked polymer immediately upon printing [49].

In addition to improvements in the structural integrity of the printed construct, further advances have been made in optimizing the deposition mechanism, in order to enhance material placement, tailor resolution, and reduce nozzle malfunctions. For instance, nozzle clogging is a common issue among traditional inkjet-based printers [30]. To address this issue, laser-assisted bioprinting (LAB) was developed as an efficient processing method that eliminates the need for a nozzle [15]. The LAB technique, pictured in Fig. 22.3, is based on a laser-induced forward transfer (LIFT) technique that allows highly defined printing of cellular patterns [50,51]. The LAB 'ribbon' is composed of multiple layers that must be prepared according to several parameters [15]. The collecting substrate is first coated with the material to be printed, which most often consists of droplets of polymer with encapsulated cells [14,15,23,51–53]. The top support must be adjusted to allow laser light in the UV or IR range to pass through the focusing lens and then the absorbing layer. In addition, there is a laser-absorbing interlayer between the top support and the bioink layers that is sensitive to the

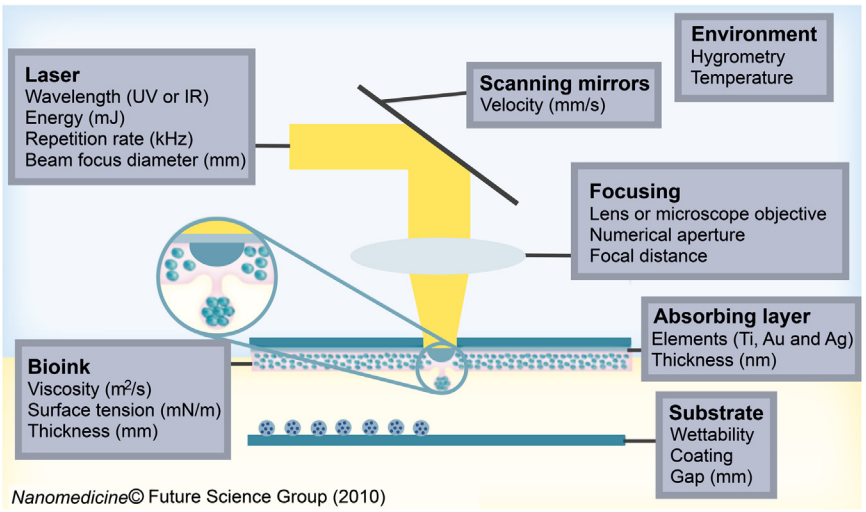

FIGURE 22.3
Laser-assisted bioprinting (LAB) parameters.
Reprinted with permission from reference [15].

optical properties of the bioink and laser wavelength and subsequently affects light transfer [15,50,54]. The interlayer is commonly made of a thin metal film, such as gold, that protects the bioink from direct interaction with the laser beam [54] and has an impact on droplet size based on its thickness [50,52]. Laser fluence — the pulse of the laser to induce printing of the bioink droplets — and the gap distance between the ribbon and the substrate are also two important parameters to define in the set-up process and are required to set the scanning speed of the galvanic mirrors before printing [15,50]. The laser light is emitted at either a UV or IR wavelength to create nanosecond pulses of energy, allowing deposition of bioink beads on a quartz disk [15]. The volume of printed material is directly dependent upon the energy of the laser pulse, and microdroplet deposition requires the achievement of a minimum threshold energy [54]. Although this method is promising in reducing nozzle clogging, height restrictions remain a current obstacle, which makes this method more appropriate for printing cell arrays instead of tissues [9,23].

While commercial bioprinters can be costly and difficult to personalize, several open-source printers have been developed to address the demand for simple, cost-effective scaffold fabrication equipment. Printers such as the RepRap and the Fab@Home systems are based on similar principles as the early inkjet printers [17], but are modular in design and allow for much easier modification based on the needs of the user [55]. The RepRap system is unique in that it has been programmed to print daughter models from an original parent model (RepRap Original Mendel, GNU General Public License). Since most of the models contain wooden parts and exposed circuitry, it is difficult to use the RepRap in sterile cell printing applications; however, models are continuously being modified with tissue engineering in mind. The Fab@Home system has already been used in preliminary studies for tissue engineering applications [5,10,32]. The Fab@Home model uses a syringe-based deposition system that extrudes liquid polymer or injectable cement solutions at ambient temperature on a platform [49]. Updated models come with multiple syringes to allow simultaneous printing of different polymer or cellular components and incorporate more design complexity and heterogeneity (Fab@Home V 2.0 — Kit for 2 Syringe System, Cornell University, Ithaca, NY). The syringe system itself is important in determining the deposition rate, the path width, and the path height, which play key roles in the final resolution [10]. The syringe tip diameter and material choice have a substantial impact on these variables, but one of the most important considerations is the lead screw threading, or the number of threads per inch [49]. Mechanically, the threading is important in determining the speed and direction of linear motion for the moving parts of the printer. For a specified syringe size, the threading also contributes to the maximum flow rate and syringe pressure. Based on the syringe tip diameter and the extrusion force, a limited range of fiber sizes may be achieved on the millimeter scale. In addition, the Fab@Home printing set-up houses a two-syringe system, placing limitations on the ability for material variance and optimal printing speed. Ideally, this set-up would be modified to accommodate four or more deposition tools to allow simultaneous or sequential printing of different types of material and avoid complications due to switching syringes. Modular components for the Fab@Home system may be added to incorporate other functionalities in the printer, including a crosslinking unit [5,55], a laser for error analysis [10], or a removable substrate platform for *in situ* applications [10], for example. These open-source systems have revolutionized the field of bioprinting by providing inexpensive, user-friendly printing systems with the capacity to be modified and improved upon by experimentalists. For example, Fig. 22.4 depicts the Fab@Home printing system, which includes a three-axis Cartesian gantry positioning system and is designed to be easily modifiable based on modular deposition parts [49]. The deposition tool is positioned on the Y-axis, which in turn rides along the X-axis. To minimize the effect of acceleration on moving parts, the Z-axis runs independently of the X- and Y-axes by moving the working stage up and down. All axes are composed of National Electrical Manufacturers Association (NEMA) size 14 bipolar motors and rotor-mounted with lead screws [49].

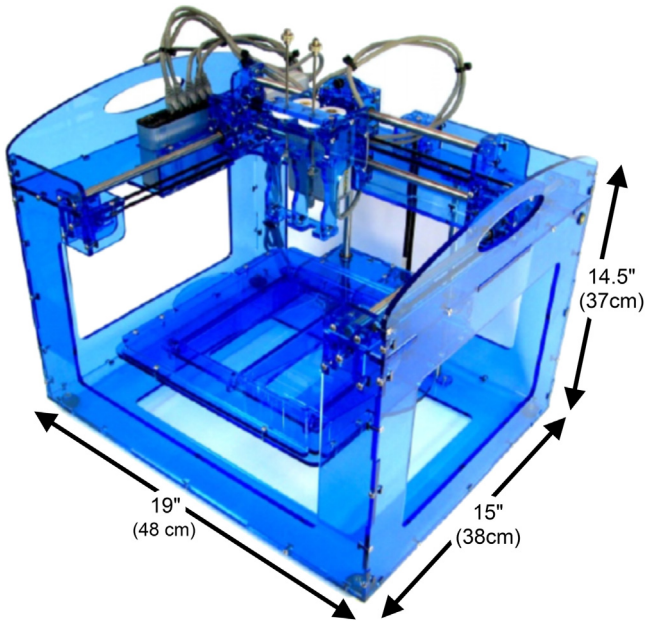

FIGURE 22.4
Dimensions and set-up of Fab@Home printer. *Adapted and reprinted with permission from reference [10].*

FINAL LAYER ARCHITECTURE AND RESOLUTION

Depending on the type of printer used, different layer or droplet sizes may be achieved due to the differences in deposition mechanisms. In general, 3DP of tissue-engineered constructs produces architectures with a high level of porosity as compared to other processing techniques, and a resolution of less than 250μm may be achieved [25]. In commercial inkjet printers, nozzle sizes range from 20–30μm, which can produce droplet sizes in the range of 10–20 pL [30]. While smaller nozzle sizes can produce fiber sizes with higher resolution [30], it is important to match the extrusion area of the nozzle to the viscosity of the material to ensure proper injectability [10]. For printers that use syringe-based deposition tools, tip diameters range from about 0.8 mm for hydrogels to 1.5 mm for ceramic pastes and produce fiber sizes of less than 2 mm in width and height [10,25]. Additionally, diameters ranging from 80–140μm have been achieved for LAB-printed droplets [50]. Overall, it has been shown that bioprinting creates an appropriate resolution for proper cellular response in a printed pattern [1].

Although precise resolutions may be achieved, there is some difficulty maintaining porous structures when stacking multiple layers, specifically the fusion of layer contact points without scaffold collapse [2,6,25]. For instance, although the vertical pores may remain intact after stacking, transverse pores in hydrogel scaffolds have the propensity to collapse due to the material softness [6]. Depending on the rigidity of the material used, there are height limitations for printing 3D scaffolds that may be mitigated by additional structural support [5]. For instance, Schuurman et al. constructed a hybrid scaffold design that alternated the printing of a structural thermoplastic polymer and a cell-laden hydrogel [7]. On the other hand, biodegradable or bioinert structural inserts may be added to guide printing and be later removed upon cellular remodeling of the matrix. Consequently, the loss of pore definition and collapse of fused layers are among the main sources of resolution error for a printed scaffold. Resolution error may readily occur during the printing process due to mechanical vibrations, slight movements of the printer head, or micrometer discrepancies in distance between the printer deposition tool and the substrate [39]. Furthermore, as fibers are extruded or droplets are deposited, the act of falling from the deposition tool to the substrate may have an impact on the material morphology and create inconsistencies in the design. Additionally, some types of printed cell-based hydrogel scaffolds that have been cultured in media for several weeks,

such as collagen and pluronic-F127, have shown shrinkage or swelling, respectively, over time [11]. All of these sources of error are important to consider when printing tissues that have a low error tolerance.

To determine whether resolution and porosity have been maintained after the scaffold has been printed, adherence to design geometry may be analyzed via micro-computed tomography (μCT) scanning [25,56] or using a charge coupled device (CCD) camera [10,20,45]. While μCT is the more time-efficient process, the use of a CCD camera appears to be a more precise method of error analysis because of the ability to evaluate each layer after it is printed [10]. A CCD camera may be used to create highly defined, low noise, real-time images of the material deposition process on the work space of the 3D printer. The use of a CCD camera for real-time visualization has several advantages. This mechanism serves as a primary method— in addition to secondary analysis with a laser distance sensor — for evaluating proper spatial resolution of each layer [10] and positioning throughout the printing process [45]. It can be used to measure parameters involving Z-positioning of the nozzle over the substrate, extrusion force on the material, fusion time between printed layers, sag length of the material over time, and work life of the material before the extrusion force is no longer adequate [10,24,45]. Because this camera allows for real-time imaging of the working area at an appropriate scale, the need for troubleshooting is much more apparent between layers, and geometric fidelity may be maintained with greater ease.

To further support CCD imaging in evaluating resolution and design flaws, a laser distance sensor may be employed as a primary mode of error analysis [57]. This sensor, as a measure of geometric conformity, has the ability to raster the sample layers post-printing in order to compare the actual resolution to the desired construction based on the computer input. As it scans the workspace, it can identify X—Y and height resolution to a certain range and measure of error. Previous work achieved a range of 104 mm and a distance measurement resolution of 0.12 mm height and 0.3 mm X—Y distance [10]. In order to achieve this high level of detection, laser placement is an important aspect in order to optimize the sensing range and to most accurately depict the printing resolution. An investigation was conducted on the sensing efficacy of a laser placed between the Y-axis carriage and the printing syringe, but it is unclear how well this placement enables optimal error analysis [10]. Ideally, the sensing mechanism should be designed to properly identify areas of heterogeneous surface texture, as well as point defects of missing or excess material. Through analysis of several determined points of each layer, it is possible to convert this information into a three-dimensional solid and export the information into an error-calculation program, such as Qualify® V11 (Geomagic Inc., Research Triangle Park, NC, USA), to compare the printed solid to the intended geometric model [10].

Error analysis of each scaffold is helpful in determining the reproducibility of the printing system by comparing error among a number of scaffolds of the same geometry and sample population and correlating these results with the intended computer model [57]. To extend printed tissue applications beyond the laboratory and into the clinic, it has been suggested to compare the calculated error to standard surgical tolerances for each respective defect in an effort to improve implant technologies [10]. Cohen et al. evaluated the efficacy of *in situ* printing in meniscal defects between the tibia and femur, as well as in osteochondral defects in a calf femur [10]. A laser sensor was then used to determine points at which the scaffold showed inconsistencies with the CAD model, in which the error was calculated and found to comply with acceptable surgical standards [10]. This result shows the ability to apply 3DP technologies to surgical procedures in order to produce highly defined, customized tissue-engineered constructs. Overall, comparison of error values from the CT image and the printing process to the surgical tolerance for defect implantation allows investigators to quantify the printing accuracy and gain a greater sense of the optimal resolution. Using simple, effective technologies such as CCD imaging and laser sensing, basic printing deposition may be

transformed into a translatable technique for designing surgical-grade defect replacements tuned specifically for each patient.

PRINTING APPLICATIONS: VASCULARITY AND ORGAN FABRICATION

While the idea of printing whole organs is still in its infancy, much work is being conducted to address this new field. When first developing tissue-engineered scaffolds, one of the most important aspects of the design is to incorporate vascularity to promote oxygen, nutrient, and waste transport throughout the tissue. Several groups have investigated vascular printing using inkjet-based printers [2,48] and syringe-based printers [5,32]. Another experimental model coupled development of entire vascular networks via bioprinting and self-assembly with computer simulations of tissue fusion [40]. Further investigation has also shown promise in developing tubular structures from functionalized hyaluronan-gelatin hydrogels [5]. It has also been shown that it is possible to print vascular architectures that support physiological flow conditions present in cardiovascular microvessels [2], which is a great improvement in the field of bioprinting and tissue engineering. Once the elements of vascularity can be implemented with printing tissues, steps toward printing whole organs will be more feasible.

In addition to work on developing vascular structures, several laboratories have also developed preliminary models toward whole and partial organ printing, including heart valves [55], skin [39], bladder [58], liver [59,60], neuroregenerative tissue [61], bone [27,44,62], and osteochondral tissue [10]. Some of these studies incorporate additional rapid prototyping techniques that may be later translated to 3DP applications. Using the Fab@Home printer, educational models of a heart and aorta have been fabricated using clear silicone, and alginate has been used to print an ovine construct for meniscal repair [5]. Bioprinting has also been suggested as a method for printing tissues with zonal architecture, such as the articular cartilage model depicted in Fig. 22.5 [3]. Taking a step beyond printing scaffolds and implanting them after cell culture, some efforts have been made toward *in situ* printing for meniscal repair [10], calvarial defects [1,45], and skin replacement [39], which may provide benefits for more efficient surgical technologies. One drawback to *in situ* printing is that the scaffolds would not be primed via mechanical stimulation prior to implantation, which may result in improper cell differentiation and subsequent difficulties in tissue regeneration. However, it is

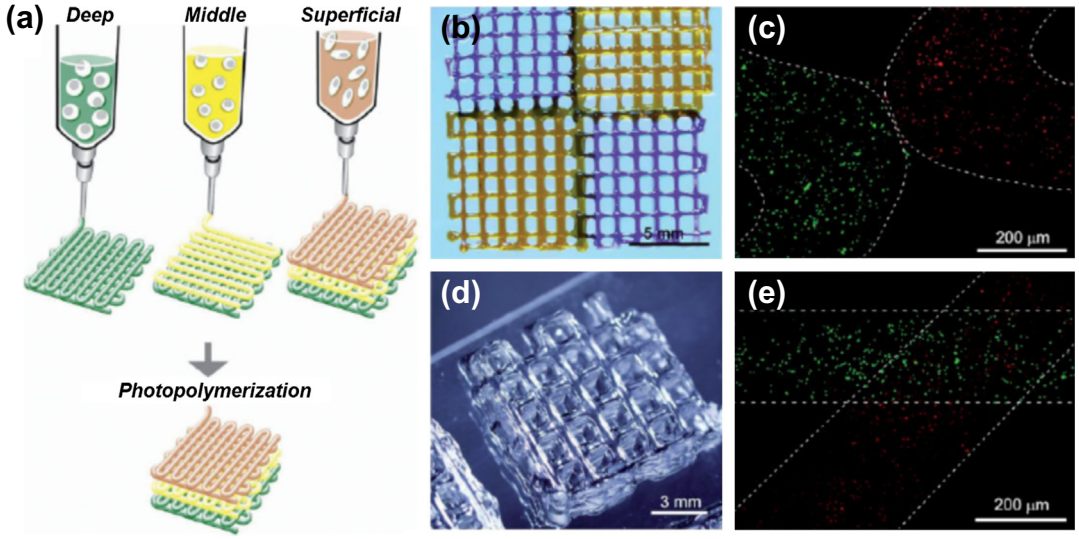

FIGURE 22.5
Printing of constructs with zonal architecture in (a). Printed hydrogel constructs in (b) and (d) show spatial and porosity control, respectively. Fluorescent images in (c) and (e) show cell confinement within zones. *Reprinted with permission from reference [3].*

still unclear whether scaffold stimulation or pre-loading is necessary to promote desired tissue formation at the defect site.

To determine whether the printed tissues actually match the form and function of biological organs, it is necessary to identify or design specific characterization techniques that detect cell-cell and cell-material interactions for *in vitro* and *in vivo* conditions. As tissues and organs are printed with varying materials, growth factors, cells, and other molecules, a collective library of these 3DP scaffolds would initially allow rapid screening of optimal parameters for tissue-engineered constructs and identify factors that are crucial for cell growth and proper function [63]. Because 3DP allows the construction of scaffolds with repeatable porous architectures, processing with this method has implications for better screening of the scaffolds themselves and in flow perfusion conditions [38]. However, current methods of evaluating 3D tissue formation are time intensive and inaccurate. For instance, individual separation of cell pellets into wells may be achieved in a more rapid manner with 3DP, but these small wells do not represent the functionality and cell-material interactions found in a full-sized tissue [38]. High-throughput scaffold arrays could also be used to identify proper microenvironments for specific tissue regeneration. One dimensional assays — such as polymer blends with varying compositions — as well as 2D assays involving polymer composition and processing temperature or surface energy and hydrophobicity may be used to characterize the effect of the material on expression of cell lineage markers [12]. Furthermore, it would be desirable to develop or utilize currently available non-destructive techniques to image and characterize the scaffold prior to implantation [20]. Although progress has been made to address the biological functionality of scaffold processing via 3DP, current technologies have yet to develop methods to increase the scaffold output for 3DP. Further modification of the printing process is necessary to meet the annual clinical demand for manufacturing artificial implants.

CONCLUSIONS

Polymer processing via three-dimensional printing is an emerging technology designed to address tissue replacement needs for individual patients. Common issues in the area of 3DP include limited scaffold size and sample number. Because this technology was originally designed to create prototypes, few efforts have been made to scale-up the manufacturing process of scaffold samples. There have also been few successes in the ability to increase scaffold size due to structural instability of the materials used, which are usually natural hydrogels. Furthermore, the process of 3DP is still time intensive due to a lack of archived patient data and the ability to create a customizable scaffold library. In addition to the time needed to create a 3D computer model of the scaffold prior to printing, the actual printing process may take hours to days, depending on the size and complexity of the construct. For printing applications that require culturing each layer before stacking, fabrication time may be even more laborious. In order to overcome this obstacle, printing materials with encapsulated cells may be more beneficial than cell seeding post-printing. Additionally, although initial development of a computer-aided design is time consuming, a scaffold library created by high-throughput combinatorial methods would enable the printing process to run much more quickly in the future and allow patient-specific modifications of implants. After optimizing characterization and scaling-up the design beyond prototypes, 3DP will become a highly viable processing method for tissue-engineering applications.

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CHAPTER 23

Biodegradable Polymers

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INTRODUCTION

The design and development of tissue-engineered products has benefited from many years of clinical utilization of a wide range of biodegradable polymers. Newly developed biodegradable polymers and modifications of previously developed biodegradable polymers have enhanced the tools available for creating clinically important tissue-engineering applications. Insights gained from studies of cell-matrix interactions, cell-cell signaling, and organization of cellular components, are placing increased demands on medical implants to interact with the patient's tissue in a more biologically appropriate fashion. While in the twentieth century, biocompatibility was largely equated with eliciting no harmful response, the biomaterials of the twenty first century will have to elicit tissue responses that support healing or regeneration of the patient's own tissue.

This chapter surveys the universe of those biodegradable polymers that may be useful in the development of medical implants and tissue-engineered products. Here we distinguish between biologically derived polymers and synthetic polymers. The materials are described in terms of their chemical composition, breakdown products, mechanism of breakdown, mechanical properties, and clinical limitations. Also discussed are product design considerations in processing of biomaterials into a final form (e.g., gel, membrane, matrix) that will effect the desired tissue response.

In this chapter, we follow the official polymer nomenclature conventions adopted by the International Union of Pure and Applied Chemistry (IUPAC). According to these rules, the correct naming of a polymer requires the simple addition of the prefix 'poly' in front of the monomer name, *if the monomer name consists of a single word*. For example, the polymer made of ethylene is simply polyethylene. There is no space between poly and ethylene. When the monomer name consists of two or more words, it is important that the entire monomer name be enclosed in parentheses. This is needed to avoid ambiguity. For example, the polymer made of lactic acid is correctly named as poly(lactic acid). Note the absence of a space between the prefix poly and the parenthesis. Polymers made of amino acids are sometimes referred to as peptides, proteins, or poly(amino acid)s. When referring to one polymer that is made of one type of amino acid, IUPAC rules require this material to be named as a poly(amino acid). When referring to several such polymers, the plural of poly(amino acid) is poly(amino acid)s. Note the placement of the plural 's' outside of the parenthesis. When a polymer is made of several different amino acids, then that polymer would be referred to as a poly(amino acids). Note the placement of the plural 's' inside the parenthesis, indicating that this is a single polymer that contains more than one type of amino acid as monomer. Unfortunately, these rules are often violated in the contemporary literature.

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BIODEGRADABLE POLYMER SELECTION CRITERIA

The selection of biomaterials plays a key role in the design and development of medical implants and tissue-engineering products. While the classical selection criterion for a safe, stable implant dictated choosing a passive, 'inert' material, it is now understood that any artificial material placed into the body of a patient will elicit a cellular response [1,2]. This means that there are in fact no artificial materials that are totally 'inert', and some of the chemically least reactive materials, such as polyethylene or polysiloxane, can cause significant inflammatory responses. Therefore, it is now widely accepted that a biomaterial must interact with tissue in a biologically appropriate manner rather than act simply as an inert body. Consequently, a major focus of biomaterials science centers around harnessing control over cellular interactions with biomaterials. Researchers now tend to explore ways to manipulate the cellular response by including biologically active components in the design of biomaterials. Specific examples of such biologically active components include protein growth factors, anti-inflammatory drugs, and gene delivery vectors [3].

It is important for the tissue-engineering product developer to have many biomaterials options available, as each application calls for a unique environment for cell-cell interactions. Such applications include:

1) Support for new tissue growth (wherein cell-cell communication and cells' access to nutrients, growth factors, and pharmaceutically active agents must be maximized);
2) Prevention of cellular activity (where tissue growth, such as in surgically induced adhesions, is undesirable);
3) Guided tissue response (enhancing a particular cellular response while inhibiting others);
4) Enhancement of cell attachment and subsequent cellular activation (e.g., fibroblast attachment, proliferation, and production of extracellular matrix for dermis repair) [4];
5) Inhibition of cellular attachment and/or activation (e.g., platelet attachment to a vascular graft);
6) Prevention of a biological response (e.g., blocking antibodies against grafted cells used in organ replacement therapies).

Biodegradable polymers are applicable to those tissue-engineering products in which tissue repair or remodeling is the goal, but not where long-term materials stability is required. Biodegradable polymers must also possess:

1) Manufacturing feasibility, including availability of sufficient commercial quantities of the bulk polymer;
2) The capability to form the polymer into the final product design;
3) Mechanical properties that adequately address short-term function and do not interfere with long-term function;
4) Low or negligible toxicity of degradation products, in terms of both local tissue response and systemic response;
5) The capability to be formulated as a drug delivery system in applications that call for prolonged release of pharmaceutically active compounds.

BIOLOGICALLY DERIVED POLYMERS

Biologically derived polymers are materials created by living organisms, as opposed to synthetic materials which are man-made. This distinction divides the universe of medically useful, biodegradable polymers into two large subgroups. However, the delineation between these groups is not always clear-cut. For example, glycolic acid is a natural metabolite and poly(glycolic acid) is naturally produced by many organisms. However, glycolic acid can also be created synthetically from oil-derived starting materials. Currently, poly(glycolic acid) is produced commercially by both fermentation and synthetic processes. Both production pathways result in the same final product, making poly(glycolic acid) either a biologically

derived polymer or a synthetic polymer. A similar situation exists in regard to polymers derived from hydroxybutyric acid and hydroxyvaleric acid. Commonly referred to as polyhydroxyalkanoates, these polymers can be derived from bacterial fermentation, as well as purely synthetic processes. In this chapter, polyhydroxyalkanoates are included among the biologically derived polymers (since the most prevalent mode of commercial production is based on bacterial fermentation), while poly(glycolic acid) and the closely related poly(lactic acid) are introduced to the reader as synthetic polymers (since the predominant mode of commercial production is currently based on oil-derived starting materials and synthetic polymerization reactions).

The biologically derived polymers can be further classified into peptides and proteins, polysaccharides, polyhydroxyalkanoates, and polynucleotides. Each of these subgroups will be discussed separately.

Peptides and proteins

Peptides and proteins are polymers derived from naturally occurring α-L-amino acids. Peptides are usually shorter chains of dozens of amino acids linked together via amide bonds, while proteins are longer chains of hundreds of individual amino acids. The amino acids are connected via hydrolytically stable amide bonds. Therefore, these materials are usually degraded via enzymatic mechanisms. The major shortcoming of peptides and proteins as starting materials for the fabrication of any medical implant is their lack of 'processibility'. This term refers to the ability of creating a shaped device by any of the conventional polymer processing methods used in the plastics industry: compression molding, extrusion, injection molding and fiber spinning. Another important limitation of peptides and proteins as biomaterials is their inherent immunogenicity. Any peptide or protein carries the risk of being recognized as foreign by the patient's immune system. For example, for many years, the safety of using bovine collagen was a hotly debated topic because of fears that the implantation of bovine collagen could provoke an immune response and predispose the patient to autoimmune diseases.

On the other hand, peptides and proteins can have outstanding biological properties and can help the tissue engineer in designing a polymer with desirable biological activities. This fact has been the driving force behind the long-standing interest in using peptides and proteins as starting materials for medical implants or tissue-engineering products. Unfortunately, most peptides and proteins have mechanical properties that are not conducive for their use in medical implants, resulting in the utilization of only a very small number of proteins as biomaterials (Table 23.1).

COLLAGEN

Collagen is the major component of mammalian connective tissue, accounting for approximately 30% of all protein in the human body. It is found in every major tissue that requires strength and flexibility. Fourteen types of collagens have been identified, the most abundant being type I [5]. Because of its abundance and its unique physical and biological properties, type I collagen has been used extensively in the formulation of biomedical materials [6]. Type I collagen is found in high concentrations in tendon, skin, bone and fascia, which are consequently convenient and abundant sources for isolation of this natural polymer.

The structure, function, and synthesis of type I collagen has been thoroughly investigated [7]. Because of its phylogenetically well-conserved primary sequence and its helical structure, collagen is only mildly immunoreactive [8]. However, many human recipients of medical or cosmetic products containing bovine collagen have anti-bovine collagen antibodies. The clinical significance of this finding is not yet fully understood.

TABLE 23.1 Proteins used as degradable biomaterials in medical implants

Type of protein	Source	Function
Collagen	Isolated from cattle, fish, and other species	Key component of tissue architecture, provides mechanical strength, supports cell attachment and growth, provides a biocompatible matrix for cell transplantation. Used extensively as a tissue expander and bulking agent in cosmetic products
Gelatin	Partially hydrolized collagen	Used in food industry, widely explored by researchers as a matrix for three-dimensional cell culture and as a component of tissue-engineering scaffolds
Elastin	Isolated from elastic tissues of cattle and birds	Key component of tissue architecture, provides elasticity to tissues
Keratin	Isolated from skin, hair and nails of cattle and birds	Key structural component of outer skin, hair and nails. Used as a matrix for cell growth and as a component in wound dressings and skin care products
Silk	Isolated from insect larvae	Used in the textile industry because of its extraordinaty strength. Also studied as a component of tissue-engineering scaffolds and as a cell culture substrate
Proteoglycans	Various tissue extracts	Used in research of cell-matrix interactions, matrix-matrix interactions, cell proliferation, cell migration

TABLE 23.2 Widely investigated polysaccharides

Type of polysaccharide	Source	Function
Cellulose	Cell wall of green plants	Main structural component of plants which keeps the stems, stalks and trunks rigid
Starch (Amylose and amylopectin)	Present in all staple foods	Important in plant energy storage
Alginate	Found in the cell walls of bacteria	Protects bacteria from engulfment by predatory protozoa or white blood cells (phagocytes)
Glycosaaminoglycans	Widely distributed	Cell-matrix interactions, matrix-matrix interactions, cell proliferation, cell migration
Chitin/chitosan	Major component of the exoskelton of insects, shells of crustaceans, cell walls of fungi	Structural component

The individual collagen molecules will spontaneously polymerize to form strong fibers that form larger organized structures. Collagen exists in tissue in the form of collagen fibers, fibrils, and macroscopic bundles [9]. For example, tendon and ligaments are comprised mainly of oriented type I collagen fibrils, which are extensively crosslinked in the extracellular space.

In vitro, collagen crosslinking can be enhanced after isolation through a number of well-described physical or chemical techniques [10]. Increasing the intermolecular crosslinks:

1) Increases biodegradation time, by making collagen less susceptible to enzymatic degradation;
2) Decreases the capacity of collagen to absorb water;
3) Decreases its solubility; and
4) Increases the tensile strength of collagen fibers.

The free amines on lysine residues on collagen can be utilized for crosslinking, or can similarly be modified to link or sequester active agents. These simple chemical modifications provide a variety of processing possibilities and consequently the potential for a wide range of tissue-engineering applications using type I collagen.

It has long been recognized that substrate attachment sites are necessary for growth, differentiation, replication, and metabolic activity of most cell types in culture. Collagen and its integrin-binding domains (e.g., RGD sequences) assist in the maintenance of such attachment-dependent cell types in culture. For example, fibroblasts grown on collagen matrices appear to differentiate in ways that mimic *in vivo* cellular activity and to exhibit nearly identical morphology and metabolism [11]. Chondrocytes can also retain their phenotype and cellular activity when cultured on collagen [12]. Such results suggest that type I collagen can serve as tissue-regeneration scaffolds for any number of cellular constructs.

The recognition that collagen matrices could support new tissue growth was exploited to develop the original formulations of artificial extracellular matrices for dermal replacements [1,2,13–15]. Yannas and Burke were the first to show that the rational design and construction of an artificial dermis could lead to the synthesis of a dermis-like structure whose physical properties 'would resemble dermis more than they resembled scar' [15]. They created a collagen–chondroitin sulfate composite matrix with a well-described pore structure and crosslinking density that optimizes regrowth while minimizing scar formation [16]. The reported clinical evidence and its simplicity of concept make this device an important potential tool for the treatment of severely burned patients [17].

The advantageous properties of collagen for supporting tissue growth have been used in conjunction with the superior mechanical properties of synthetic biodegradable polymer systems to make hybrid tissue scaffolds for bone and cartilage [18,19]. These hybrid systems show good cell adhesion, interaction and proliferation compared to the synthetic polymer system alone. Collagen has also been used to improve cell interactions with electrospun nanofibers of poly(hydroxy acids) such as poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone) and their copolymers [18–22].

In recent years, the combination of collagen scaffolds with active biological entities, such as cells, growth factors, platelet rich plasma [23] and various autologous or allogenic cell types [24] have provided significant opportunities for researchers to achieve tendon and ligament regeneration.

GELATIN

Gelatin is commonly used for pharmaceutical and medical applications because of its enzymatic biodegradability and biocompatibility in physiological environments [25–27]. Of the two types, acidic and alkaline gelatin, the former has an isoelectric point similar to collagen. The isoelectric point depends on its extraction procedure from collagen, and variations in it allow gelatin to bind with either positively or negatively charged therapeutic agents. Based on this, the acidic gelatin, with an isoelectric point of 5.0, could be used as a carrier for basic proteins *in vivo*, while basic gelatin, with an isoelectric point of 9.0, could be used for the sustained release of acidic proteins under physiological conditions. The advantage of gelatin as a carrier for controlled drug release is that the therapeutic agent can be loaded into the gelatin matrix under mild conditions. Gelatin hydrogels have been used as controlled release devices for a variety of growth factors known to enhance bone formation. Yamada et al. successfully incorporated bFGF (basic fibroblast growth factor) into acidic gelatin hydrogels, which were implanted into a rabbit skull defect to allow for the localized release over 12 weeks [28,29].

ELASTIN

Elastin is an extracellular matrix protein and is most abundant in tissues where elasticity is of major importance, such as blood vessels (50% of dry weight), elastic ligaments (70% of dry weight), lungs (30% of dry weight) and skin (2–4% of dry weight) [30,31].

The incorporation of elastin into biomaterials was a major topic of research in the 1970s and 1980s. The basic structure-function-activity correlations of various elastin sequences were discovered by Urry and his associates [32]. It is important to note that elastin is not a single, well-defined molecule. Rather, the name 'elastin' is associated with a wide range of elastic peptide and protein sequences that exist in different lengths and with different compositions. A common feature of all elastin sequences is that they are rich in glycine, proline, and lysine. In humans, elastin is synthesized early in life. By age 40 (approximately) elastin biosynthesis in humans slows down to a trickle. The appearance of skin wrinkles and other aging processes are directly related to the loss of elastin biosynthesis. This fact has been exploited as a marketing gimmick by cosmetic companies, who add elastin to a wide range of anti-aging products. However, there is overwhelming evidence that externally applied elastin is not able to pass through the skin and is not able to slow the loss of elastin from aging tissues.

When elastin was used as a component in heart valve prosthetic devices, the deposition of calcium-rich precipitates was a significant problem. This process, often referred to as 'calcification', limited the utility of elastin-containing biomaterials in cardiovascular prosthetic implants [33]. This experience illustrates that the use of natural substances is not necessarily a guarantee for clinically successful device performance.

Another important point is that not all biologically derived polymers are good cell growth substrates. While collagen is highly cell adhesive, elastin tends to discourage cell attachment and growth. This has been a concern in the biomedical community, since many potential applications of elastin (for example in blood vessel and ligament regeneration) would require a highly cell adhesive surface. This challenge can be addressed using the tools of protein engineering to incorporate cell adhesive peptide sequences within the elastin structure [32].

Overall, elastin is a highly versatile biomaterial that continues to inspire the imagination and curiosity of biomedical engineers. Recently, the ability of elastin to self-assemble into large supramolecular structures was used to fabricate sponges, scaffolds, sheets and tubes from human tropoelastin [34]. These promising studies are still ongoing.

KERATIN

Keratin is the name for a family of structural proteins which are abundant in the outer layer of human skin, in hair, and in nails. Keratin is rich in the amino acid cysteine, and it has the ability to self-assemble into bundles of fibers. Within these fiber bundles, individual strands are further crosslinked through S-S (sulfur-sulfur) bonds involving the cysteine side chains. In this way, keratin forms particularly tough, insoluble structures that are among the strongest non-mineralized tissues found in nature. The only other non-mineralized tissue that resembles the toughness of keratin is chitin (the material found in the exoskeleton of insects and the outer shell of shellfish).

Since human hair is rich in keratin, this protein is added to many hair care products. However, as in the case of the misleading marketing claims made for elastin-containing anti-aging skin products, there is no evidence that externally applied keratin can penetrate into the hair structure. While keratin was mostly studied for use in cosmetic (hair care) products, the exploration of keratin's properties also led to the development of keratin-based biomaterials for use in biomedical applications. The unusual mechanical properties and strength of keratin and its ability to self-assemble were the driving force for these biomedical studies.

The history of keratin research illustrates another major challenge when using biologically derived polymers as biomaterials. Based on the animal source used, and depending on the exact extraction procedure employed, individual keratin preparations can differ significantly from each other. The variability and irreproducibility of individual preparations is a general feature of most biologically derived polymers. Early research, mostly by Yamauchi's laboratory, focused on the preparation of protein films from keratin extracted from wool and human hair. These pure protein films were brittle and weak. The researchers could not reproduce in the laboratory the outstanding strength of keratin formed *in vivo*. Only when glycerol was added as a plasticizer, could keratin films be obtained that were relatively strong and flexible [35]. Fujii et al. also demonstrated that hair keratins were useful for preparing protein films and described a rapid casting method [36]. This research also revealed the feasibility of incorporating bioactive molecules such as alkaline phosphatase into the keratin films for controlled release applications. The films, however, had poor strength and flexibility [36]. Recently, significant progress was made by researchers at Wake Forest University, where keratin extracted from human hair was used as a matrix for the regeneration of peripheral nerves. This is currently one of the most advanced applications of keratin as a biomaterial [37].

SILK

On a weight basis, natural silk fibers can be stronger than high-grade steel. However, the strength of natural silk fibers is difficult to reproduce in the laboratory once pure silk has been extracted from the cocoons of silkworm larvae or from spider's webs. Still, the unique mechanical properties of natural silk fibers have fascinated scientists for more than a century. Recent work has demonstrated the biocompatibility of various silk protein preparations [38]. Another very interesting fact is that the degradation rate of laboratory-made silk fibers can be tailored to be from months to years after implantation *in vivo*, based on the processing procedure employed during the material's formation [39]. Finally, the thermal stability of silk biomaterials allows processing over a wide range of temperatures up to about 250°C, as evidenced by the ability to autoclave silk biomaterials without loss of functional integrity. Min and colleagues investigated the potential of electrospun silk matrices for accelerating early stages of wound healing [40]. In another study, chitin was blended with silk fibroin to fabricate composite fibrous scaffolds for skin tissue engineering. The rationale of these experiments was to combine the good mechanical properties of silk fibers with the wound healing effects of chitin [41]. The chitin/silk fibroin blends were electrospun to form nanofibrous matrices and evaluated for initial cell attachment and spreading [42]. *In vitro*, increased adhesion of keratinocytes was observed on chitin/silk blend matrices compared to pure chitin matrices, but the significance of these results for wound healing *in vivo* has not yet been established.

Overall, the exploration of silk as a biomaterial has not yet produced any commercially available medical products. While silk is a fascinating material, the studies performed so far have not discovered truly compelling reasons for the use of silk as a medical implant material.

PROTEOGLYCANS

Proteoglycans (PGs) are a major component of the extracellular matrix (ECM). They consist of one or more glycosaminoglycan (GAG) chains that are attached, via a tetrasaccharide link, to serine residues within a core protein [43,44]. GAGs are long chains of repeating disaccharide units that are variably sulfated. There are four main classes of GAGs — hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS). PGs exhibit great structural diversity because each type of PG may contain different kinds of GAGs, different numbers and lengths of GAG chains, modifications in the repeating patterns of the disaccharides by a complex pattern of sulfate groups, and a different core protein structure. PGs can be present in monomeric form or can form very large aggregates. Both the core protein and the GAG chains of PGs play key roles in tissue remodeling, intracellular signaling, uptake of proteins, cell migration, and many other crucial functions in

native tissues [43,44]. The fact that PGs seem to be involved in so many crucial cell signaling pathways is the main reason for the intense interest of biomedical engineers in understanding their properties.

To replicate the biological functions of PGs in tissue scaffolds, PGs or their GAG chains have frequently been grafted to the polymers used in fabricating tissue scaffolds, or they have been grafted onto the surface of tissue-engineering scaffolds. Most notably, chondroitin sulfate GAGs have been used to create collagen–GAG hybrid materials which seem to be particularly effective in skin regeneration.

PGs are sometimes used alone or in combination with other matrix proteins such as fibrin, collagen, or chitosan to create hybrid materials [45–47]. The rationale for exploring such complex mixtures is usually an attempt to 'mix and match' biological properties with appropriate mechanical properties. For example, a mixture of PGs and extracellular matrix proteins are part of the widely used Matrigel™ scaffold. Among the various combinations, collagen–GAG scaffolds are often preferred for tissue-engineering applications, because collagen provides a very cell friendly matrix environment and the specific GAG used can affect (and regulate) cell behavior. Collagen–GAG hybrids can be readily sterilized using heat, and can be manufactured with a variety of pore structures and a wide range of degradation rates [48]. The first commercially used tissue-engineered product, an artificial skin scaffold, developed by Integra LifeSciences, a New Jersey company, is a collagen–GAG hybrid.

Biomimetic materials

Biomimetic materials are synthetic (man-made) materials that mimic natural materials or that follow a design motif derived from nature. In the previous section, a number of peptides and proteins were discussed. In general, peptides and proteins are isolated from natural sources and are therefore listed among the biologically derived polymers. However, significant research breakthroughs were made when scientists started to create mimics of natural polymers by semi-synthetic or totally synthetic means. An excellent example for this type of research approach is the work of Urry and coworkers, who used peptide synthesis methods to create artificial variants of elastin [49,50]. Using a combination of solid-phase peptide chemistry and genetically engineered bacteria, they synthesized several polymers which contained homologies of the elastin repeat sequences valine-proline-glycine-valine-glycine (VPGVG). These biomimetic polymers had better engineering properties than their natural equivalents, making it possible to create films and fibers that could then be further modified by crosslinking. The resultant films had intriguing mechanical responses, such as a reverse phase transition which results in contraction with increasing temperature [50], resembling the action of muscles. The exact transition temperature could be varied by variations in the polymer's amino acid composition [50]. Several medical applications are under consideration for this system, including musculoskeletal repair mechanisms, ophthalmic devices, and mechanical and/or electrically stimulated drug delivery.

Other investigators, notably Tirrell and Cappello, have combined techniques from molecular and fermentation biology to create novel protein-based biomaterials [51–54]. These researchers had the remarkably innovative idea of creating genetically-engineered microorganisms that would produce exactly those polymers that the researchers liked to study. In this way, completely new variations of biologically derived peptides and proteins could be prepared. These protein polymers were based on repeat oligomeric peptide units, which were controlled via the genetic information inserted into the producing bacteria. It has been shown that the mechanical properties and the biological activities of these protein polymers can be pre-programmed, suggesting a large number of potential biomedical applications [55].

Another approach to elicit an appropriate cellular response to a biomaterial is to graft active peptides to the surface of a biodegradable polymer. For example, peptides containing the RGD

sequence have been grafted to various biodegradable polymers to provide active cell-binding surfaces [56]. Similarly, Panitch et al. incorporated oligopeptides containing the REDV sequence to stimulate endothelial cell binding for vascular grafts [57].

Polysaccharides

Polysaccharides are polymers made of various sugar (saccharide) units. The most common monosaccharides are glucose and fructose. Sucrose is the chemical name of the widely used table sugar. Sucrose is a disaccharide composed of glucose and fructose. Scientists who are not experts in sugar chemistry are often unaware of the exquisite structural variability of these molecules. For example, the important human food, *starch*, and the structural polymer of all plants, *cellulose*, are both polymers of glucose. The only difference between starch and cellulose is the way in which the individual glucose units are linked together. Considering the many different isomeric variations possible for saccharides, and the different chemical bonds that can be used to link individual saccharide units together, the number of structurally different polysaccharides is extremely large. In fact, the chemistry of polysaccharides is as rich in diversity and variability as protein chemistry. It is therefore not surprising that various saccharides and polysaccharides play an important role in fine-tuning the responses of cells to their environment. As outlined above, proteoglycans (PGs) and glucosaminoglycans (GAGs) are critical in regulating key cell functions. In contrast, the industrially used polysaccharides (such as starch and cellulose) are polymers comprising exclusively various sugar (saccharide) units as monomers. These polysaccharides can be extremely large polymers containing millions of monomers, and they are mostly used in nature for cellular energy storage or as a structural material. As a general rule, most of the natural polysaccharides are not biodegradable when implanted in any mammalian species due to the lack of digestive enzymes. Therefore, without further chemical modification, most polysaccharides are not obvious material choices for use in biomedical applications.

CELLULOSE

Cellulose is the most abundant polymeric material in nature. In its most common form, it is a fibrous, tough, water-insoluble material that is mostly found in the cell walls of plants, mainly in stalks, stems or trunks. Cellulose is the major ingredient of wood. Cellulose is composed of D-glucose units that are linked together by β-(1→4) glycosidic bonds. In nature, cellulose is formed by a simple polymerization of glucose residues from a substrate such as UDP-glucose [58]. Cellulose possesses high strength in the wet state [59]. The major commercial applications of cellulose are in the paper, wood, and textile industries [60] where millions of tons of cellulose are processed annually worldwide.

The major limitation of cellulose as a biomaterial is that it is not biodegradable, due to the lack of digestive enzymes for cellulose in the human organism [61,62]. However, a number of cellulose derivatives, such as methylcellulose, hydroxypropylcellulose and carboxymethylcellulose are used as biomaterials, due to the useful material properties exhibited by these synthetically created derivatives [63].

Cellulose is not an obvious choice for biomedical applications and there is no scientific rationale to expect cellulose to have unique or useful properties as a cell growth substrate or tissue-regeneration scaffold. This, however, has not prevented a number of laboratories from exploring cellulose or cellulose derivatives as a drug delivery implant, as a barrier for the prevention of surgical adhesions, or as a scaffold in cartilage tissue engineering [64,65]. *In vivo* studies have been performed to assess the biocompatibility of a bacterial cellulose scaffold by subcutaneous implantation in rats. While there were no macroscopic or microscopic signs of inflammation around the implants, and no fibrotic capsule or giant cells were observed, the limited biodegradability of cellulose will most probably prevent the use of this material in most biomedical applications [65].

STARCH

Whereas cellulose is composed of D-glucose units that are linked together by β-(1→4) glycosidic bonds, starch is composed of D-glucose units that are linked together by α-(1→4) glycosidic bonds. This change makes starch digestible, allowing its use as a major human nutrient. The chemistry of starch is complicated by the fact that starch consists of linear and branched chains, referred to as amylose and amylopectin respectively [66]. The relative abundance of these two natural ingredients can significantly influence the material properties of starch. Consequently, literally hundreds of different starches exist, each with its own particular composition of amylose and amylopectin.

Starch can be totally water insoluble, or partially soluble at room temperature, depending on the proportions of amylose and amylopectin present. Water-soluble starches can be dispersed in water, forming clear solutions upon heating. Upon cooling, soluble starch forms a highly viscous solution at low concentrations and a stiff hydrogel at higher concentrations. This is the basis of the thickening action of soluble corn starch, which is used extensively in the food industry.

Although enzymes present in the human gut can digest starch, it is not readily biodegradable when implanted into human tissues. The cellular energy storage polymer in humans is glycogen and not starch. Consequently, starch is not an obvious choice for biomedical applications. However, some starch-based polymers are biodegradable in human tissues and biocompatible. Among many potential applications, starch-based polymers were used to prepare scaffolds for cartilage regeneration [67]. These scaffolds did not perform better than other types of scaffolds. Starch was also used for the design of implantable drug delivery systems. In spite of the research conducted by several laboratories worldwide, starch-based polymers are not likely to find major biomedical applications in the near future.

ALGINATE

Alginate is a natural anionic polysaccharide found in seaweed, which is composed of β-(1−4) linked D-mannuronic acid and α-L-guluronic acid units. Along its polymer chain, alginate has regions rich in sequential mannuronic acid units, guluronic acid units and regions in which both monomers are equally prevalent.

The most important use of alginate in biomedicine is as a cell-compatible hydrogel. Alginate can form strong hydrogels in the presence of divalent cations (such as Ca^{2+} or Ba^{2+}) that interact with the carboxylic groups present in the alginate backbone to form ionic crosslinks. In a typical experiment, a solution of alginate is added to cells suspended in physiologic buffer solution. This mixture is then dropped slowly into a solution of calcium chloride. As each drop of the cell suspension touches the calcium chloride solution, the alginate forms a hydrogel that encapsulates and captures the suspended cells. This process has been used *in vitro* to encapsulate human articular chondrocytes in the presence of recombinant human BMP-2 [68].

Alginate has a well-characterized structure, which allows for a range of comparative studies to be performed. Cells do not readily attach and grow on or within alginate hydrogels. This is a common feature of most unmodified polysaccharides. However, the carboxylic groups in its guluronic acid residues provide an easy handle for the chemical modification of alginate [69]. This feature makes it possible to attach biologically active ligands (such as the important RGD peptide) to the alginate backbone [69].

Alginate's major disadvantage is the difficulty of its isolation from contaminated seaweed, which leads to the presence of mitogenic, cytotoxic and apoptosis inducing impurities in the final processed material. Although such molecules can be removed by further purification steps, it is a time consuming and costly process [70].

GELLAN GUM

Gellan gum is another anionic polysaccharide, similar in utility profile to alginate. Gellan gum can be easily processed into transparent gels that are resistant to heat. Gellan gum is not cytotoxic [71]) and can be injected into tissues. It has been used *in vivo* in humans as an ocular drug delivery vehicle [4]. Gellan gum is still relatively unknown in the biomedical community and only a few studies have explored this material for tissue engineering [72]. Like alginate, gellan gum can be used for the encapsulation and *in vitro* culture of cells [71]. Gellan gum hydrogels were able to support the development of nasal chondrocytes, and injectable gellan gum hydrogels were efficient in the encapsulation and support of human articular chondrocytes, while also enabling active synthesis of ECM components.

GLYCOSAMINOGLYCANS

Glycosaminoglycans (GAGs), which consist of repeating disaccharide units in linear arrangement, usually include a uronic acid component (such as glucuronic acid) and a hexosamine component (such as N-acetyl-D-glucosamine). The predominant types of GAGs attached to naturally occurring core proteins of proteoglycans include chondroitin sulfate, dermatan sulfate, keratan sulfate, and heparan sulfate [73,74]. The GAGs are attached to the core protein by specific carbohydrate sequences containing three or four monosaccharides.

The largest GAG, hyaluronic acid (hyaluronan), is an anionic polysaccharide with repeating disaccharide units of N-acetylglucosamine and glucuronic acid, with many unbranched units ranging from 500 to several thousand. Hyaluronic acid can be isolated from natural sources (e.g., rooster combs) or via microbial fermentation [75]. Because of its water-binding capacity, dilute solutions of hyaluronic acid form viscous solutions.

Like collagen, hyaluronic acid can be easily chemically modified, for instance by esterification of the carboxyl moieties, which reduces its water solubility and increases its viscosity [75,76]. Hyaluronic acid can be crosslinked to form molecular weight complexes in the range 8 to 24 $\times$ 10^6 or to form an infinite molecular network (gel). In one method, hyaluronic acid is crosslinked using aldehydes and small proteins to form bonds between the C—OH groups of the polysaccharide and the amino or imino groups of the protein, thus yielding high molecular weight complexes [77]. Other crosslinking techniques include the use of vinyl sulfone, which reacts to form an infinite network through sulfonyl-bis-ethyl crosslinks [78]. The resultant infinite network gels can be formed into sheaths, membranes, tubes, sleeves, and particles of various shapes and sizes. No species variations have been found in the chemical and physical structure of hyaluronic acid. The fact that it is not antigenic, eliciting no inflammatory or foreign body reaction, makes it desirable as a biomaterial. Its main drawbacks in this respect are its residence time and the limited range of its mechanical properties.

Because of its relative ease of isolation and modification and its superior ability in forming solid structures, hyaluronic acid has become the preferred GAG in medical device development. It has been used as a viscoelastic during eye surgery since 1976, and has undergone clinical testing as a means of relieving arthritic joints [79]. In addition, gels and films made from hyaluronic acid have shown clinical utility in preventing formation of post-surgical adhesions [80—82].

The benzyl ester of hyaluronic acid, sold under the trade name HYAFF-11, has been studied for use in vascular grafts [83—88].

CHITOSAN

Chitosan is a biosynthetic polysaccharide that is the deacylated derivative of chitin. Chitin is a naturally occurring polysaccharide that can be extracted from crustacean exoskeletons or generated via fungal fermentation processes. Chitosan is a β-1,4-linked polymer of 2-amino-2-deoxy-D-glucose; thus it carries a positive charge from amine groups [89]. It is hypothesized

that the major path for chitin and chitosan breakdown *in vivo* is through lysozyme, which acts slowly to depolymerize the polysaccharide [90]. The biodegradation rate of the polymer is determined by the amount of residual acetyl content, a parameter that can easily be varied. Chemical modification of chitosan produces materials with a variety of physical and mechanical properties [91–93]. For example, chitosan films and fibers can be formed utilizing crosslinking chemistries adapted from techniques for altering other polysaccharides, such as treatment of amylose with epichlorohydrin [94]. Like hyaluronic acid, chitosan is not antigenic and is a well-tolerated implanted material [95].

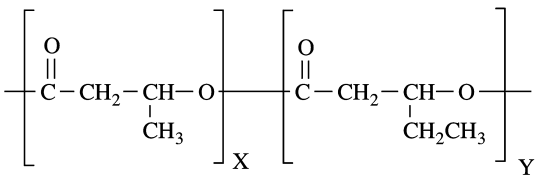
Chitosan has been formed into membranes and matrices suitable for several tissue-engineering applications [96–99] as well as conduits for guided nerve regeneration [100,101]. Chitosan matrix manipulation can be accomplished using the inherent electrostatic properties of the molecule. At low ionic strength, the chitosan chains are extended via the electrostatic interaction between amine groups, whereupon orientation occurs. As ionic strength is increased, and chain-chain spacing diminishes; the consequent increases in the junction zone and stiffness of the matrix result in increased average pore size. Chitosan gels, powders, films, and fibers have been formed and tested for such applications as encapsulation, membrane barriers, contact lens materials, cell culture, and inhibitors of blood coagulation [102].

Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a group of copolymers of hydroxybutyric acid and hydroxyvaleric acid. These linear polyesters are intracellular energy storage polymers whose function is to provide a reserve of carbon and energy [103] in certain microorganisms. These polyesters are slowly biodegradable, biocompatible, thermoplastic materials [104,105]. Depending on growth conditions, bacterial strain, and carbon source, the molecular weights of these polyesters can range from tens into the hundreds of thousands. Although the structures of PHA can contain a variety of *n*-alkyl side chain substituents (see Structure 23.1), the most extensively studied PHA is the simplest: poly(3-hydroxyburtyrate) (PHB).

ICI developed a biosynthetic process for the manufacture of PHB, based on the fermentation of sugars by the bacterium *Alcaligenes eutrophus*. PHB homopolymer, like all other PHA homopolymers, is highly crystalline, extremely brittle, and relatively hydrophobic. Consequently, the PHA homopolymers have degradation times *in vivo* on the order of years [106,104]. The copolymers of PHB with hydroxyvaleric acid are less crystalline, more flexible, and more readily processable, but suffer from the same disadvantage of being too hydrolytically stable to be useful in short-term applications, in which resorption of the degradable polymer within less than one year is desirable.

PHB and its copolymers with up to 30% of 3-hydroxyvaleric acid are now commercially available under the trade name Biopol, and are mostly used as environmentally friendly polymers that degrade slowly when disposed of in a landfill. It was previously found that a PHA copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate, with a 3-hydroxyvalerate content of about 11%, may have an optimum balance of strength and toughness for a wide range of possible applications. PHB has been found to have low toxicity, in part due to the fact that it degrades *in vivo* to D-3-hydroxybutyric acid, a normal constituent of human blood. Applications of these previously tested polymers, and others now under development, include



hydroxybutyric acid (HB) hydroxyvaleric acid (HV)

STRUCTURE 23.1
Poly(b-hydroxybutyrate) and copolymers with hydroxyvaleric acid. For a homopolymer of HB, Y = 0; commonly used copolymer ratios are 7, 11, or 22 mole percent of hydroxyvaleric acid.

controlled drug release, artificial skin, and heart valves, along with industrial applications such as paramedical disposables [107–109]. Among the biomedical applications, sutures are the main product where polyhydroxyalkanonates are used, although a number of clinical trials for other applications may still be ongoing [110].

Polynucleotides

Gene delivery from the surfaces of tissue scaffolds represents a new approach to manipulating the local environment of cells [111]. Gene therapy approaches can be employed to increase the expression of tissue inductive factors or block the expression of factors that would inhibit tissue formation [112]. A biomaterial can enhance gene transfer by localized expression of the genetic material and by protecting the genetic material against degradation by nucleases and proteases. Sustained delivery of DNA from a polymer matrix may transfect large numbers of cells at a localized site and lead to the production of a therapeutic protein that could enhance tissue development. The work by Yao [113] serves as a specific example for this approach: Yao investigated the potential of chitosan/collagen scaffolds with pEGFP-TGFβ1 as a gene vector candidate in cartilage tissue engineering.

SYNTHETIC POLYMERS

The concept of a 'polymer' evolved from the study and commercial development of biologically derived macromolecules such as cellulose derivatives (celluloid, cellulose acetate) and vulcanized rubber in the nineteenth century. In 1907, the first totally synthetic polymer, Bakelite, was invented. At that time, the macromolecular structure of Bakelite and all other polymers was still not understood. It was only in 1922 that Hermann Staudinger proposed that the properties of polymers can be best explained by assuming that they consist of long chains of monomers linked together via regularly repeating bonds. The shortage of natural materials (in particular rubber) during World War II was the driving force behind the development of totally synthetic polymers. During World War II, Nylon, Teflon, various polyesters, and synthetic rubber emerged. The tremendous improvements in virtually all consumer products since 1945 would not have been possible without the development of hundreds of specialized polymers.

World War II left an additional legacy: the medical needs of millions of injured war fighters across the globe stimulated the development of new surgical procedures and innovative medical devices. Often, advances in the material sciences produced and enabled commensurate advances in medical practice. A new polymer, referred to as 'Vinyon N', was developed during World War II for use in parachutes. In 1952, vascular surgeons noticed the stretchiness and elasticity of this material and developed the first vascular graft using Vinyon N. This effort became the starting point for the development of polymer-based medical implants and devices.

The systematic development of polymers for medical implants started with a focus on inert, biostable materials to be used in implants that lasted for the lifetime of the patient. It was only in 1969 that the first biodegradable polymer, poly(glycolic acid), was used to create the first, synthetic degradable suture line – a breakthrough that ended the use of suture lines made from the intestines of animals. Due to the efforts of many research groups, a number of different polymeric structures and compositions have been explored as degradable biomaterials. However, commercial efforts to develop these new materials for specific medical applications have been limited. Thus, detailed toxicological studies *in vivo*, investigations of degradation rate and mechanism and careful evaluations of the physicomechanical properties have so far been published for only a very small fraction of those polymers. This leaves the tissue engineer with just a relatively limited number of promising polymeric compositions to choose from. The following section is focused on a review of the most commonly investigated classes of biodegradable, synthetic polymers.

Aliphatic polyesters

Aliphatic polyesters made of hydroxy acids, such as glycolic acid, lactic acid, and ε-hydroxycaproic acid, have been utilized for a variety of medical product applications. As an example, bioresorbable surgical sutures made from poly(α-hydroxy acids) have been in clinical use since 1969 [114–116]. Other implantable devices made from these versatile polymers (e.g., internal fixation devices for orthopedic repair) are now part of standard surgical protocol [56,117,118].

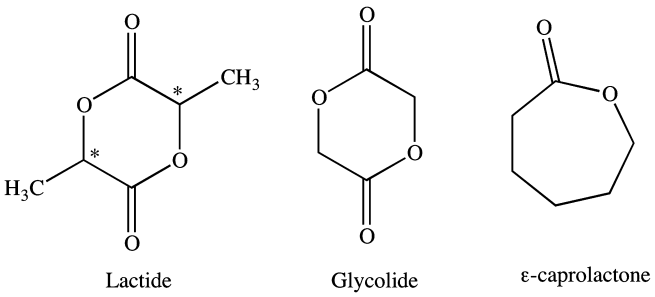
Although polyesters can be synthesized by polycondensation of hydroxy acids such as lactic acid [119], it is difficult to achieve high molecular weights and control the molecular weight, molecular weight distribution, and architecture of the polymer in this process. In most cases, biodegradable polyesters are synthesized in a two-step procedure: first, the hydroxy acids are transformed into intramolecular lactones (see Structure 23.2), which are then used as monomers in ring-opening polymerizations.

The ester bond of the aliphatic polyesters is cleaved by hydrolysis, which results in a decrease in the polymer molecular weight of the implant [120]. This initial degradation occurs until the molecular weight of the resulting oligomers is less than 5000 Da, at which point the oligomers become water soluble and the degrading implant starts to lose mass as well. The final degradation and resorption of the polyester implants may also involve inflammatory cells (such as macrophages, lymphocytes, and neutrophils). Although this late-stage inflammatory response can have a deleterious effect on some healing events, these polymers have been successfully employed as matrices for cell transplantation and tissue regeneration [121,122]. The useful lifetime of implants made from these polymers is determined by the initial molecular weight, exposed surface area, crystallinity, and (in the case of lactide-glycolide copolymers) by the ratio of the monomers.

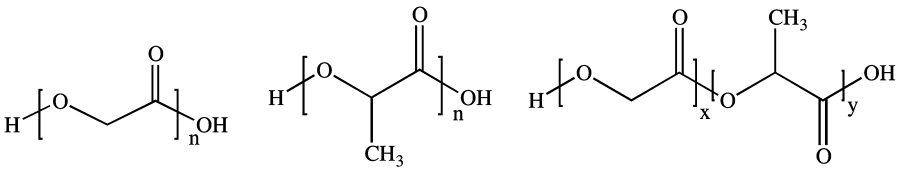
Aliphatic polyesters have a modest range of thermal and mechanical properties and a correspondingly modest range of processing conditions. The polymers can generally be formed into films, tubes, and matrices using such standard processing techniques as molding, extrusion, solvent casting, and spin casting. Ordered fibers, meshes, and open-cell foams have been formed to fulfill the surface area and cellular requirements of a variety of tissue engineering constructs [56,117,123]. The aliphatic polyesters have also been combined with other components, e.g., poly(ethylene glycol), to modify the cellular response elicited by the implant and its degradation products [124].

POLY(GLYCOLIC ACID), POLY(LACTIC ACID), AND THEIR COPOLYMERS

Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) are the most widely used synthetic degradable polymers in medicine (see Structure 23.3). Of this family of linear aliphatic polyesters, PGA has the simplest structure and is more hydrophilic than PLA. Since PGA is highly crystalline, it has a high melting point and low solubility in organic solvents. PGA was used in the development of the first totally synthetic absorbable suture [114]. The crystallinity of PGA in surgical sutures is typically in the range 46–52% [125]. Due to the hydrophilic nature and quick water uptake, surgical sutures

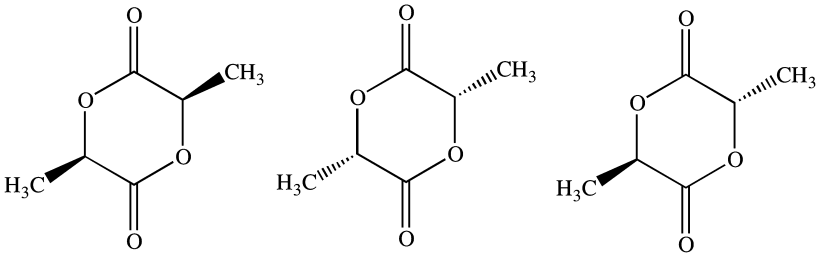


STRUCTURE 23.2
Cyclic esters used in ring-opening polymerizations. *Lactide has two optically active carbon atoms in the ring.



Poly(glycolic acid) Poly(lactic acid) Poly(glycolic acid-co-lactic acid)

STRUCTURE 23.3
Poly(glycolic acid), poly(lactic acid) and their copolymer.



D-lactide L-lactide meso-lactide

STRUCTURE 23.4
Lactide enantiomers: d-lactide, l-lactide and meso-lactide.

made of PGA lose their mechanical strength typically over a period of 2 to 4 weeks post implantation [126].

In order to adapt the materials properties of PGA to a wider range of possible applications, researchers undertook an intensive investigation of copolymers of PGA with the more hydrophobic poly(lactic acid) (PLA) (see Structure 23.3). Alternative sutures composed of copolymers of glycolic acid and lactic acid are currently marketed under the trade names Vicryl.

Due to the presence of an extra methyl group in lactic acid, PLA is more hydrophobic than PGA. The hydrophobicity of high molecular weight PLA limits the water uptake of thin films to about 2% [125] and results in a rate of backbone hydrolysis lower than that of PGA [126]. In addition, PLA is more soluble in organic solvents than PGA.

It is noteworthy that there is no linear relationship between the ratio of glycolic acid to lactic acid and the physicomechanical properties of their copolymers. Whereas PGA is highly crystalline, crystallinity is rapidly lost in PGA-PLA copolymers. These morphological changes lead to an increase in the rates of hydration and hydrolysis. Thus, copolymers tend to degrade more rapidly than either PGA or PLA [125,126].

Since lactic acid is a chiral molecule, it exists in two stereoisomeric forms that give rise to four morphologically distinct polymers. D-PLA and L-PLA are the two stereoregular polymers, D,L-PLA is the racemic polymer obtained from a mixture of D- and L-lactic acid, and meso-PLA can be obtained from D,L-lactide. The polymers derived from the optically active d and l monomers are semi-crystalline materials, while the optically inactive D,L-PLA is always amorphous.

The differences in the crystallinity of D,L-PLA and L-PLA have important practical ramifications: Since D,L-PLA is an amorphous polymer, it is usually considered for applications such as drug delivery, where it is important to have a homogeneous dispersion of the active species within a monophasic matrix. On the other hand, the semi-crystalline L-PLA is preferred in applications where high mechanical strength and toughness are required — for example, sutures and orthopedic devices [127–129].

L-PLA (PLLA) and D-PLA (PDLA) are semi-crystalline polymers with a glass transition temperature (Tg) of approximately 60°C, and a peak melting temperature (Tm) of approximately 180°C. D,L-PLA (PDLLA) is amorphous with a Tg of approximately 55°C. Both semi-crystalline PLLA and amorphous PDLLA polymers are rigid materials. Their Young's modulus

and stress at break values are close to 3.5 GPa and 65 MPa, respectively. However, these polymers are relatively brittle with an elongation at break of less than 6% [130–132].

These polymers degrade by hydrolysis in which naturally occurring lactic acid is formed. Degradation of the polymers starts with water uptake, followed by random cleavage of the ester bonds in the polymer chain. The degradation is throughout the bulk of the material [133]. Upon degradation, the number of carboxylic end groups increases, which leads to a decrease in pH and an autocatalytic acceleration of the rate of degradation [134]. During the degradation of semi-crystalline PLLA, crystallinity of the residual material increases as hydrolysis preferentially takes place in the amorphous domains [135]. In general, the rate of degradation and erosion of amorphous PDLLA is faster than that of PLLA [136].

Ikada et al. were the first to report on the stereocomplexation of enantiomeric PLLA and PDLA polymers during co-precipitation of mixed polymer solutions in non-solvents [222]. Since then, they have shown that stereocomplexation of PLLA and PDLA can take place in dilute and concentrated solutions, during solvent evaporation and spinning, and during annealing of their mixtures prepared in the melt [137]. Other groups have also reported the formation of poly(lactic acid) stereocomplexes [138,139].

Upon stereocomplexation, the melting temperature of the poly(lactide) stereocomplexes increases to about 230°C. This is 50°C higher than the melting temperature of the enantiomeric PLLA and PDLA polymers. Stereocomplexation influences the mechanical properties of poly(lactide) films [133]. When stereocomplexes were formed from PLLA and PDLA their films were stiffer, stronger, and tougher than films prepared from the enantiomeric polymers. In addition, under hydrolytic degradation conditions, the degradation of thin films of stereocomplexes of PLLA and PDLA is slower than that of the enantiomeric PLLA and PDLA homopolymers [140]. These findings are of great interest to biomedical engineers as they provide a simple way to increase the mechanical strength of these polymers with a concomitant increase in hydrolytic stability.

Recently, PLA, PGA, and their copolymers have been combined with bioactive ceramics, such as Bioglass particles or hydroxyapatite, that stimulate bone regeneration while greatly improving the mechanical strength of the composite material [141]. It was also reported that composites of polymer and Bioglass are angiogenic (e.g., they supported the growth of blood vessels), suggesting a novel approach for providing a vascular supply to implanted devices [142].

Some controversy surrounds the use of these materials for orthopedic applications. According to one review of the clinical outcomes for over 500 patients treated with resorbable pins made from either PGA or PGA:PLA copolymer, 1.2% required reoperation due to device failure, 1.7% suffered from bacterial infection of the operative wound, and 7.9% developed a late noninfectious inflammatory response that warranted operative drainage [143]. This delayed inflammatory reaction represents the most serious complication of the use of PGA or PLA in orthopedic applications. The mean interval between device implantation and the clinical manifestation of this reaction is 12 weeks for PGA and can be as long as three years for the more slowly degrading PLA [143]. Whether avoiding reoperation to remove a metal implant outweighs an approximately 8% risk of severe inflammatory reaction is a difficult question; in any event, an increasing number of trauma centers have suspended the use of these degradable fixation devices. It has been suggested that the release of acidic degradation products (glycolic acid for PGA, lactic acid for PLA, and glyoxylic acid for polydioxanone) contributes to the observed inflammatory reaction. Thus, the late inflammatory response appears to be a direct consequence of the chemical composition of the polymer degradation products [143]. The incorporation of alkaline salts or antibodies to inflammatory mediators may diminish the risk of a late inflammatory response [144]. A more desirable solution to these problems for orthopedic (and perhaps other) applications requires the development of new polymers that do not release acidic degradation products upon hydrolysis.

Using biodegradable PGA mesh scaffolds and a biomimetic perfusion system, Niklason et al. have successfully engineered small-diameter vessel grafts using either endothelial cells (ECs) and smooth muscle cells (SMCs) obtained from vessels in various species, or mesenchymal stem cells derived from adult human bone marrow [145,146]. In this approach, *ex vivo* culture resulted in the formation of a tissue-engineered vascular structure composed of cells and ECM while the PGA degraded during the same period. Cellular material was removed with detergents to render the grafts non-immunogenic. Tested in a dog model, grafts demonstrated excellent patency and resisted dilatation, calcification, and intimal hyperplasia [147].

POLY(ε-CAPROLACTONE)

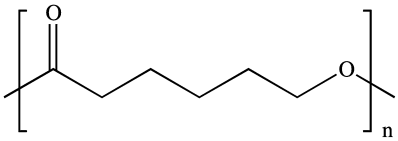
Poly(ε-caprolactone) (PCL) (Structure 23.5) is an aliphatic polyester that has been intensively investigated as a biomaterial. The discovery that PCL can be degraded by microorganisms led to evaluation of PCL as a biodegradable packaging material; later, it was discovered that PCL can also be degraded by a hydrolytic mechanism under physiological conditions [148–150]. Under certain circumstances, crosslinked PCL can be degraded enzymatically, leading to what can be called enzymatic surface erosion [148,149]. Low molecular weight fragments of PCL are reportedly taken up by macrophages and degraded intracellularly, with a tissue reaction similar to that of the other poly(hydroxy acids) [150]. Compared with PGA or PLA, the degradation of PCL is significantly slower. PCL is therefore most suitable for the design of long-term, implantable systems such as Capronor, a one-year implantable contraceptive device [151].

Poly(ε-caprolactone) exhibits several unusual properties not found among the other aliphatic polyesters. Most noteworthy are its exceptionally low glass transition temperature of about −60°C and its low melting temperature of 57°C. Another unusual property is its high thermal stability. Whereas other tested aliphatic polyesters had decomposition temperatures (Td) between 235 and 255°C, poly(ε-caprolactone) has a Td of 350°C, which is more typical of poly(ortho esters) than aliphatic polyesters [152].

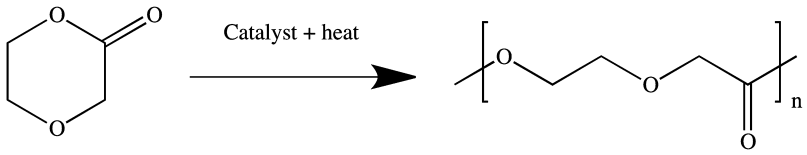
A useful property of PCL is its propensity to form compatible blends with a wide range of other polymers [153]. In addition, ε-caprolactone can be copolymerized with numerous other monomers (e.g., ethylene oxide, chloroprene, THF, δ-valerolactone, 4-vinylanisole, styrene, methyl methacrylate, vinylacetate). Particularly noteworthy are copolymers of ε-caprolactone and lactic acid, which have been studied extensively [149,154]. PCL and copolymers with PLA have been electronspun to create nanofibrous tissue-engineered scaffolds that show promise for vascular applicaions [155,20–22]. The toxicology of PCL has been extensively studied as part of the evaluation of Capronor. Based on a large number of tests, the monomer ε-caprolactone, and the polymer PCL, are currently regarded as non-toxic and tissue compatible materials. Early clinical studies [156] of the Capronor system were started around the year 2000 and resulted in a commercial implant used in Europe, but not in the USA.

POLY(P-DIOXANONE)

Poly(p-dioxanone) (PDS) is prepared by the ring-opening polymerization of p-dioxanone (Structure 23.6). It is a relatively weak, rapidly biodegrading polymer that was introduced into the market in the 1980s as a new degradable suture material. When compared on a weight basis (for example per 1 gram of implant), poly(p-dioxanone) releases degradation products



STRUCTURE 23.5
Poly(ε-caprolactone).



p-dioxanone polydioxanone (PDO)

STRUCTURE 23.6
p-dioxanone and polydioxanone.

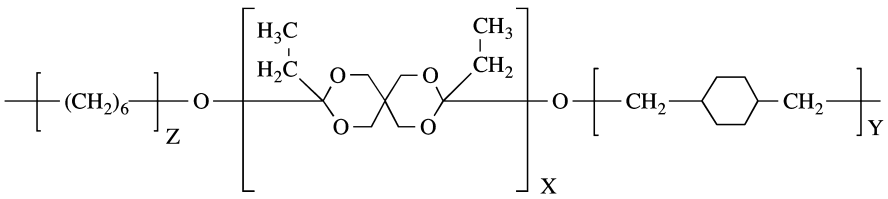
that are less acidic than the degradation products released by PGA or PLA. This is a potential advantage for orthopedic applications, which led to the development of small bone pins for the fixation of fractures in non-load bearing bones. Bone pins based on poly(p-dioxanone) are marketed under the trade name Orthosorb in the USA and under the name Ethipin in Europe. Poly(p-dioxanone) is also found in suture clips.

Unfortunately, poly(p-dioxanone) is relatively weak and lacks the stiffness and strength required for most orthopedic applications. Its range of applications is therefore limited. Academic laboratories have investigated poly(p-dioxanone) mostly as a polymer for implantable drug delivery applications. Little work has been done to explore this polymer as a scaffold for tissue engineering, probably because the polymer offers no clear advantages in terms of cell attachment and tissue compatibility over the widely used poly(lactic acid) and the copolymers of lactic and glycolic acid.

POLY(ORTHO ESTERS)

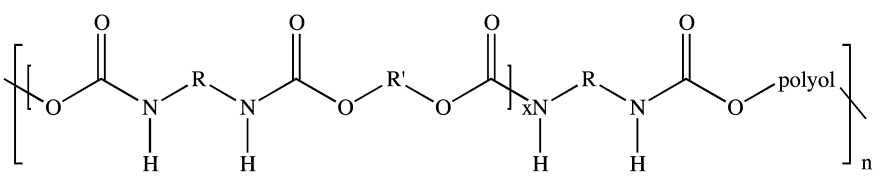
Poly(ortho esters) are a family of synthetic degradable polymers that have been under development since 1970 [157,158]. Devices made of poly(ortho esters) can be formulated in such a way that the device undergoes surface erosion — that is, the polymeric device degrades at its surface only and will thus tend to become thinner over time rather than crumbling into pieces. Since surface-eroding, slab-like devices tend to release drugs embedded within the polymer at a constant rate, poly(ortho esters) appear to be particularly useful for controlled release drug delivery [159]; this interest is reflected by the many descriptions of these applications in the literature [160].

There are two major types of poly(ortho esters). Originally, poly(ortho esters) were prepared by the condensation of 2,2-diethoxytetrahydrofuran and a dialcohol [161]) and marketed under the trade names Chronomer and Alzamer. Upon hydrolysis, these polymers release acidic by-products that autocatalyze the degradation process, resulting in degradation rates that increase with time. More recently, Heller et al. [162]) synthesized a new type of poly(ortho ester) based on the reaction of 3,9-bis(ethylidene 2,4,8,10-tetraoxaspiro {5,5} undecane)



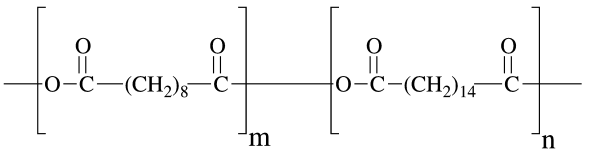
1,6-HD DETOSU t-CDM

STRUCTURE 23.7
Poly(orthoesters). The specific composition shown here is a terpolymer of hexadecanol (1,6-HD), trans-cyclohexyldimethanol (t-CDM), and DETOSU.

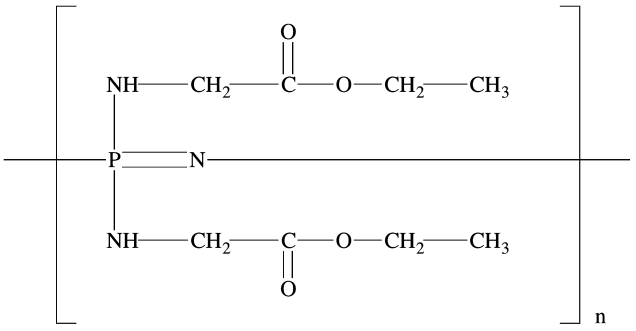


hard segment soft segment

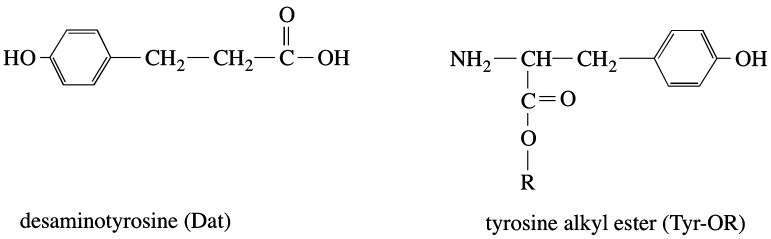
STRUCTURE 23.8
General structure of a segmented poly(urethane) prepared from diisocyanate, OCN-R-NCO; chain extender, HO-R'-OH; and polyol building blocks.

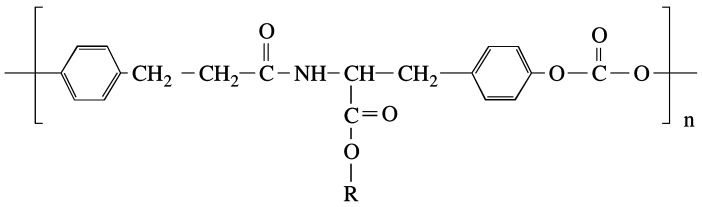


STRUCTURE 23.9
Poly(SA-HDA anhydride). This composition represents one of many polyanhydrides that were explored. The clinically relevant polyanhydrides are copolymers of sebacic acid and p-carboxy-phenoxypropane.



STRUCTURE 23.10
Polyphosphazene. Shown here is a polymer containing an amino acid ester attached to the phosphazene backbone.





STRUCTURE 23.11
A poly(amide carbonate) derived from desaminotyrosyl tyrosine alkyl esters. This is an example for a group of new, amino acid derived polymers.

(DETOSU) with various dialcohols (Structure 23.7). These poly(ortho esters) do not release acidic by-products upon hydrolysis and thus do not exhibit autocatalytically increasing degradation rates. By selecting diols having different degrees of chain flexibility, polymers can be obtained that range from hard, brittle materials to materials that have a gel-like consistency. A drug release system for mepivacaine (a treatment for post-operative pain), was tested in Phase 2 clinical trials between 2000 and 2005 [158], and as of 2012, a fourth generation, poly(ortho ester)-based drug delivery system for prevention of chemotherapy-induced nausea and vomiting was in Phase 3 clinical trials.

Biodegradable polyurethanes

Polyurethanes, polymers in which the repeating unit contains a urethane moiety, were first produced by Bayer in 1937. These polymers are typically produced through the reaction of a

diisocyanate with a polyol. Conventional polyols are polyethers or polyesters. The resulting polymers are segmented block copolymers with the polyol segment providing a low glass transition temperature (i.e., <25°C) soft segment and the diisocyanate component, often combined with a hydrocarbon chain extender, providing the hard segment (Structure 23.8). A wide range of physical and mechanical properties have been realized with commercial polyurethanes.

Polyurethanes have been used for nearly 50 years in biomedical applications, particularly as blood-contacting material in cardiovascular devices. Intended as non-biodegradable coatings, polyurethanes fell out of favor with the failure of pacemaker leads and breast implant coatings. Subsequent studies, as reviewed by Santerre et al., have clarified much about the behavior of polyurethanes in biological systems [163]. Elucidation of the biodegradation mechanism and its dependence on the polyurethane structure and composition have led to the development of biodegradable polyurethanes for a variety of tissue-engineering applications such as meniscal reconstruction [164], myocardial repair [165] and vascular tissues [166]. Design of biodegradable polyurethanes has required alternative diisocyanate compounds. The traditional aromatic diisocyanates are putative carcinogenic compounds. Biodegradable polymers are made from biocompatible diisocyanates such as lysine-diisocyanate or hexamethylene diisocyanate that release non-toxic degradation products.

The urethane bond is essentially non-degradable under physiological conditions. Therefore, biodegradable poly(urethane)s can only be obtained when the employed soft polyol segments are degradable [163]. As an example, aliphatic poly(ester urethane)s containing random 50/50 ε-caprolactone/L-lactide copolymer segments, 1,4-butanediol and 1,4-butanediisocyanate were synthesized and used to prepare porous structures for meniscus reconstruction [167]. In these polymers, the biodegradation mechanism involves the hydrolytic cleavage of the ester bonds in the ε-caprolactone/L-lactide copolymer segments, resulting in the formation of low molecular weight blocks of the non-degradable hard segments which are ultimately excreted from the body via the liver and/or kidney.

An interesting applicaton of polyurethanes was developed by Santerre et al. where fluoroquinolone antimicrobial drugs were incorporated into the polymer as hard-segment monomers [163]. This led to the design of drug polymers (trade name: Epidel) that release the drug when degraded by enzymes generated by an inflammatory response. This is an example of a 'smart' system in that antibacterial agents are released only while inflammation is present. Once healing occurs, the enzyme level drops and the release of drug diminishes.

Polyanhydrides (Structure 23.9)

Polyanhydrides were first investigated in detail by Hill and Carothers [168] and were considered in the 1950s for possible applications as textile fibers [169]. Their low hydrolytic stability, the major limitation for their industrial applications, was later recognized as a potential advantage by Langer et al. [170], who suggested the use of polyanhydrides as degradable biomaterials. A study of the synthesis of high molecular weight polyanhydrides has been published by Domb et al. [171].

A comprehensive evaluation of the toxicity of the polyanhydrides showed that, in general, they possess excellent *in vivo* biocompatibility [172]. Their most immediate applications are in the field of drug delivery, although tissue-engineering applications are also being developed. Drug loaded devices are best prepared by compression molding or microencapsulation [173]. A wide variety of drugs and proteins, including insulin, bovine growth factors, angiogenesis inhibitors (e.g., heparin and cortisone), enzymes (e.g., alkaline phosphatase and b-galactosidase) and anesthetics have been incorporated into polyanhydride matrices, and their *in vitro* and *in vivo* release characteristics have been evaluated [174]. One of the most aggressively-investigated uses of the polyanhydrides is for the delivery of chemotherapeutic agents. An example of this application is the delivery of BCNU (bis-chloroethylnitrosourea) to

the brain for the treatment of glioblastoma multiformae, a universally fatal brain cancer [175]. For this application, polyanhydrides derived from bis-p-(carboxyphenoxy propane) and sebacic acid received Food and Drug Administration (FDA) regulatory clearance in the fall of 1996 and are currently being marketed under the name Gliadel®.

Polyphosphazenes (Structure 23.10)

Polyphosphazenes consist of an inorganic phosphorous-nitrogen backbone, in contrast to the commonly employed hydrocarbon-based polymers [176a]. Consequently, the phosphazene backbone undergoes hydrolysis to phosphate and ammonium salts, with the concomitant release of the side group. Of the numerous polyphosphazenes that have been synthesized, those that have some potential use for medical products are substituted with amines of low pKa, and those with activated alcohol moieties [177–179]. Singh and colleagues have modified the side groups to tune polyphophazine properties such as glass transition temperature, degradation rate, surface wettability, tensile strength and elastic modulus, enabling these polymers to be considered for a wider range of biomedical applications [180]. The most extensively studied polyphosphazenes are hydrophobic, having fluoroalkoxy side groups. In part, these materials are of interest because of their expected minimal tissue interaction, which is similar to Teflon.

Aryloxyphosphazenes and closely related derivatives have also been extensively studied. One such polymer can be crosslinked with dissolved cations such as calcium to form a hydrogel matrix because of its polyelectrolytic nature [181]. Using methods similar to alginate encapsulation, microspheres of aryloxyphosphazene have been used to encapsulate hybridoma cells without affecting their viability or their capacity to produce antibodies. Interaction with poly(L-lysine) produced a semi-permeable membrane. Similar materials have been synthesized that show promise in blood contacting and with novel drug delivery applications.

Poly(amino acids) and 'pseudo'-poly(amino acids)

Since proteins are composed of amino acids, many researchers have tried to develop synthetic polymers derived from amino acids to serve as models for structural, biological and immunological studies. In addition, many different types of poly(amino acids) have been investigated for use in biomedical applications [176]. Poly(amino acids) are usually prepared by the ring-opening polymerization of the corresponding *N*-carboxy anhydrides, which are in turn obtained by reaction of the amino acid with phosgene [182].

Poly(amino acids) have several potential advantages as biomaterials. A large number of polymers and copolymers can be prepared from a variety of amino acids. The side chains offer sites for the attachment of small peptides, drugs, crosslinking agents, or pendant groups that can be used to modify the physicomechanical properties of the polymer. Since these polymers release naturally occurring amino acids as the primary products of polymer backbone cleavage, their degradation products may be expected to show a low level of systemic toxicity.

Poly(amino acids) have been investigated as suture materials [178], as artificial skin substitutes [183] and as drug delivery systems [184]. Various drugs have been attached to the side chains of poly(amino acids), usually via a spacer unit that distances the drug from the backbone. Combinations of poly(amino acid)s and drugs that have been investigated include poly(L-lysine) with methotrexate and pepstatin [185], and poly(glutamic acid) with adriamycin and norethindrone [186]. Short amino acid sequences such as RGD and RGDS, strong promotors of specific cell adhesion, have been linked to the polymer backbone to promote cell growth in tissue-engineering applications [187,188].

Despite their apparent potential as biomaterials, poly(amino acids) have actually found few practical applications. *N*-carboxy anhydrides, the starting materials, are expensive to make and difficult to handle because of their high reactivity and moisture sensitivity. Most

poly(amino acids) are highly insoluble and non-processible materials. Since poly(amino acids) degrade via enzymatic hydrolysis of the amide bond, it is difficult to reproduce and control their degradation *in vivo*, because the level of relevant enzymatic activity varies from person to person. Furthermore, the antigenicity of poly(amino acids) containing three or more amino acids limits their use in biomedical applications [176]. Because of these difficulties, only a few poly(amino acids), usually derivatives of poly(glutamic acid) carrying various pendent chains at the γ-carboxylic acid group, have been identified as promising implant materials [189].

As an alternative approach, Kohn et al. have replaced the peptide bonds in the backbone of synthetic poly(amino acids) by a variety of 'nonamide' linkages such as ester, iminocarbonate, urethane, and carbonate bonds [190,191]. The term *pseudo-poly(amino acid)* is used to denote this new family of polymers in which naturally occurring amino acids are linked together by nonamide bonds (Structure 23.11).

The use of such backbone-modified pseudo-poly(amino acids) as biomaterials was first suggested in 1984 [192]. The first pseudo-poly(amino acids) investigated were a polyester from *N*-protected trans-4-hydroxy-L-proline and a poly(iminocarbonate) from tyrosine dipeptide [193,194]. Several studies indicate that the backbone modification of conventional poly(amino acids) generally improves their physicomechanical properties [190,195,196]. This approach is applicable to, among other materials, serine, hydroxyproline, threonine, tyrosine, cysteine, glutamic acid, and lysine; it is only limited by the requirement that the nonamide backbone linkages give rise to polymers with desirable material properties. Additional pseudo-poly(amino acids) can be obtained by considering dipeptides as monomeric starting materials. Hydroxyproline-derived polyesters [194,197], serine-derived polyesters [198], and tyrosine-derived polyiminocarbonates [199] and polycarbonates [200] represent specific embodiments of these synthetic concepts.

COMBINATIONS (HYBRIDS) OF SYNTHETIC AND BIOLOGICALLY DERIVED POLYMERS

Biologically derived polymers have important advantages over synthetic materials. These advantages often include reduced toxicity, since they generally originate from molecules that occur naturally in the body and biodegrade under physiological conditions [201,202], and they also exhibit improved *bioactivity*. In this context, the term bioactivity refers to the ability of a material to elicit specific cellular responses. Collagen, for example, is a bioactive material because of its ability to support cell attachment, growth and differentiation. However, the usefulness of biologically derived polymers is often limited by their poor engineering properties. Important disadvantages of biologically derived polymers are:

1) High batch-to-batch variability due to complex isolation procedures from inconsistent sources,
2) Poor solubility and processibility, preventing the use of industrial manufacturing processes,
3) Risk of contamination by pyrogens or pathogens,
4) Poor or limited materials properties such as strength, ductility, elasticity, or shelf life, and
5) Their high cost.

In overall terms, synthetic materials offer a wider range of useful engineering properties (polymer composition, architecture, mechanical properties, etc.) but they generally fail to promote cell growth and differentiation to the same degree as some of the biologically derived polymers [203]. To address these shortcomings, it is an obvious idea to combine biologically derived polymers with synthetic materials. As a general rule, the purpose of such hybrid materials is to combine the bioactivity of biologically derived polymers with the superior engineering properties of synthetic materials.

As an example, a clinically useful cornea replacement material was obtained when collagen was incorporated into a synthetic, polyacrylate-based hydrogel [204]. Another example of this approach is provided by a hybrid material derived from silk fibroin (as the hydrophobic, bioactive material) and poly(vinyl alcohol) (as the hydrophilic, synthetic component) [205]. Poly(vinyl alcohol) (PVA) can be crosslinked under mild conditions by exposure to UV (ultraviolet) radiation. This crosslinking method allows PVA and fibroin to form a highly biocompatible hydrogel with adjustable engineering properties [205].

A final example of this approach is provided by a hybrid material composed of poly(N-isopropylacrylamide) (pNIPAM) as the synthetic component and various natural polysaccharides as the biologically derived component. pNIPAM undergoes a thermally induced phase transition around 32°C that causes the soluble material to precipitate. This property allows the design of a product that is liquid and injectable at room temperature and that precipitates and forms a hydrogel at body temperature, facilitating the formation of a solid implant while eliminating the need for surgical insertion. The disadvantage of pNIPAM as a biomaterial is its inability to support cell growth and its lack of biodegradability. This can be overcome by crosslinking pNIPAM with natural polysaccharide precursors, leading to cell-permissive formulations that will biodegrade *in vivo*.

USING POLYMERS TO CREATE TISSUE-ENGINEERED PRODUCTS

The medical implant of the future will not be an artificial prosthetic device that replaces tissue lost due to trauma, disease, or aging, but a tissue scaffold that integrates with the surrounding healthy tissue and assists in the regeneration of lost or damaged tissue. As one possible implementation of this concept, the biomedical literature currently envisions such tissue scaffolds as consisting of bioactive polymers that may be shaped as porous sponge-like structures. These tissue scaffolds may either be preseeded with specific cell populations outside of the body of the patient prior to implantation into the patient, or the tissue scaffolds may be implanted into the body of the patient first, and then be colonized by the patient's own cells, leading to the regeneration of the desired tissue. In either case, one can formulate several design criteria that can guide the biomedical engineer in the process of selecting an appropriate biomaterial for the intended application.

First and foremost, one must be concerned with the bulk polymer properties. The biomaterial must have an appropriate range of physicomechanical properties, biodegradation rates, and biocompatibility appropriate for the intended application. Next, it must be possible to create the desired tissue scaffold. This requires that the desired device shape and architecture can actually be created by cost-effective fabrication methods. For example, electrospinning may be the preferred fabrication method to create an ECM-like scaffold that consists of nano-sized fibers. Since electrospinning requires that the biomaterial is soluble in non-toxic solvents, any insoluble biomaterial would not meet a key design requirement. Finally, it is important to consider the biological properties of the biomaterial. Tissue-engineering products require specific cellular responses. For example, in a bone regeneration scaffold, the ability to support the attachment and growth of osteoblasts in combination with the ability to adsorb and concentrate endogenous bone morphogenic protein on the polymer surface may be important design requirements (Figure 23.1). There is a wide range of additional requirements that impose further limitations on the selection of the biomaterial and/or the architecture of the implant. For example, whenever functional tissue has to form within the tissue-engineering scaffold, a lack of proper vascularization will impede a favorable tissue response. For encouraging angiogenesis within the scaffold, details such as pore size, pore structure and pore connectivity are often critical [206].

Once an appropriately shaped tissue scaffold has been conceptionalized, it is time to consider a wide range of secondary design criteria relating to device shelf life, ability of the device to be

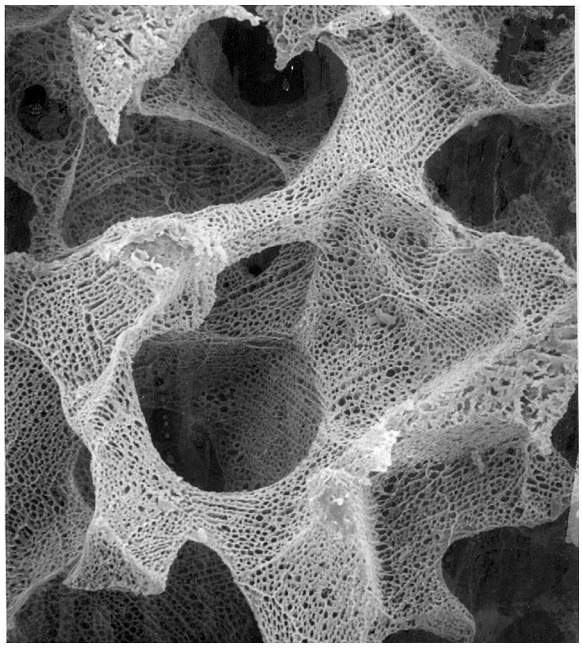


FIGURE 23.1
Bone regeneration scaffold from the New Jersey Center for Biomaterials. The scaffold has a delicate and carefully optimized pore structure consisting of large macropores (200—400 μm) and linearly aligned micropores (5—20 μm). This particular pore architecture is believed to facilitate the regeneration of bone *in vivo. (Photo: Courtesy of the Laboratory of Professor Joachim Kohn, Rutgers-the State University of New Jersey)*

sterilized and packaged for clinical use, and overall cost. Here, being able to create a terminally sterilized device is a factor often overlooked by academic researchers, who fail to consider the fact that most FDA-recognized sterilization methods cannot be applied to products that contain living cells. Thus, the requirement to ensure device sterility has been a major hurdle in translating research concepts from the laboratory to clinical practice.

Barriers: membranes and tubes

Design formats requiring cell activity on one surface of a device while precluding transverse movement of surrounding cells onto that surface calls for a barrier material. For example, peripheral nerve regeneration must allow for axonal growth, while at the same time precluding fibroblast activity that could produce neural-inhibiting connective tissue. Structures such as collagen tubes can be fabricated to yield a structure dense enough to inhibit connective tissue formation along the path of repair while allowing axonal growth through the lumen [207]. More recently, conduits composed of a silk fibroin scaffold coupled with biologics have shown improvement in performance [208]. Similarly, collagen membranes for periodontal repair provide an environment for periodontal ligament regrowth and attachment while preventing epithelial ingrowth into the healing site [209]. Anti-adhesion formulations using hyaluronic acid, which prevent ingrowth of connective tissue at a surgically repaired site, also work in this way [80].

Gels

Gels are used to provide a hydrogel scaffold, encapsulate, or provide a specialized environment for isolated cells. For example, collagen gels for tissue engineering were first used to maintain fibroblasts, which were the basis of a living skin equivalent [210]. Gels have also been used for the maintenance and immunoprotection of xenograft and homograft cells such as hepatocytes, chondrocytes, and islets of Langerhans used for transplantation [211—213]. Semipermeable gels have been created to limit cell-cell communication and interaction with surrounding tissue, and to minimize movement of peptide factors and nutrients through the implant. Injectable biodegradable gel materials that form through crosslinking *in situ* show promise for regeneration of bone and cartilage. Temenoff and Mikos review a number of injectable systems that demonstrate appropriate properties for these applications [214].

Lee and colleagues discuss the use of biodegradable polyester dendrimers (highly branched, synthetic polymers with layered architectures) to form hydrogels for tissue-engineering applications such as corneal wound sealants [215]. In general, non-degradable materials are used for cell encapsulation to maximize long-term stability of the implant. In the future, however, it may be possible to formulate novel 'smart' gels in which biodegradation is triggered by a specific cellular response instead of simple hydrolysis.

Matrices

It has been recognized since the mid-1970s that three-dimensional structures are an important component of engineered tissue development [13,14]. Yannas and his coworkers were the first to show that pore size, pore orientation, and fiber structure are important characteristics in the design of cell scaffolds. Several techniques have subsequently been developed to form well-defined matrices from synthetic and biologically derived polymers, and the physical characteristics of these matrices are routinely varied to maximize cellular and tissue responses [216–219]. Examples of engineered matrices that have led to several resorbable templates are oriented pore structures designed for regeneration of trabecular bone [220,221].

CONCLUSION

Research using currently available biomaterials and research aimed at developing novel biodegradable polymers has helped to advance the field of tissue engineering. Throughout most of the twentieth century, most research and development efforts relied on a small number of biodegradable polymers that had a history of regulatory approval, making poly(lactic acid) the most widely used biodegradable polymer. Research in the twenty first century aims to develop advanced biodegradable polymers that elicit predictable and useful cellular responses. To achieve this goal, current research efforts focus on creating bioactive materials that combine the superior engineering properties of synthetic polymers with the superior biological properties of natural materials. The extremely complex material requirements of tissue scaffolds pose significant challenges and require a continued research effort toward the development of new bioresorbable polymers.

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CHAPTER 24

3D Scaffolds

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INTRODUCTION

A key concept in tissue engineering (TE) is using material-based porous three-dimensional (3D) scaffolds to provide physical support and local environment for cells to enable and facilitate tissue development [1]. Over the past two decades, 3D scaffolds have evolved to serve complex functions to guide cellular behavior with spatial and temporal controls of the material and structural properties for different TE applications. Scaffolds can be seeded with embryonic or adult stem cells, progenitor cells, mature differentiated cells, or co-cultures of cells to induce tissue formation *in vitro* and *in vivo*; scaffolds can also be directly implanted *in vivo* with capabilities to deliver soluble/insoluble biomolecules and temporal/spatial cues to guide functional regeneration in defective tissues and organs. While the specific functions vary with tissue type and clinical need, scaffolds may potentially coordinate biological events at the molecular, cellular and tissue levels, on time and dimension scales ranging from seconds to weeks and nanometers to centimeters, respectively [2]. A central task in designing tissue engineering scaffolds is to understand the correlations between scaffold properties and biological functions.

During tissue development, morphogenetic factors are present in the extracellular environment in a dynamic way. In response, cells make decisions on how to divide, differentiate, migrate, degrade/produce extracellular matrix (ECM) and orient themselves. Based on our understanding of cell-ECM interactions in native tissues, it should theoretically be possible to design scaffolds with the capacity to modulate tissue formation via logical, systematic variations in one or more chemical and/or physical properties. Living cells and tissues, however, are not simple, linear systems. The roles that ECM structures play in orchestrating the biological events are cell-specific and kinetically complex. Moreover, the performance of any scaffold property must be evaluated, not only in the relatively controlled *in vitro* environment, but also in the context of the tissue's physiological function (e.g., under mechanical or electrical stimulation), and ultimately *in vivo*. The dynamic superposition of multiple scaffold properties with multiple environmental factors can profoundly impact the outcome of the tissue formation process. Correlations between individual scaffold properties and certain

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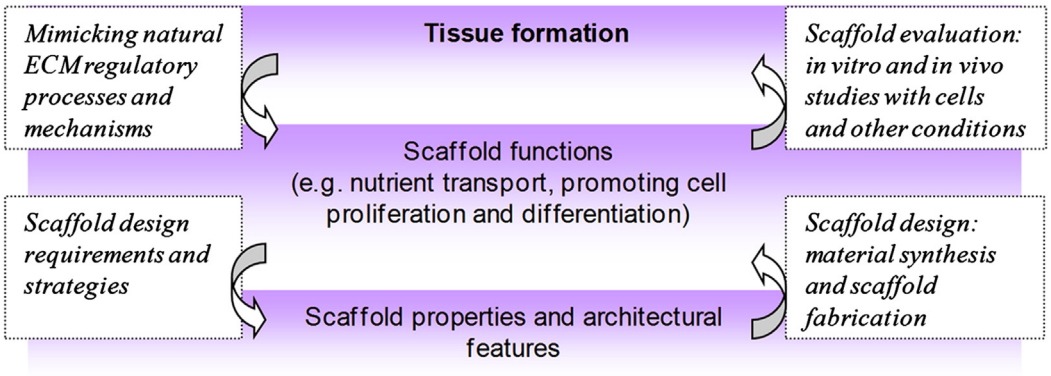

FIGURE 24.1

Scaffold synthesis consists of processes to understand the design principles and develop enabling methodologies. On one hand, scaffold design is a deductive process entailing the proposition and testing of candidate scaffold properties, and the cumulative elucidation of their independent and coupled reactions with cells and tissues. On the other hand, scaffold design is an inductive process, an exercise in recapitulating the wealth of tissue functions and cellular mechanisms observed throughout the body.

aspects of tissue formation (e.g., cellular phenotype and concomitant proliferation or ECM production) can be derived, and have even been applied to the optimization of scaffold designs [3]. From an engineering stand-point, it is desirable that scaffold properties and their regulatory functions be reproducibly integrated within a scaffold design, such that tissue formation can be induced and controlled in a stepwise manner *in vitro* and/or at the site of implantation *in vivo* [4,5] (Fig. 24.1).

Owing to the innate complexity of biological systems, reproducing natural processes and engineering tissues with the aid of scaffolds is a formidable task. Complications can arise on many fronts, such as from our incomplete understanding of native cell-ECM interactions, the difficulty of analyzing and standardizing biological systems, and even from our increasing technical capabilities of synthesizing scaffolds, which may outstrip our understanding of how to best apply them. More questions may be raised — such as to what degree of complexity a tissue-engineering scaffold should be designed, how to coordinate different types of cellular and tissue events, and how scaffolds can be designed to function robustly in response to patient-specific variations and different clinical situations. In this chapter, we summarize current scaffold design approaches and essential principles that may guide the rational multifunctional design of scaffolds for tissue engineering. Design variables are decoupled and analyzed in terms of how they have been controlled using different materials and fabrication techniques, and what implications they have in controlling the tissue formation process.

3D SCAFFOLD DESIGN AND ENGINEERING

From the perspective of material properties, mass transport, mechanics, electrical conductivity, surface chemistry and topology all mediate cell behavior at the nano, micro and macroscopic scales. These properties are often correlated with scaffold chemical compositions and structures in an interdependent way. For example, while a higher porosity may result in a beneficial increase in the mass transport capabilities of a scaffold, it may also compromise the scaffold's mechanical strength. Achieving optimal scaffold functional performance for a particular tissue-engineering application, therefore, requires balancing different factors.

An understanding of the fundamental scaffold properties and functions must form the foundation of any higher order hierarchal scaffold design involving temporal and/or spatial controls. Temporally, the scaffold properties at the onset of *in vitro* seeding (i.e., time zero) or during the acute phase of the host response following direct implantation *in vivo*, will provide an

initial set of environmental conditions for cells and tissues. These initial scaffold properties and associated functions will then evolve over time in response to passive and active interactions with the culture environment and cells, respectively. Temporal features, mainly enabled by scaffold degradation and the release of bioactive factors, are obligatory for presenting time-appropriate signals corresponding with each particular stage of tissue development. Tissues and organs are 3D structures having a characteristic organization consisting of patterns of different types of cells and ECM constituents. Spatially, anisotropic biochemical and structural properties can be incorporated into scaffolds to induce specific cell-cell interactions, cell and ECM orientations, and to guide multi-scale interactions up to the gross tissue or organ level.

Mass transport and pore architectures

The mass transport characteristics of a scaffold affect how fluid, solutes and cells move in and out of a tissue construct. As a porous 3D substrate, the mass transport in a scaffold at the initial stage is correlated with the scaffold chemical compositions and the structural parameters of its pores or voids, characterized mainly by pore size, porosity, pore interconnectivity/tortuosity, and surface area. These scaffold characteristics interdependently affect the diffusive or convective behavior of soluble nutrients, growth factors and cytokines. In addition to cell functions, the local biochemical and cellular environment can also influence the scaffold behavior [e.g., pH dependence of the hydrolysis of poly(lactic-co-glycolic acid) (PLGA) scaffolds]. In scaffolds designed to deliver bioactive soluble factors, the release profile and distribution pattern of these factors will be influenced by the mass transport characteristics of the scaffold. Compared to the vascularized tissues containing convective flows, diffusion is often the primary mass transport mechanism in engineered tissue constructs cultivated *in vitro* and prior to inosculation with the host vasculature following implantation *in vivo*. Theoretical models to simulate and predict the nutrient, protein and cell diffusion processes in scaffolds have been developed [6,7].

Once a scaffold is impregnated with cells, the solute diffusion and distribution profiles are mainly influenced by the cellular environment. In metabolically active tissues *in vivo*, most cells reside within 100 μm of a capillary. In engineered tissue constructs cultivated in static culture without medium perfusion, neo-tissue formation is generally limited to the peripheral 100–200 μm of the scaffold due to diffusion limitations [8–10]. Subsequently, the organization and density of cells in this peripheral region can further influence the distribution and availability of nutrients to cells within the scaffold interior [6]. Although certain types of cells may tolerate nutrient deficiency to some extent (such as chondrocytes under hypoxia), the induction of rapid vascularization following implantation is considered essential to establishing nutrient exchange and to the retention of a viable cell population within an engineered tissue construct. The structural characteristics of the pores and void spaces in scaffolds are the primary variables governing the initial cell distribution and organization in scaffolds.

One main parameter that affects the efficiency of initial cell impregnation is the pore size of the scaffold. The resulting geometries and spatial characteristics of individual cells or cell aggregates within the void space of scaffolds direct the subsequent cell proliferation, differentiation and tissue formation events. As observed in non-woven fibrous scaffolds made of poly(ethylene terephthalate) (PET) and seeded with human placenta trophoblast cells *in vitro*, the size of cell aggregates increased with larger pore volumes between fibers [11]. Variations in cell behavior were also observed, with attenuated differentiation and suppressed proliferation activities in larger cell aggregates compared to smaller ones [11,12]. Optimal scaffold pore sizes have been suggested for regenerating various types of tissues *in vivo*. The critical pore size was found to be above 500 μm for rapid fibro-vascularization in poly(L-lactic acid) (PLLA) scaffolds with cylindrical pores [13]. In ECM analog scaffolds (made of crosslinked collagen and glycosaminoglycans [GAG]), the average pore diameters required to induce dermis and peripheral nerve regeneration were 20–120 μm and 5–10 μm, respectively [14]. In bone engineering,

early studies by Hulbert and coworkers showed that a minimum pore size of 100 μm was required to allow bone tissue ingrowth in ceramic scaffolds [15]. Further investigations were carried out to understand the pore size requirement for bone tissue engineering. Although the optimal pore size varied with scaffold material and other parameters such as tortuosity, the general consensus is that larger pore sizes (e.g., >100 μm) may favor higher alkaline phosphatase activity and more bone formation [16,17].

Cell transport and vascularization as a result of scaffold pore size can also affect the tissue types and tissue formation process in scaffolds. When bone morphogenetic proteins were loaded into honeycomb-shaped hydroxyapatite scaffolds to induce osteogenesis, it was found that smaller diameters (90–120 μm) induced cartilage followed by bone formation, whereas those with larger diameters (350 μm) induced bone formation directly [18]. The difference was likely caused by the different onset time of vascularization and cell differentiation.

In addition to pore size, cell transport parameters such as diffusion, attachment, and migration are also affected by porosity (the fraction of pore volume), pore interconnectivity, and available surface area in scaffolds. While a high porosity is often desired, it is inversely related to the surface area available for cell attachment in 3D scaffolds. Achieving an optimal cell density in scaffolds therefore necessitates a high surface area-to-volume ratio. In order to facilitate the transport of cells and bioactive chemicals, scaffolds may also need to have pores at both macro and micro scales; features which may be difficult to obtain via traditional scaffold fabrication techniques such as particle leaching, gas foaming and phase separation. Rapid prototyping techniques such as solid free form fabrication (SFF) are emerging as important methods for generating highly controlled scaffold structures. Compared to scaffolds fabricated with traditional methods, the pore size and tortuosity in rapid-prototyped scaffolds have much narrower variations in structural distribution. Local topologies can also potentially be optimized by computational algorithms to control the permeability and mechanical properties [3]. Studies have been carried out to compare scaffolds fabricated by controlled processes with those containing irregular structures fabricated by conventional methods. In one of these studies, scaffolds with similar porosities were prepared by particle leaching or by 3D fiber deposition, and their ability to support cartilage tissue growth was compared [19] (Fig. 24.2). A significantly higher glycosaminoglycan (GAG) content was observed in the scaffolds fabricated by the controlled 3D fiber deposition process. Besides the fibrous morphology and highly accessible pores, more uniform and efficacious cell diffusion/attachment may also have contributed to the observed upregulation of GAG production and the better scaffold function. Advanced scaffolds fabricated by tightly controlled methods, with more uniform and controllable structures and properties, therefore hold promise in promoting the reproducible formation of functional engineered tissues.

Mechanics

The mechanical properties of the natural ECM are of paramount importance in dictating macroscopic tissue functions (e.g., bearing load) and regulating cellular behavior via mechanotransduction signaling. In designing tissue constructs, mechanical properties of the scaffold which resemble native tissue properties are often sought. Most importantly, in the acute phase following implantation, the scaffold must fulfill the key mechanical functions of the tissue which is being replaced. For example, the earliest TE blood vessels based on cell-contracted collagen gels were not strong enough to withstand physiologic blood pressures, and thus had to be reinforced by a tubular synthetic polymer mesh to ensure structural integrity [20]. More recent TE blood vessels based on relatively strong non-woven poly(glycolic acid) (PGA) scaffolds exhibited burst pressures exceeding physiological requirements upon implantation (>2000 mmHg) [21]. In addition to appropriately matching gross tissue mechanical properties, the scaffold must also provide an internal micromechanical environment conducive to the *de novo* synthesis and organization of ECM. For example, while non-woven PGA scaffolds have been successfully employed in blood vessel [21] and heart valve

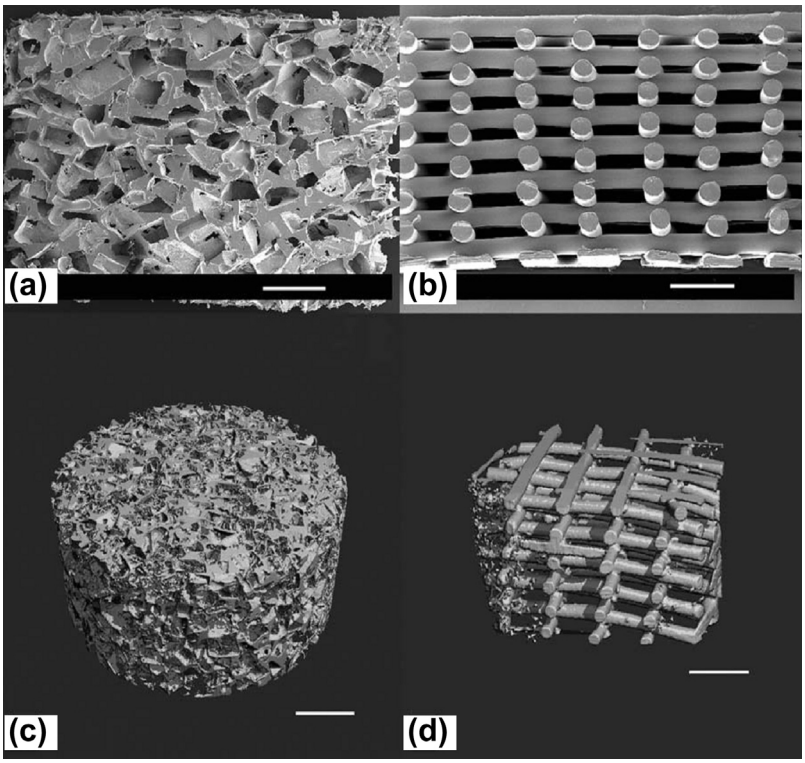

FIGURE 24.2

Designer scaffolds offer opportunities to control scaffold properties such as pore structures, surface area and mechanical strength. Electron micrographs of scaffolds fabricated by conventional method of compression-molding and particle leaching (a) and 3D fiber deposition (b); (c) and (d), 3D images of scaffolds in (a) and (b), respectively. *Reprinted from [19], with permission from Elsevier.*

TE [22], their application to myocardial tissue has been comparatively challenging [23]. In contrast to the predominant load-bearing function of vessels and valves, the primary function of myocardial tissue is cyclic contraction. While the out-of-plane compressive modulus of a typical non-woven PGA scaffold is relatively low ($\sim 6.7 \pm 0.5$ kPa [24]), the in-plane tensile and compressive moduli resisting cardiomyocyte-mediated contraction are comparable at $\sim 284 \pm 34$ kPa [25]. To understand the role of material elasticity on cell behavior, myoblasts were cultured on collagen strips attached to glass or polymer gels of varying elasticity [26]. Cells were found to differentiate into a striated, contractile phenotype only on substrates within a very narrow range of muscle-like stiffness (i.e., 8–11 kPa) (Fig. 24.3). To optimize a scaffold design for a particular application requires consideration of the gross organ and tissue level functional requirements, as well as the micromechanical requirements for appropriate tissue formation at the cell-level.

The mechanical properties of TE scaffolds are determined in part by the bulk properties of their constituent materials (e.g., modulus of elasticity, degradation rate). For example, most hydrogel materials exhibit a much lower strength and stiffness compared to hydrophobic polyester materials. As traditional PLGA-based scaffolds have a limited subset of mechanical properties, new biodegradable materials have been developed, such as poly(hydroxyalkanoates) and poly(glycerol sebacates) to improve scaffold toughness and elasticity [27,28]. Because of the high porosity and concomitant low material content, the mechanical properties of TE scaffolds are very often primarily dictated by the structural arrangement of their constituent materials (e.g., pore size, fiber diameter and orientation; Table 24.1) and associated modes of structural degeneration (e.g., fiber fragmentation, bond disruption). For example, in a recent study the effective stiffness (E) (equivalent to initial

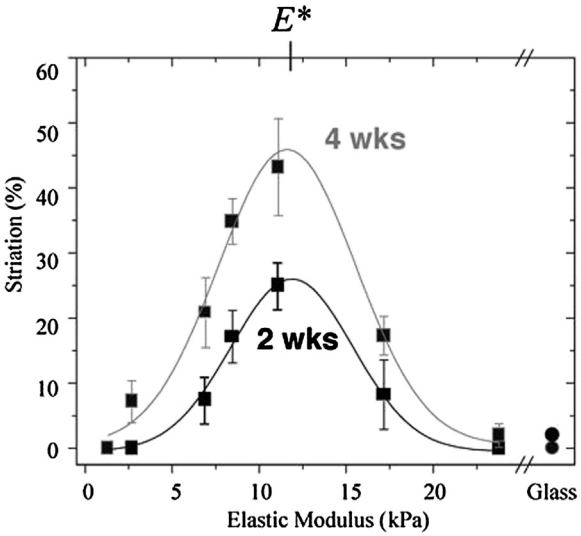

FIGURE 24.3
To understand the role of material elasticity on cell behavior, myoblasts were cultured on collagen strips attached to glass or poly(acrylamide) gels of varied elasticity. Substrate stiffness was found to have a profound influence on myocyte differentiation, with optimal differentiation (as assessed by striation) occurring within a very narrow range of muscle-like stiffness (i.e., 8–11 kPa) [26]. These results suggest that cell differentiation within 3D tissue-engineering scaffolds may exhibit a similar sensitivity to the local micromechanical properties. *Figure reproduced with permission by Rockefeller University Press.*

tensile modulus) of non-woven PGA scaffolds was predictably modulated by tuning the fiber diameter via NaOH-mediated hydrolysis [25].

In addition to the initial structure imparted during the fabrication of the TE scaffold, the modes of structural degeneration manifested by the scaffold need to be considered. For example, while 50:50 blend PGA/PLLA scaffolds do not undergo significant mechanical degeneration over a period of 3 weeks [35], scaffolds dip-coated with the biologically-derived thermoplastic poly(4-hydroxybutyrate) (P4HB) incur a rapid loss of rigidity with cyclic flexural mechanical loading as the P4HB bonds between fibers are disrupted [36]. Depending on the kinetics of scaffold hydrolysis, structural degeneration may be more pronounced and thus represent a more important consideration in scaffold design.

Because the foremost role of the scaffold following implantation is to temporarily fulfill the key mechanical functions of the replaced tissue, it is essential to consider the physiological loading state of the native tissue. While the physiological loading state may be highly complex and virtually impossible to reproduce *ex vivo*, certain mechanical testing configurations are generally more relevant than others. For example, the physiological loading state of

TABLE 24.1 Dependence of a scaffold mechanical property (initial tensile modulus) on the bulk material mechanical property and scaffold structure[a]

Material	Initial tensile modulus (MPa)		
	Fibrous scaffold	Foam scaffold	Bulk material
Poly(glycolic acid) PGA	0.284 ± 0.034 (non-woven) (29)	0.919 ± 0.067[b] (salt leach) [30]	18,780 ± 3430 (fiber) [29]
Poly(ester urethane) urea PEUU	8±2 (electrospun) [31]	~1.4[c] (TIPS[d]) [32]	60 ± 10 (film) [31]
Poly(glycerol sebacate) PGS	N/A	0.004052 ± 0.0013 (salt leach) [33]	0.282 ± 0.0250 (film) [34]

[a] *Several order-of-magnitude differences in modulus can be realized by starting with different bulk materials and/or by converting the bulk material into different porous scaffold structures (e.g., foam or fibrous). For comparison, the initial tensile modulus of a typical passive muscle tissue was reported to be 0.012 ± 0.004 MPa [26].*
[b] *Aggregate modulus obtained from creep indentation testing of PGA-PLLA scaffold.*
[c] *Estimated from PEUU1020 stress-strain curve (Fig. 24.3 [32]).*
[d] *Thermally induced phase separation.*

a semilunar heart valve leaflet depends on time-varying solid-fluid coupling (i.e., leaflet tissue-blood), and includes multi-axial flexural, tensile, and fluid shear stress components. In light of the strong planar anisotropy and tri-layered structures exhibited by native leaflet tissues, biaxial tensile testing [37] and flexural testing [38] have been employed to characterize their behavior.

Because engineered tissues based on synthetic polymer scaffolds inherently begin development as composite materials, their effective mechanical properties will be determined by the combined effects of the cells, ECM, scaffold, and their unique micromechanical interactions. Thus, the appropriate formulation and validation of a mathematical model to simulate and/or predict the mechanical properties of a scaffold is a critical prerequisite for developing a mathematical model to simulate and/or predict the mechanical properties of an engineered tissue. While standard phenomenological models may be useful in characterizing the gross mechanical behavior of a scaffold or engineered tissue construct (i.e., for meeting organ and tissue level functional requirements), only an appropriately formulated structural-based model can be used to design the micromechanical environment presented at the cell-level. Structurally-based models can either be computationally driven, as in the work of Hollister and colleagues [3,39], or purely analytical, as in the case of a model for non-woven scaffolds by Engelmayr and Sacks [25]. Irrespective of the solution method, the goals of structure-based modeling are both to bridge the gap between the disparate length scales of cells, tissues, and scaffolds, and in particular to accurately simulate the micromechanical environment presented at the cellular level. For example, while traditional rule of mixtures theories accounting for the volume fractions and orientations of individual composite constituents are often invoked in describing native and engineered tissues [40], higher order reinforcement effects observed via the structurally-based modeling of commercially available non-woven PGA and PLLA scaffolds preclude the use of rule of mixtures approaches. These higher order reinforcement effects, which yield proportional increases in fiber effective stiffness with increased ECM stiffness, predict a very different micromechanical environment to that predicted by a rule of mixtures, highlighting the importance of an accurate micromechanical representation of the TE scaffold.

Electrical conductivity

Electrical conduction is an important mechanism that enables cellular signaling and function in many types of tissues. The cardiac electrical conduction system is essential for maintaining the synchronous beats that pump blood in an ordered fashion. In the process of bone regeneration, naturally-occurring piezoelectric properties of the apatite crystal are hypothesized to generate electric fields involved in bone remodeling. The nervous system possesses a well-known system of electrochemical signaling. Much research has been carried out using materials to record from and influence bioelectric fields. In making tissue scaffolds, electrically conductive biomaterials have been studied to understand their abilities to interface with bioelectrical fields in cells and tissues to replicate normal electrophysiology.

A wide variety of electrically conductive polymers are available to the tissue engineer, each with differing characteristics that may direct choice for a given application. These organic compounds include poly(pyrrole), poly(vinylidene fluoride), poly(tetrafluoroethylene), poly(aniline), poly(thiophene), and poly(acetylene). Such polymers generally contain delocalized pi bonds and can be considered semiconductors, with conductivity determined by degree of doping. Polypyrrole, an aromatic heterocycle, is perhaps the most widely studied conductive biomaterial, due to both its ease of fabrication and demonstrated biocompatibility. In contrast to the static charges seen in conductive materials like poly(pyrrole), piezoelectric materials such as poly(vinylidene fluoride) display a transient charge in response to mechanical deformation. For transplantation purposes, it is often desirable to have the material biodegrade after cell delivery and incorporation into the host environment have been accomplished. Degradable conductive polymers have been synthesized using several different

approaches, including incorporation of 3-substituted hydrolysable side groups [41], degradable ester linkages connecting oligomers of pyrrole and thiophene [42] and emulsion/precipitation of poly(pyrrole) in poly(D,L-lactide) [43].

In order to tailor conductive biomaterials to a given application, scalable technologies have been developed to fabricate conductive structures of arbitrary geometry. Due to horizontal and vertical growth of polypyrrole during electropolymerization, two-dimensional photolithographically defined gold layers can be used to pattern three-dimensional structures [5,44]. Hollow polymer tubes ranging from the nano to the micro scale have been fabricated using a range of techniques, including polymerization via electrodes on opposite ends of a silicone tube [45] or via oxidation on platinum wire molds followed by reduction [46].

There are a growing number of reports demonstrating synergistic effects of conductive biomaterials and electrical stimulation. Neonatal cardiomyocytes cultured on collagen sponges and matrigel show synchronized contraction in response to applied electric fields [47,48]. Applied potentials can also be used to change the surface properties of conductive polymers, altering cell shape and function, including DNA synthesis and extension [49]. Others have shown that electrical stimulation promotes neurite outgrowth on conductive polymers beyond that seen on indium tin oxide, an inorganic conductive substrate [50]. Composite conductive polymer films have been manufactured to include substrates that direct cell function, such as hyaluronic acid to promote angiogenesis [51].

The mechanistic basis for each of these electrical-material-tissue interactions is not fully understood and will be an important area for future study. However, some leading hypotheses have emerged. This enhanced function of engineered cardiac tissues may be due to greater ultra-structural organization in response to electric fields [47]. Increased neurite outgrowth with electrical stimulation may be caused by better ECM protein adsorption [52] rather than direct effects on the cell itself although there is ample evidence of the latter. Electrophoretic redistribution of cell surface receptors likely governs the galvanotropic response of neurons to a horizontally oriented two-dimensional applied field [53]. Such mechanisms do not fully explain the alterations in cell function which occur in response to stimulation applied to the substrate or material relative to the medium or a distant ground. For such depolarization, signaling through voltage-gated calcium channels can activate ubiquitous second messengers such as cAMP and alter gene transcription affecting learning, memory, survival, and growth [54]. Such secreted gene products might be used to enhance the survival of host cells surrounding an implanted scaffold. Given that applied electric fields can so profoundly affect cell function, conductive three-dimensional scaffolds will be important tools for harnessing this interaction to create functional tissues.

Surface properties

Cells interact with scaffolds primarily through the material's surface, which can be dominated by the surface chemical and topological features. The surface chemistry here refers to the insoluble chemical environment that the scaffold surface presents to cells, which can differ from the biochemical compositions of the bulk, and/or the substances derived from surface adsorption or chemical reactions. Besides mediating cell behavior and functions inside scaffolds, controlled surface properties are of central importance in directing the inflammatory and immunological response. Controlled surface properties may be useful for ameliorating the foreign body reaction at the host-scaffold interface *in vivo* [55,56].

SURFACE CHEMISTRY

Each type of synthetic, natural, or composite scaffold gives rise to a set of distinct surface chemical characteristics governed by the material chemistry and its physical form (such as crystallinity, charge, and topology). Although numerous efforts have been made to tailor the scaffold surface, the chemical environment can exhibit extremely complicated patterns within

the biological milieu. Complex processes such as the spontaneous adsorption of a diversity of proteins from biological fluids to the scaffold surface, and the protein surface conformation are difficult to analyze, but exert profound effects on the scaffold performance. To tailor the scaffold chemical properties, the interactions of scaffolds with different environmental factors need to be considered.

Scaffolds derived from natural ECM materials such as collagen, fibrin, hylauronic acid (HA), proteoglycans, or their composites have the advantage of directly containing innate biological ligands that cells can recognize and provide natural mechanisms for tissue remodeling. ECM analogs have been created to emulate an appropriate tissue regeneration environment. For example, as an essential ECM component in natural cartilage tissue, collagen type II scaffolds may have better biochemical properties for maintaining chondrocyte phenotype and enhance the biosynthesis of glycosaminoglycans compared to collagen type I [57]. Fibrin, the native provisional matrix of blood clots, can provide ligands to initiate cell attachment and ECM remodeling [58]. HA plays a role in morphogenesis, inflammation and wound repair, and the cell-ECM interactions mediated through receptors such as CD44 and RHAMM (receptor for hyaluronan-mediated motility) can be activated in scaffolds with HA constituents [58]. The natural cell-ECM interactions, however, can also alter after purification, manufacturing and scaffold fabrication processes. For example, acid-treated type I collagen polymers contained only 5% residual crystallinity compared to native collagen. The loss of crystalline structure led to platelets binding with diminished degranulation, which in turn limited the myofibroblast numbers and the related inflammatory response at the injured site [14].

Synthetic scaffolds offer a variety of mechanisms to modulate cell behavior. Chemical reactions with biological fluids remodel the scaffold surface and affect tissue growth through both reaction dynamics and kinetics. Studies have shown that bioactive glasses (Class A bioglass composed of 45–52% SiO_2, 20–24% CaO, 20–24% Na_2O and 6% P_2O_5) have superior osteoproductive properties to either bioactive hydroxyapatite, or bioinert metals and plastics. The difference was found to be due to the surface reaction kinetics in physiological fluids. The rapid rate of the reaction that converts amorphous silicate to polycrystalline hydroxyl-carbonate apatite on the bioglass surface is the key to positively regulating the cell cycle and bone formation [59] (Fig. 24.4a). Physical processes also play active roles in

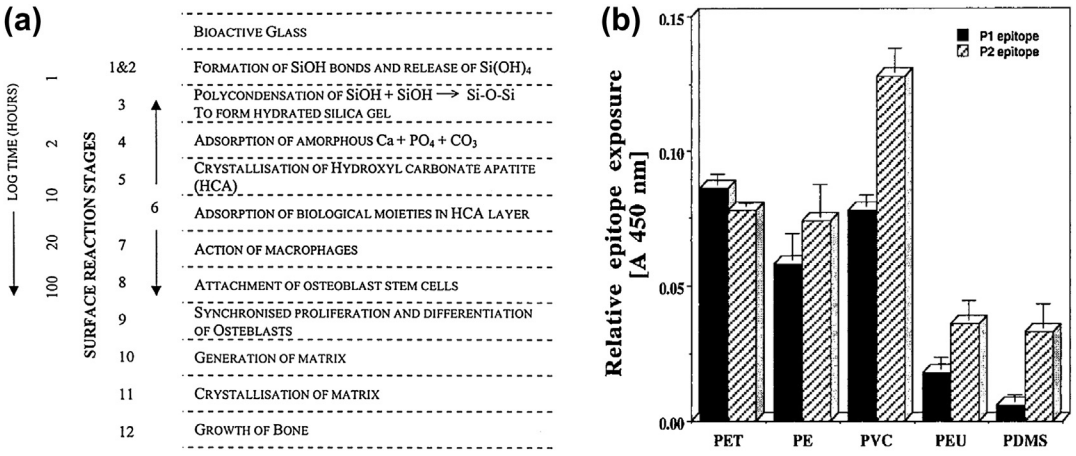

FIGURE 24.4

Understanding the molecular basis of surface interactions is essential to controlling surface functions. a) A series of surface reactions on Class A bioglass [59]. *Figure reproduced with permission from Springer.* **b)** Dynamic and kinetic mechanisms of material interactions with proteins can lead to different cell response at the scaffold surface. The example shows the exposure of two epitopes (P1 and P2) in fibrinogen on a surface varied with the substrate materials (PET, PE, PVC, PEU, and PDMS disks coated with human fibrinogen) [56]. *Figure reproduced with permission from American Society of Hematology.*

controlling the material-cell interface. Because of the adsorbed protein moieties from serum or body fluid, many polyester-based scaffolds, such as those made from poly(α-hydoxy esters), exhibit adequate adhesion to support cell attachment and tissue growth in some *in vitro* and *in vivo* applications. Methods that alter the surface hydrophobicity, e.g., by changing monomer compositions or by chemical surface treatment, can potentially improve the scaffold performance [60–62]. For example, biodegradable foams of hydrophobic polymers (e.g., PLLA and PLGA) can be efficiently wet by two-step immersion in ethanol and water. This surface treatment could overcome the hindered entry of water into air-filled pores to facilitate cell seeding [60].

Surface modifications of scaffolds have been developed to generate surface chemical specificity and recognition. The surface chemistry can be created by either incorporating bioactive moieties directly in the scaffold bulk, or by modifying the surface. These moieties bound to scaffolds trigger desired specific intracellular signaling. In particular, many synthetic and natural hydrogel materials (e.g., poly(ethylene glycol), poly(vinyl alcohol), alginate and dextran) are protein-repellent, and immobilizing biomolecules to such hydrogel scaffolds may be especially useful in tailoring the surface chemistry for cell-material interactions at the molecular level.

Cell adhesion mediated through extracellular adhesive proteins is involved in many intracellular signaling pathways which regulate most fundamental cell behaviors, including differentiation, proliferation, and migration. Enriching scaffold surfaces with specific ECM-derived adhesion proteins has been widely applied to scaffold modification. PLGA-based scaffolds have been coated with fibronectin by physical adsorption for supporting growth and differentiation of human embryonic stem cells in 3D [64]. Fibronectin was covalently attached to PVA (poly(vinyl alcohol)) hydrogels for improved cell adhesion, proliferation, and migration [65]. Fibrinogen was also denatured and fused into the backbone of a PEG (poly(ethylene glycol)) hydrogel material [66] to elicit cellular responses. Elucidating the underlying molecular mechanisms on scaffold surface however is not a trivial task. Fundamental studies have been carried out to understand how the adsorption and denaturing of proteins can lead to different cellular responses at the material surface and may provide a molecular basis to control cell-material interaction for rational design of scaffolds [56] (Fig. 24.4b).

Immobilizing peptide ligands derived from the active domains of ECM adhesion proteins to scaffolds is another major approach to generating specific surface-bound biological signals. For example, integrins, the principal adhesion receptor mediating cell-ECM attachment, comprise a family of more than 20 subtypes of heterodimeric transmembrane proteins. Each of them recognizes and interacts with certain types of ECM adhesion proteins to activate a cascade of signaling pathways which regulate essential cell activities and functions [67]. Integrins can be activated by short peptides in similar ways, e.g., Arg-Gly-Asp (RGD) from fibronectin and Tyr-Ile-Gly-Ser-Arg (YIGSR) from laminin. Provided that peptides are relatively stable and economical to use, incorporating them into scaffolds has become an important way of generating surface biomimicry and enhancing tissue regeneration [68,69]. To introduce peptide moieties, the scaffold materials need to contain appropriate functional groups, which may not be available in most hydrophobic polyesters. Methods have been developed to functionalize polyesters. For example, poly(lactic acid-co-lysine) has been synthesized, and the RGD peptide can be immobilized through the side-chain amine groups of the lysine residues [70,71].

Like the biomolecules in natural ECM, the functions of immobilized bioactive ligands in modulating membrane receptors and intracellular signaling are influenced by their spatial characteristics. For example, integrin affinity to ECM affects cell attachment and migration; as a result, 3D neurite migration demonstrated a bi-phasic dependence on RGD concentration, with intermediate adhesion site densities (between 0.2 and 1.7 mol of peptide/mol of fibrinogen) yielding maximal neurite extension compared with higher densities which

inhibited outgrowth [72]. In another study, integrin clustering, a prerequisite to many integrin-mediated signaling pathways, was recapitulated by RGD nanoclusters immobilized on a comb-polymer substrate [73].

SURFACE TOPOGRAPHY

The material-cell interactions mediated through topographical features have traditionally been studied through planar substrates. Surface modification techniques, including photolithography, contact printing and chemical treatments have been developed to generate micro- and nano-scale surface topographical features. Surface topographical features such as ridges, steps, and grooves have been found to guide cytoskeletal assembly and cell orientation. Surfaces with textures such as nodes, pores or random patterns are often associated with marked changes in cell morphology, cell activities and the production of autocrine/paracrine regulatory factors compared to smooth surfaces [74]. In general, surface roughness increases cell adhesion, migration, and the production of ECM. Cells sense and respond to topographical features in a dimensionally-dependent way. As demonstrated on titanium surfaces, microtextures increased osteoblast attachment and growth, but only the presence of nano-scale roughness led to enhanced cell differentiation in connection with elevated growth factor production [75].

Current fabrication techniques can be used to generate a wide variety of topographical features in scaffolds. Scaffolds can be randomly packed with regular or irregular geometries and shapes (e.g., particles, pellets and fibers), or condensed with amorphous structures (e.g., foam and sponge), or fabricated with specifically designed architectures. Based on the cell-material interactions, scaffolds provide different topographical properties correlated with the dimensions of scaffold geometries and shapes. When the feature size is larger or comparable in size to cells, e.g., the fiber diameter in a non-woven mesh, and pores and walls in a foam, the scaffold may provide curved surfaces for cell attachment. Pore size and surface area constitute the major topological features in an extracellular environment. As demonstrated in a study of mesenchymal stem cells seeded onto polymeric fibrous fabrics, increased fiber diameter favored cell attachment and proliferation by providing more surface area [76]. Surface treatment techniques, such as sodium hydroxide etching, have been used to generate nano-scale roughness to increase cell adhesion, growth, and ECM production [77].

The size scale of most natural ECM components, e.g., fibrous elastin and collagen, fall into the range of several to tens of nanometers. The extracellular environment is dominated by nano-scale topographical features, such as nano pores, ridges, fibers, ligand clusters and high surface area-to-volume ratios in 3D. Such native topographies can be recapitulated to a degree in scaffolds made of natural ECM polymers such as collagen and elastin. As synthetic materials have the advantage of greater control over scaffold properties, interest is growing in developing synthetic nano fibrous scaffolds. 3D PLLA-based scaffolds containing nanofibers have been produced by thermally induced phase separation processes [78,79], and selective surface adsorption of adhesion proteins was observed. In a more versatile method, electrospinning techniques have been used to fabricate a variety of synthetic and natural materials with different hydrophobicities into fibers with diameters ranging from a few to hundreds of nanometers [80]. Another important approach involves building scaffolds from the bottom up. Polypeptides made of 12–16 amino acids have been designed to form hydrogel scaffolds through β-sheet assembly [81]. Amphiphilic molecules consisting of a hydrophilic peptide head and a hydrophobic alkyl tail self-assemble into nanocylinders to form inter-woven scaffolds [82,83].

Nanofibrous scaffolds have demonstrated abilities to support cell and tissue growth. For example, when cardiomyocytes were cultured in meshes made of electrospun poly(ε-caprolactone) nanofibers, they expressed cardiac-specific markers and were contractile in 3D scaffolds [84]. In a scaffold based on self-assembled peptide amphiphilic molecules

containing the laminin epitope IKVAV, neural progenitor cells selectively differentiated into neurons [82]. The potential of designing nano-scale topographies in scaffolds remains largely unknown with regard to exactly how cell cycle, gene expression patterns, and other cell activities are regulated. Some possible mechanisms may be related to cell receptor regulation (clustering, density, and ligand-binding affinity) on nanofibers, nutrient gradients in nanoporous matrices, mechanotransduction induced by the unique matrix mechanics, and the conformation of adhered proteins for cellular recognition sites.

Temporal control

SCAFFOLD DEGRADATION

Unlike permanent or slowly degrading implants which may serve to augment or replace organ function (e.g., hip implants, artificial hearts, or craniofacial plates), tissue-engineering scaffolds serve as temporary devices to facilitate tissue healing and regeneration processes. The regeneration of a fully functional tissue ideally coincides in time with complete scaffold degradation and resorption. Controlling degradation mechanisms allows scaffolds to temporally cooperate with cell and tissue events via changes in scaffold properties and functions. Tuning the scaffold degradation rate to make it kinetically match with the evolving environment during tissue healing and regeneration is an important design criterion. Due to the multiple roles of scaffolds, the interrelations between scaffold property variables, and the different wound conditions in individual patients, it remains a challenge to design degradation properties that can be tailored to meet various clinical tissue regeneration requirements.

During scaffold degradation, some scaffold properties and functions may weaken or diminish with time. In general, there exist lower and upper limits to the optimal degradation rate, which may vary with cellular or tissue processes, scaffold chemical compositions, and scaffold functions. For example, scaffolds often need to serve mechanical functions; such as bone implants which need to support compressive loading while maintaining an environment permissive to new bone formation. If a material degrades prior to transferring mechanical load to the new tissue, the therapy would fail [85]. Alternatively, materials in bone implants that degrade too slowly may cause stress shielding, thereby impeding the regeneration process and potentially endangering surrounding tissues [86]. In skin wound models, healing can be compromised if the scaffold degradation occurs too quickly, whereas scar tissue occurs when the degradation is too slow [14]. The optimal skin synthesis and prevention of scar formation could be achieved when the template was replaced by new tissue in a synchronous way, i.e., the time constant for scaffold degradation (t_d) and the time constant for new tissue synthesis during wound healing (t_h) were approximately equal [14]. Matching tissue formation with material degradation thus requires coupling of specific temporal aspects of tissue formation processes with chemical properties of the scaffold.

Scaffold degradation can occur through mechanisms that involve physical or chemical processes, and/or biological processes that are mediated by biological agents such as enzymes in tissue remodeling. In the passive degradation mode, the degradation is often triggered by reactions that cleave the polymer backbone or crosslinks within the polymer network. Many polyester scaffolds made of lactic acid and glycolic acid, e.g., PLLA and PLGA, undergo bulk backbone degradation due to their wettability and water penetration through the surface. Hydrophilic scaffolds such as hydrogels made of natural or synthetic materials crosslinked by hydrolysable bonds (e.g., ester, carbonate, or hydrazone bonds) also convert to soluble degradation products predominantly through the bulk [87,88]. Chemical degradation can be conveniently varied through scaffold physical and chemical properties such as the backbone hydrophobicity, crystallinity, glass transition temperature, and crosslink density. Because of this flexibility, the degradation rate can principally be engineered for optimal tissue regeneration [85,89].

Scaffolds degrading through passive mechanisms exhibit limited capabilities to match with tissue growth and wound healing. In bulk degradation, the accumulation of degradation products may exert adverse effects on tissue, e.g., acidic products from PLGA degradation. In order to exert more control over the degradation properties of a scaffold and to attempt to tailor the degradation of the scaffolds, consideration of pertinent wound healing and tissue regeneration mechanisms is required. For example, it is known that orchestrated cascade of events occurs during the wound healing process and typically involves inflammation, granulation tissue formation, and remodeling of the ECM. Scaffolds should be designed to degrade *in vivo* during the formation of granulation tissue and/or during the remodeling process. Many inorganic scaffolds studied for bone tissue engineering exhibit biodegradable and bioresorbable characteristics that matches and promotes the process of new tissue formation [63,90,91].

To integrate natural biological mechanisms of ECM remodeling, a new class of hydrogels that degrade in response to proteases have been developed [92,93]. In these scaffolds, degradation occurs through cellular proteolytic activities mediated by enzymes such as collagenase and plasmin. In one of these studies, a PEG hydrogel modified with adhesion ligands was cross-linked with molecules containing matrix metalloproteinase (MMP) peptide substrates. Migration of human primary fibroblasts inside the gel was observed and found depending on the substrate sensitivity to the enzyme [94]. When used for delivering recombinant human bone morphogenetic protein-2 (rhBMP-2) into critical-sized defects in rat crania, the PEG hydrogel matrix was remodeled through the MMP mediated mechanism and supported bone regeneration within five weeks [95]. The approach demonstrated a paradigm of how scaffold degradation and intervention can be engineered to synchronize with wound healing and new tissue synthesis via natural mechanisms.

DELIVERY OF SOLUBLE BIOACTIVE FACTORS

The incorporation of delivery systems in 3D scaffolds offers an indispensable platform for enabling temporal and spatial control in tissue constructs. Compared to systemic administration, using a local controlled release system to deliver soluble inductive and therapeutic factors has the advantages of preventing rapid factor clearance, metering factors in a desired pharmacokinetic manner, and allowing therapeutic doses for an appropriate duration while limiting side effects at unwanted body locations. The release of soluble factors from scaffolds can be mediated through single or multiple mechanisms, e.g., by diffusion, dissolution, scaffold or carrier degradation, or external stimuli. In particular, delivery of growth factors has been studied for various tissues, due to their important roles in instructing cell behavior.

Built upon established particle-based delivery systems, one common method for controlling the release from a scaffold involves prefabricating biofactor-loaded particles and embedding them into a scaffold matrix [96,97]. The release of biofactors in these systems can be delayed with a minimized burst effect, compared to the particles used alone. Typical particle carriers include PLGA and hydrogel microspheres. This method takes advantage of established systems, but involves double matrices which influence the release profile. Alternatively, soluble factors may be incorporated directly into the scaffold itself without a secondary carrier/matrix. This often requires the scaffold to be fabricated under mild physiological conditions to preserve the bioactivity of proteins or other biofactors. Growth factors and proteins have been incorporated in scaffolds through surface coating [98], emulsion-freezing drying [99], gas foaming/particulate leaching [100,101] and nanofiber electrospinning [80,102]. Different delivery profiles of growth factors or DNA plasmids were achieved. Due to their hydrophilic and biocompatible nature, hydrogel scaffolds are amenable to incorporating proteins and plasmid DNA, yielding both higher loading efficiencies and bioactivity compared to PLGA-based materials. Biofactors have been immobilized in hydrogel matrices via physical interactions and/or covalent chemical bonds for prolonging retention time and controlling release via designed mechanisms [103,104].

Scaffolds that integrate controlled release methods have been used in conjunction with scaffolds for a variety of purposes, including enhancing tissue formation, stimulating angiogenesis, guiding cell differentiation and facilitating wound healing [103,105]. Delivering growth factors from scaffolds has demonstrated advantages over using the free form directly [106]. Synergistic effects on accelerating tissue regeneration have been observed when scaffolds, cells, and growth factors are combined. For example, autologous bone marrow-derived cells transplanted with scaffolds containing bone morphogenetic protein-7 resulted in the greatest bone formation compared to constructs without either growth factor or cells [107]. A major challenge in delivering biofactors involves achieving meaningful pharmacokinetic delivery. The dosage, release kinetics, and duration time should be optimized and tailored to tissue growth/healing mechanisms. For example, vascular endothelial growth factor (VEGF) acts as an initiator of angiogenesis while platelet-derived growth factor (PDGF) provides essential stimuli for blood vessel maturation. To simulate the process, PLGA-based scaffolds have been developed to deliver these two angiogenic factors with distinctive kinetics for rapid formation of a mature vascular network [108].

Instead of releasing soluble growth factors directly into the environment, they can also be initiated by cellular activities. Many growth factors are tightly sequestered in the ECM as inactive precursors, and released through their interaction with cells via specific protease mediated mechanisms. To simulate the biological process, growth factors were covalently conjugated to hydrogel matrices via proteolytically cleavable linkages. The immobilized growth factors remained active and their release was elicited by cells through plasmin activity [109,110]. In these systems, the mode of growth factor release could be varied and controlled by cellular activities to achieve precise temporal control in different clinical situations.

Spatial control

Tissues consist of hierarchically ordered structures of cells and ECM; an important tissue engineering design principle is incorporating spatial cues into 3D scaffolds to guide structural tissue formation. Such guidance involves designing anisotropic scaffold properties. By generating directional variations in cell-cell and cell-ECM communications, various cell behavior and new ECM depositions can potentially be guided.

At the macroscopic or tissue level, scaffolds are configured to have appropriate geometries that correspond to tissue/organ anatomical features. The scaffolds are seeded with cells and/or direct the ingrowth of cells from host tissues to promote spatially compartmentalized new tissue growth and wound healing. One of the first methods introduced to generate macroscopic, anatomical shapes utilized highly interconnected pore structures from laminating porous membranes of PLLA and PLGA [111]. In another example, polymeric conduits were constructed for growing blood vessels and guiding nerve tissue regeneration. Smooth muscle cells and endothelial cells have been seeded onto tubular biodegradable PGA scaffolds as an approach for engineering vascular grafts [21]. Nerve guidance channels are used for connecting damaged nerve stumps. The entubulation strategy has demonstrated abilities to guide axonal spouting, directing growth factor diffusion, and blocking undesired fibrous-tissue ingrowth. To fabricate scaffolds with more complex shapes, rapid prototyping techniques use tissue or defect images recorded by medical imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) [3,112,113]. Another approach to shape scaffold materials to fit into individual tissue defects relies on liquid-solid transformation process. *In situ* forming hydrogels solidify upon external stimuli (e.g., chemicals, light, and pH), and have been designed as injectable scaffolds for minimally invasive cell and biomolecule transplantation. For example, methacrylated PEG and HA polymers were able to photopolymerize in situ with chondrocytes and support neocartilage formation *in vivo* [114,115].

At the microscopic or cellular level, various techniques have been developed to couple cell/tissue guidance mechanisms to regenerate tissues that require directional cell growth

(e.g., nerve). Anisotropic characteristics in pore architectures, mechanics, surface properties, degradation, and delivery can potentially generate signals recapitulating haptotaxis and chemotaxis for guiding cell behavior and ECM deposition in 3D. Various scaffolds have been developed containing aligned longitudinal regions. For example, PLGA conduit devices containing physical channels have been created by low-pressure injection molding; oriented lumen surfaces facilitated and guided Schwann cell attachment for peripheral nerve regeneration [116]. Guidance can also be generated through fibrous topographical features. Aligned nanofibers made of poly(L-lactide-co-ε-caprolactone) were fabricated by an electrospinning technique and the oriented fiber structure elicited directional growth of smooth muscle cells. Fibrils in natural scaffold materials, such as collagen and fibrin, have been aligned using magnetic fields [117,118]. The resulting scaffolds increased the rate and depth of axonal elongation *in vitro* and improved sciatic nerve regeneration *in vivo* as compared to scaffolds with random fiber orientations [118].

Creating heterogeneous chemistry in scaffolds is another approach that has been explored to achieve spatial control for tissue guidance. Adhesive RGD peptides have been photo-immobilized in selected regions of agarose hydrogel matrices [119]. The patterns of adhesive and non-adhesive regions induced oriented axonal elongation and migration from dorsal root ganglion cell aggregates *in vitro*. Studies have also been carried out to incorporate chemical gradients in scaffolds. Gradients of proteins play important roles in tissue formation/remodeling during embryogenesis and wound healing. Combining fluidic systems and *in situ* forming hydrogel materials, concentration gradients of peptides and proteins have been generated in 3D matrices and exhibited abilities to modulate cell functions. For example, entrapment of nerve growth factor in a poly(2-hydroxyethyl methacrylate) hydrogel induced directional axonal growth from PC12 cells *in vitro* [120]. A microfluidic device was used to create gradients of immobilized molecules and crosslinking densities in photocrosslinked hydrogels; the gradients of immobilized RGD adhesion ligands modulated the spatial distribution of attached endothelial cells [121].

Creating spatial features involves processing of scaffolds to integrate different control mechanisms. To this end, biomaterials with improved processing may need to be combined with different fabrication techniques, so that architectural structures, biomolecules, and cells can all be combined in a desired manner. For example, to generate complex tissue patterns, it is desirable to position cells with defined microstructures. Encapsulation of cells using photopolymerizable hydrogel materials have been combined with stereolithography in rapid prototyping to create programmed cell organization in 3D [122]. In designing devices for spinal cord injury repair, as molecular, cellular, and tissue level treatments are discovered, the combinations of such treatments will be necessary to synergistically promote tissue regeneration. Multiple-channel, biodegradable scaffolds have been fabricated with capabilities to locally deliver molecular agents and control cell spatial distribution for transplantation (Moore, Friedman et al. 2006).

CONCLUSIONS

Tissue-engineering scaffolds need to be built with functions to interact with cells at different spatial and temporal scales to invoke complex, tissue-like patterns. Over the past two decades, scaffold design criteria have evolved from simply inducing tissue formation to explicitly controlling tissue formation. Tissue engineers have at their disposal an ever broadening array of techniques to fabricate scaffolds which incorporate spatially and temporally varying biochemical and physical cues. Based on our collective understanding of natural cellular and tissue processes, optimal integration of these scaffold structures and properties should in principle allow us to explicitly control the tissue formation process. The challenge, therefore, is to develop a system-level understanding of how fundamental scaffold properties (e.g., mass transport, mechanics, electrical conductivity, and surface properties)

are inter-related in affecting cell behavior, and how they can be rationally programmed – spatially and temporally – to provide the necessary signals at the right time and place to aid tissue formation/regeneration.

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PART 5

Transplantation of Engineered Cells and Tissues

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CHAPTER 25

The Role of the Host Immune Response in Tissue Engineering and Regenerative Medicine

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INTRODUCTION

Tissue engineering and regenerative medicine-based approaches commonly employ a combination of scaffolds, cells, and bioactive molecules. Regardless of the approach used for each clinical application, the objective is the same; specifically, the restoration of structure and function. Similarly, regardless of which strategy is selected, the response of the host to the implanted construct will dictate the success or failure of the eventual outcome. The present chapter briefly reviews the default mechanisms of wound healing, the foreign body response to non-degradable permanent implants, and the host response to various strategies employed within tissue-engineered constructs. Particular emphasis is placed upon newly defined paradigms surrounding the host macrophage response.

WOUND HEALING

All materials implanted into a mammalian recipient are subject to response by the host's innate immune system. Such response occurs immediately and is dependent in part upon both the anatomical context of the implanted construct and the constituents of the implanted construct.

The default mammalian response following tissue injury is characterized by a well-documented series of events that typically result in the deposition of dense fibrous connective tissue (i.e., scar tissue) [1–3]. Only select tissues in adult mammals have the ability to regenerate following injury; among which are the bone marrow, liver, intestinal epithelium and epidermis of the skin. The default response to tissue injury has been described as occurring in four stages: hemostasis, inflammation, proliferation and remodeling [2]. Each of these stages is reviewed briefly in a general context below.

Hemostasis

Following tissue injury and associated damage to the vasculature, platelets are activated by released tissue factor, resulting in the activation of a cascade of clotting factors which causes the

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formation of a provisional fibrin clot with entrapped erythrocytes. The provisional matrix provides a substrate for further cell migration into the site of injury and a medium for cell signaling. In addition to their role in hemostasis and provisional matrix formation, platelets also release cytokines, including platelet derived growth factor (PDGF), TGF-β, chemokine C-X-C ligand 4 (C-X-C L4), IL-1β, and the CD47 ligand thrombospondin [4–8]. These factors, among others, contribute to the initial repair process via recruitment of multiple cell types including neutrophils, macrophages, fibroblasts and other tissue-specific cells to the injury site [6].

Inflammation

Neutrophils are the first inflammatory cell type to arrive at the wound site. Neutrophils phagocytose and destroy foreign material, bacteria or cell debris that may be present, and provide additional signaling molecules that recruit macrophages to the injury site [4]. Mast cells also participate in the early stages of wound healing by releasing granules rich in enzymes, histamine, and other factors that modulate the inflammatory response [2,9]. By 48–72 hours post-injury, however, macrophages typically dominate the cell population at the site of injury [10]. These cells are of a predominantly pro-inflammatory phenotype, and they secrete cytokines and chemokines that promote the further recruitment of leukocytes to the site of injury [4,6]. Macrophages also remove apoptotic neutrophils, the phagocytosis of which may lead to a change toward a more reparative macrophage phenotype (discussed in further detail below) and the resolution of the inflammatory phase of wound healing [11–13]. The T lymphocyte population also plays an important late regulatory role in the resolution of the inflammatory process through local secretion of cytokines and chemokines, many of which are known to affect macrophage polarization [14].

Proliferative phase

The proliferative phase of wound healing involves cellular proliferation, angiogenesis, new ECM (extracellular matrix) deposition, and the formation of granulation tissue – processes that are largely mediated via the effects of the local microenvironment, including pH and oxygen tension, and the cytokine milieu secreted by macrophages, T lymphocytes and other cells within the wound site [6,15,16]. These cytokines include EGF, b-FGF, TGF-α, TGF-β, VEGF and others depending on the nature of the injured tissue [2]. Importantly, macrophage participation and phenotypic polarization during this proliferative stage or the injury response may have significant downstream remodeling effects.

Remodeling phase

Following the deposition of significant amounts of new extracellular matrix (ECM rich in collagens type I and III) during the proliferative phase, the remodeling phase of wound healing begins. This phase is characterized by matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP)-mediated degradation and remodeling of the newly deposited matrix components with eventual scar tissue formation/maturation [2,17]. In some cases, dysregulation of the default healing process can result in prolonged inflammation and remodeling, leading to subsequent fibrosis or hypertrophic scar formation [2,18–20].

Although these wound healing events are described as part of the default response to tissue injury, selected components of this response should also be legitimately considered as part of a constructive remodeling process. That is, the selective activation of components of the inflammatory, proliferation, and remodeling phases can result in a constructive and functional outcome as opposed to scar tissue formation. In particular, the role of macrophages in promoting a constructive remodeling outcome following implantation of tissue-engineered constructs is discussed in further detail below.

THE FOREIGN BODY RESPONSE

The foreign body reaction is a well-described tissue response in the context of classical biomaterials [21,22]; specifically, those materials which are composed of non-degradable synthetic and metallic components and intended for long-term implantation. In general, the foreign body response is considered to be an adverse occurrence, with negative implications for material longevity and local tissue structure/function relationships. While many of the materials used in the fabrication of tissue-engineered constructs are transient (degradable) or composed of biologic materials which are not necessarily subject to the foreign body response as described below, it is nonetheless important to fully understand the foreign body response. In the sections which follow, additional responses which do not fall under the category of a foreign body response and their implications for tissue engineering and regenerative medicine will be discussed.

The host response following the implantation of a non-degradable synthetic or metallic biomaterial involves a series of overlapping processes that include: blood-material interaction with deposition of a protein film on the biomaterial, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrosis and capsule development [22,23]. While many of these processes are similar to those described above for the default host response to tissue injury, there are a number of key differences.

Blood-material interaction and provisional matrix formation

The surgical implantation of a biomaterial is invariably associated with tissue damage and disruption of the vasculature at the surgical site. Release of blood into the wound site results in degranulation of platelets, formation of a provisional matrix as described above for wound healing, and signaling that recruits inflammatory cells (i.e., neutrophils and macrophages) to the surgical site. Blood contact also results in adsorption of proteins to the surface of the biomaterial within seconds of implantation [24]. The proteins that adsorb to a biomaterial may include components of the coagulation system (fibrinogen and tissue factors), complement cascade (C5), and other plasma-derived proteins (albumin and IgG) [22,25]. These proteins provide a substrate with which the inflammatory cells arriving at the site of injury interact at the surface of the biomaterial. The specific proteins that attach and the behavior of the attached cells are dependent on the physical and functional nature of the biomaterial surface and on an adsorption/desorption process that is governed by the affinity of the proteins for the biomaterial surface (known as the Vroman effect) [24,25]. As described briefly below, interactions of cells with the proteins adsorbed to the surface of the biomaterial may lead to a variety of cellular responses, including adherence, activation or triggering of phagocytic pathways, among others, depending on the cell type and the proteins involved [26,27].

Acute inflammation

Acute inflammation, consisting of the emigration of neutrophils from the vasculature into the implant site, follows formation of the provisional matrix and the release of chemoattractant factors by platelets and other cells within the inflammatory site, much like the process described above for default wound healing. However, upon arrival within the wound site, neutrophils interact with the proteins adsorbed onto the biomaterial surface through integrin receptors specific for the adsorbed proteins [22]. For example, the adsorption of fibronectin and IgG plays significant roles in the Mac-1-mediated attachment of neutrophils and macrophages to biomaterial surfaces during the acute phase of inflammation [28]. Complement and serum immunoglobulin adsorption to a pathogen (termed opsonization) leads to phagocytosis by neutrophils and/or macrophages, or destruction of the pathogen via the complement pathway. In comparison, an opsonized biomaterial elicits either phagocytosis from neutrophils (and later macrophages), or will be subjected to frustrated phagocytosis,

depending on the nature of the biomaterial and its size [22,29]. The process of frustrated phagocytosis involves the extracellular release of microbicidal contents at the surface of a foreign body. This release may cause the erosion of implanted materials, and may eventually lead to failure of the material to perform as intended.

Chronic inflammation

The chronic inflammation phase associated with the implantation of a biomaterial is typically characterized by the presence of activated macrophages. This process of macrophage accumulation may occur for a period of days to months depending on the nature of the implanted material and the adsorbed proteins. A meshwork of new ECM is deposited around the biomaterial and an accompanying angiogenic process is prominent. The continued presence of macrophages at the site of biomaterial implantation is often the precursor to the formation of granulation tissue, the foreign body giant cell response, and the eventual encapsulation of the biomaterial within a dense layer of collagenous connective tissue as described below.

Granulation tissue formation, foreign body reaction, and tissue encapsulation

Chronic inflammation can progress to a granulation tissue phase, in which the deposition of new ECM and the growth of vasculature into the implantation site through the process of angiogenesis are conspicuous. The persistence of granulation tissue combined with the presence of a non-degradable biomaterial is eventually associated with the formation of foreign body giant cells. The classic histologic description of a foreign body reaction consists of macrophages and foreign body giant cells, formed through fusion of macrophages, which are typically located at or near the interface of the host tissue with the biomaterial [22,30]. There are a number of factors, including the chemical composition and surface topography, which determine protein adsorption to the biomaterial, and the subsequent degree to which a material elicits a foreign body giant cell response [31–33]. As previously stated, macrophages generally interact with protein adsorbed surfaces through cell surface integrin receptors, the ligation of which induces intracellular signaling cascades that regulate macrophage behavior. Depending on the type of signaling elicited and the immunologic microenvironment, macrophages may fuse with adjacent macrophages forming foreign body giant cells. The exact mechanisms of foreign body giant cell formation are highly complex and have yet to be fully described. An in-depth discussion of the process of foreign body giant cell formation is beyond the scope of this chapter, however, the topic of foreign body giant cell formation as it relates to biomaterials has been reviewed elsewhere [22]. In the final stage of the host response following the implantation of a biomaterial, an increasingly dense layer of collagenous connective tissue is deposited around the surface of the material, thus isolating or 'encapsulating' it from the surrounding healthy tissue.

THE HOST RESPONSE IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

The foreign body response with an outcome of tissue encapsulation is, without question, considered an undesirable outcome for tissue engineering and regenerative medicine strategies which seek to promote functional recovery. There are a number of factors which dictate the host response to engineered tissues and regenerative medicine constructs, and aspects of both the innate and acquired immune response are invariably present. Among the determinant factors are the choice of biomaterial, the types of cells included within a cell-seeded construct, and the bioactive factors or pharmaceutical interventions used as part of or in concert with the implanted construct. Each of these factors elicit some degree of response from the host, which together dictate the nature of the overall response with implications for the success or failure of the strategy of choice. While it is beyond the scope of this chapter to consider each of these

factors in depth, the reader can apply the general concepts presented below to any of the many strategies presented throughout the rest of this text.

Biomaterials

In general, tissue engineering and regenerative medicine strategies will employ degradable naturally occurring or synthetically-derived biomaterials. These materials, due to their transient nature, elicit a distinctly different response than those intended for long term implantation without degradation. The effects of these materials upon the overall host response will be profound, and the selection of material must be considered carefully. The benefits of a particular material, its structure, mechanical properties, and ability to support the growth of cells *in vitro* must be balanced against the tissue reaction it will elicit following placement. Interactions of host immune cells with the material will result in reactions which initiate a cascade of inflammatory processes, which in turn dictate the course of tissue remodeling events. Therefore, it is likely that the physical properties, such as mechanical strength or porosity, which characterize the device at the time of implantation will begin changing immediately after surgical placement *in vivo*.

There are a wide range of biomaterial-associated factors which determine the host response following its implantation, including the chemistry of the material, its hydrophobicity/hydrophilicity, ability to degrade, surface and bulk architecture, and—in the case of some naturally derived biomaterials—the processing methods for manufacturing the constructs and the presence of an endogenous ligand landscape, among a number of others. The effects of these individual properties has been described elsewhere [34,35]. A variety of strategies have been suggested for avoidance of the foreign body reaction; however, many of these are effective only in the short term and therefore have met with only modest success at best. The host response to implanted materials and constructs is both unavoidable and an essential part of a constructive remodeling response which can facilitate material integration with surrounding tissue. Examples of materials based strategies that modulate the host innate immune response to improve remodeling outcomes are discussed in the sections which follow.

Cells

In addition to biomaterials, many tissue-engineering approaches utilize cells as part of a cultured construct. The biomaterials are typically intended either as a delivery vehicle for the cells or to provide mechanical and functional support immediately upon implantation. A number of recent approaches have demonstrated that it is possible to culture and deliver scaffold free constructs consisting only of cells [36,37]. These strategies have met with varying levels of success, as is described elsewhere within this text. Of the highest importance in these applications is the source of cells. Ideally, the cells used in tissue engineering and regenerative medicine are autologous, to minimize potentially detrimental activation of the adaptive immune system. However, it is well understood that autologous sources of tissue specific cells may be limited depending upon the application. A few approaches have utilized allograft sources, and even fewer xenogeneic cell-based applications exist due to the associated immune and regulatory complications. Allograft and xenograft cells may be recognized by the host through mechanisms which have been described for organ transplantation. Recently, induced pluripotent stem cells (iPSC) have emerged as a potential source of autologous cells for tissue engineering and regenerative medicine [38,39]. While iPSC hold great promise as a source for potentially unlimited potent autologous cells, the effects of their reprogramming upon the host response to these cells following placement is currently unknown. It is logical to assume that, in all cases, recognition and resultant formation of a Th1 polarized rejection response will have negative effects upon the success of any tissue engineering or regenerative medicine strategy.

Recent evidence suggests that much of the benefit of the inclusion of cells within tissue-engineered constructs may be the ability to promote construct integration through early

paracrine mechanisms. In particular, mesenchymal stromal cells (MSCs) have become well known for their immunomodulatory properties [40]. It is highly unlikely, however, that newly formed tissues will include the initially implanted population of cells for any extended period of time. Therefore, it is also important to carefully consider the fate of the cells and the potential mechanisms of cell death. Necrotic cell death may play an important role in determining the response of immune cells to transplanted cells. In particular the specific effects of cellular debris and damage associated molecular pattern molecules released during necrotic cell death upon the immune response to implanted constructs remains largely unknown.

Bioactive factors

Bioactive factors such as growth factors, cytokines and chemokines have been shown to have wide ranging effects upon the ability of tissue-engineered constructs to support cellular growth and differentiation *in vitro*. Growth factor and cytokine delivery have been shown to have wide ranging effects upon the success of implantable constructs *in vivo*. Additional strategies may use pharmaceutical interventions in concert with the delivery of tissue-engineered constructs to improve outcomes. These strategies may be aimed at modulating and maintaining tissue specific cell phenotypes, or at modulating the local host immune response. Obviously, the inclusion of cytokines or chemokines with immunomodulatory properties will have effects upon the host response to the implanted construct and thus, effects upon the downstream course of remodeling events. Consideration of the effects of each of the potential growth factor, cytokine, chemokine, or pharmaceutical interventions which may be used is beyond the scope of this chapter. However, an example of the effects of modulation of macrophage phenotype using a cytokine loaded construct is provided in the following section.

ACQUIRED AND INNATE IMMUNITY IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

As described above, both the innate and adaptive immune responses are important in the processes of natural wound healing and in the response to implantable tissue-engineered constructs. It is well recognized that there is an important interplay between the innate and adaptive immune system which can lead to feedback and feed forward loops of inflammation — which in turn can affect outcomes. This complexity of the immune response provides an explanation, at least in part, for the failure of strategies which seek to avoid host immune cell interactions. The following sections briefly describe the role of both the acquired and innate immune responses to tissue-engineered constructs, with a focus upon newly described paradigms for the host macrophage response.

Acquired immunity in tissue engineering and regenerative medicine

The mechanisms of the host cellular and humoral response to whole organ transplantation are well known. Xenogeneic and allogeneic cellular antigens are recognized by the host, elicit immune activation and cause the production of pro-inflammatory mediators with downstream cytotoxicity and transplant tissue rejection. This Th1 type response is commonly associated with rejection. Conversely, Th2 responses are characterized by production of a different set of chemokines, and are more commonly associated with the process of transplant acceptance. The effects of the Th1 and Th2 pathways upon transplant and cell rejection are well described and readers are referred to basic immunology texts for further descriptions [41]. It is logical to assume that tissue-engineering strategies which utilize cell sources of an allogenic or xenogenic nature may elicit a destructive Th1 type response. A number of strategies have been proposed for the protection or encapsulation of cells within a construct, thereby protecting them from immune recognition [42,43]. These strategies may have utility in protecting cells which produce therapeutic molecules, such as insulin production by islet cells. However, these approaches do not promote the integration of such cells into the tissue or organ of interest and

are, therefore, often only effective in the short term. Such recognition of non-self by the host will obviously have deleterious effects upon the ability of the tissue-engineered construct to integrate within the host tissues and otherwise perform as intended.

In addition to the recognition of cellular constituents of tissue-engineered constructs, it is increasingly being demonstrated that the components of the Th1 and Th2 pathways can be activated by materials alone; especially those derived from natural sources as these may contain potentially immune-activating cellular epitopes or other biologic ligands. For example, ECM-based scaffold materials, though composed of components which are highly conserved across mammalian species, have been demonstrated to elicit a robust immune response, including not only components of the innate immune system but also activation of the acquired immune system [44,45]. These materials, though, have consistently been shown to evoke a more Th2-type T cell response, the occurrence of which is thought to play an important role in the process by which ECM scaffold materials promote constructive remodeling. Briefly, tissue cytokine analysis following ECM implantation in experiments with mice has revealed that the ECM group elicited expression of IL-4 and suppressed the expression of IFN-γ compared to groups receiving xenogeneic tissue implants. The ECM group elicited the production of an ECM-specific antibody response, however it was restricted to the IgG1 isotype. Reimplantation of the mice with another ECM scaffold led to a secondary anti-ECM antibody response that was also restricted to the IgG1 isotype and there was no evidence of the formation of a Th1 type response. Further investigation confirmed that the observed responses were in fact T cell dependent. Several synthetic biomaterials have been shown to elicit strong differential pro-inflammatory Th1 cytokine panels. For example, in a recent study, polyethylene was found to be more pro-inflammatory than a number of other synthetic scaffolds as it induced high levels of Th1 cytokines and chemokines but low levels of pro-wound healing cytokines [46].

It should be noted that both the acquired and innate immune systems are tightly coupled and that activation of the acquired immune system, its direct effects upon the tissue-engineered construct notwithstanding, will also drive an innate immune response. Together, the combined effects of the acquired and innate immune systems will dictate the eventual success or failure of a particular strategy. It is also important to note that, while the present section has focused primarily upon the T cell response to implanted constructs and materials, other responses, including hypersensitivity, immunotoxicity and allergy, may also play an important role in the host's response to implanted constructs [22,34].

The role of macrophages in the host response to tissue-engineered constructs

In general, an intense mononuclear cell reaction is seen within the site of implantation of any biomaterial or tissue-engineered construct within days of placement. This response is both a component of the normal response to the injury created during surgical placement of the construct and a component of the response to the implanted construct itself. Ideally, this response resolves quickly without detrimental effects upon the viability of the construct, its material properties, and its function. To date, prolonged macrophage interactions with biomaterial surfaces have been associated with detrimental effects and the precursor to the foreign body response as described above. Without question, the long-term presence of pro-inflammatory cells can have negative effects upon the ability of the construct to function as intended, especially if this response proceeds to a foreign body type response. However, recently a number of studies have demonstrated that the host macrophage response is an essential component of the remodeling process, which leads to constructive and functional remodeling of implanted constructs.

For example, ECM-based scaffold materials, which have now been shown to promote the formation of site-appropriate and functional host tissues when implanted in a number of preclinical and clinical applications [47–50], elicit a robust mononuclear response by

approximately 2–3 days post-implantation [51,52]. This response, which is histologically indistinguishable from the early stages of a classic chronic inflammatory response, may persist at the site of implantation for a month or more depending upon the application [51,52]; however, the response does not progress to a foreign body response. Rather, functional restoration has been observed to occur as the device degrades [47–49,53–61]. When these same materials are treated with chemical crosslinking agents which prevent their degradation, implantation results in a histologically similar acute response, but results in a markedly divergent outcome compared to the non-crosslinked material [51,62]. That is, implantation of materials which have been chemically crosslinked does not result in constructive remodeling, but rather chronic inflammation and a foreign body reaction. This dichotomous response, in which the same material altered only by the treatment with crosslinking agents results in histologically similar early outcomes but distinctly different outcomes at later time points, suggest phenotypic differences among the host innate immune cells which respond following implantation of a biomaterial.

Macrophage polarization

Initially described in the contexts of the host response to pathogen and cancer immunology, macrophages have been shown to have heterogeneous phenotypes ranging from M1 (classically activated, pro-inflammatory) to M2 (anti-inflammatory, homeostatic, wound healing) [63–68], mimicking the Th1/Th2 pathways as described above. M1 macrophages are activated by well-known pro-inflammatory signals such as IFN-γ and lipopolysaccharide (LPS), produce characteristic pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23 and TNF-α, have low production of anti-inflammatory cytokines such as IL-10; produce high levels of reactive oxygen species (ROS), are efficient antigen-presenting cells, and cause the formation of inducer and effector cells in the Th1 pathway. In contrast, M2 cells are activated by molecular cues such as IL-4, IL-13, IL-10 and immune complexes; produce high levels of IL-10 and have increased expression of scavenger, mannose and galactose receptors; produce ornithine and polyamines in place of ROS; and are involved in polarized Th2 type reactions. It should be noted that the term M2 macrophage has evolved to encompass virtually all macrophages that do not fit the M1, classically activated, description [67–69]. However, this segregation into two distinct phenotypes is a marked simplification of the *in vivo* reality. Macrophage phenotype is more accurately considered as a spectrum between the M1 and M2 extreme, in which any given cell may express certain components of either phenotype.

Macrophages, unlike T cells, appear to possess remarkable plasticity once activated. M1 to M2, and M2 to M1 phenotype switching have been observed in multiple studies [70–73]. This plasticity likely represents a protective mechanism by which the host can mount an appropriate host response to a given pathogen, but also effectively resolve such a response without excessive local or systemic damage. Dysfunction of macrophage phenotype, and dysfunction of macrophage plasticity in particular, has been proposed as an underlying mechanism of a number of diseases, including cancer, atherosclerosis, insulin resistance, inflammatory bowel disease and fibrosis [18–20,65,74–80]. Macrophage phenotype has also been found to be an important modulator of the tissue remodeling process which occurs following injury in skin, skeletal muscle, cardiac tissue and the central nervous system among others [81–90]. In general, an initial M1 type response is mounted to destroy potential pathogens within the wound site and to debride the wound site of dead cells and damaged tissue. Transition to an M2 phenotype is associated with tissue remodeling resulting in either scar tissue or constructive remodeling as an outcome depending on the timing of the phenotype switch. Prolonged M1 polarization or overly exuberant transition to an M2 phenotype may lead to excessive scarring or a delay in wound healing, respectively. A description of the unique role of macrophage polarization in each of these diseases and tissue remodeling in each of these organs has been reviewed elsewhere [18,74], but it is important to note that an effective and timely switch in macrophage polarization is almost always a key component of a positive outcome.

Implications for tissue engineering and regenerative medicine

This paradigm, when applied to the host response which follows implantation of a tissue-engineered construct, has been shown to have important implications for the ability to promote constructive remodeling. For example, when applied to the host response to ECM scaffold materials, it has been shown that non-crosslinked materials elicit a more M2 type and less M1 type macrophage response than do chemically crosslinked materials [62,91–94]. A recent study which investigated the response to 15 commercially available, ECM-based, bioscaffold materials demonstrated that there were significant effects of the source tissue and methods used in the production of the material upon the ability to support a constructive remodeling outcome when implanted within the abdominal wall of rats. The phenotype of the host macrophage responses to these materials at early time points was a significant predictor of downstream tissue remodeling outcomes associated with their implantation [62].

Similar phenomena have been observed for certain synthetic materials. Recent studies have demonstrated that porous biomaterials of the same composition as those which, in their non-porous form, elicit an FBR and encapsulation heal with less encapsulation and more vascularity when implanted into either the dermis or into cardiac tissues [95–98]. To assess the importance of porosity to healing, a series of materials were made in which pores were spherical, interconnected and of uniform size. It was noted that those materials possessing pores of 30–40 μm healed with minimal fibrosis and the highest vascularity. These pores were also found to be heavily infiltrated by macrophages, but not foreign body giant cells. Further, when compared to the non-porous control, implants with 30–40 μm pores elicited a host response which was characterized by significantly higher ratios of M2/M1 macrophages [97]. Similarly, recent studies have also shown potential modulating effects of biomaterial surface topography upon macrophage polarization [95,99].

Other strategies, which seek to alter macrophage phenotype through the delivery of cytokines, have also met with increasing success in recent studies. For example, when macrophage polarization was modulated using either IFN-γ and LPS or IL-4 within polymeric nerve guidance conduits, divergent outcomes were observed [100]. Polarization of macrophages towards the M2 phenotype was shown to improve outcomes in a critically sized rat sciatic nerve gap defect model. It was further shown that the M2 phenotype is associated with increase Schwann cell chemotaxis and faster axonal outgrowth.

The studies described above are provided as an example of how a greater understanding of the host innate immune system may influence the design criteria for next generation strategies for tissue engineering and regenerative medicine. In particular, an evolving view of cellular plasticity within the innate immune system and increasing evidence which links particular macrophage phenotypes to the success of biomaterials, both for long-term implantation and/or in tissue engineering and regenerative medicine, appear to be crucial considerations in design.

CONCLUSIONS

Regardless of the composition of a tissue-engineered construct, it will undergo interactions with the host immune system which begin immediately upon implantation. These interactions will invariably include components of the host acquired and innate immune system; however, the specific components and their relative importance in determining success or failure will be construct and context dependent. Regardless of the strategy employed, it is unlikely that activation of the host innate immune system can be avoided. Therefore, designs which incorporate an in-depth understanding of the host immune response together with other requisite criteria will likely meet with improved success and downstream functionality.

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CHAPTER 26

Tissue Engineering and Transplantation in the Fetus

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INTRODUCTION

The fetus is, arguably, the ideal tissue-engineering subject, both as a host and as a donor. The developmental and long-term impacts of tissue implantations into a fetus, along with the many unique characteristics of fetal cells, add new dimensions which greatly expand the reach of tissue engineering, to extents unmatched by most other age groups. Indeed, perhaps not surprisingly, attempts at harnessing these prospective benefits started long before the modern eras of transplantation and regenerative medicine. The first reported transplantation of human fetal tissue took place almost a century ago, in 1922, when a fetal adrenal graft was transplanted into a patient with Addison's disease [1]. A few years later, in 1928, fetal pancreatic cells were transplanted in an effort to treat *Diabetes mellitus* [2]. In 1957, a fetal bone marrow transplantation program was first undertaken [3]. All those initial experiments involving human fetal tissue transplantation failed. It was only since the late twentieth century that fetal tissue transplantation in humans has started to yield favorable outcomes. A number of therapeutic applications of fetal tissue have already been explored, with variable results. Although the majority of studies to date have involved simply fetal cell, tissue, or organ transplantation, various engineered open systems using fetal cells have been explored in animal models and their first clinical applications are justifiably expected in the not too distant future.

Fetal tissue has also been utilized as a valuable investigational tool in biomedical science, since the 1930s. Embryologists, anatomists and physiologists have long studied fetal metabolism, feto-placental unit function, premature life support and brain activity in pre-viable fetuses [4,5]. *In vitro* applications of fetal tissue are well established and somewhat common. Cultures of different fetal cell lines, as well as commercial preparations of human fetal tissue, have been routinely used in the study of normal human development and neoplasias, in genetic diagnosis, in viral isolation and culture and to produce vaccines. Biotechnology, pharmaceutical and cosmetic companies have employed fetal cells and extra-embryonic structures such as placenta, amnion and the umbilical cord to develop new products and to screen them for toxicity, teratogenicity and carcinogenicity. Fetal tissue banks have been operating in the United States and abroad for many years as source of various fetal cell lines for research.

Considering that a large body of data has come out of research involving fetal cells or tissues, and that attempts at engineering virtually every mammalian tissue have already taken place, comparatively less has been done on the 'true' engineering of fetal tissue, through culture and

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placement of fetal cells into matrices or membranes, or through other *in vitro* manipulations prior to implantation. Controlled human trials of open system tissue engineering have yet to be performed and a relatively small number of animal experiments have been reported thus far. Fetal cells were first used experimentally in engineered constructs by Vacanti et al. [6]. Interestingly, this investigation was part of the introductory study on selective cell transplantation using bioabsorbable, synthetic polymers as matrices. This same group performed another study involving fetal cells in 1995 [7]. Both experiments, in rodents, did not include structural replacement, or functional studies. The use of fetal constructs as a means of structural and functional replacement in large animal models was first reported experimentally only in 1997 [8,9].

This chapter offers an outlook of the infantile field of fetal tissue engineering, along with a general overview of fetal cell and tissue transplantation.

GENERAL CHARACTERISTICS OF FETAL CELLS

Immunological rejection (in non-autologous applications), growth limitations, differentiation and function restraints, incorporation barriers and cell/tissue delivery difficulties are all well known complications of tissue engineering. Many of those problems can be better managed, if not totally prevented, when fetal cells are used. Due to their properties both *in vitro* and *in vivo*, fetal cells are excellent building blocks for tissue engineering.

In vitro

Compared with cells harvested postnatally, most fetal cells multiply more rapidly and more often in culture. Depending on the cell line considered, however, this increased proliferation is more or less pronounced, or, in a few cases, not evident at all. Due, at least in part, to their proliferation and differentiation capacities, fetal cells have long been recognized as ideal targets for gene transfers.

Because they are very plastic in their differentiation potential, fetal cells respond better than mature cells to environmental cues. Data from fetal myoblasts and osteoblasts, and from amniotic mesenchymal stem cells (aMSCs) suggest that purposeful manipulations in culture or in a bioreactor can be designed to steer fetal cells to produce improved constructs. Younger mesenchymal stem cells (MSCs) from mid-gestational fetal tissues are more plastic and grow faster than adult, bone marrow-derived MSCs. MSCs have also been isolated earlier in fetal development, from first-trimester blood, liver, and bone marrow. These cells are biologically closer to embryonic stem cells and have unique markers and characteristics not found in adult bone marrow MSCs, which are potentially advantageous for cell therapy. Fetal MSCs may express Human Leukocyte Antigen (HLA) class I but not HLA class II. The presence of interferon gamma in the growth medium may initiate the intracellular synthesis and cell surface expression of HLA class II, but neither undifferentiated nor differentiated fetal MSCs tend to induce proliferation of allogenic lymphocytes in mixed cultures. Actually, fetal MSCs treated with interferon-gamma typically suppress alloreactive lymphocytes in this setting. These and other data indicate that both undifferentiated and differentiated fetal MSCs may not elicit much alloreactive lymphocyte proliferation, thus potentially rendering these cells particularly suitable for heterologous transplantation.

Fetal cells can survive at lower oxygen tensions than those tolerated by mature cells and are therefore more resistant to ischemia during *in vitro* manipulations. They also commonly lack long extensions and strong intercellular adhesions. Probably because of those characteristics, fetal cells display better survival after refrigeration and cryopreservation protocols when compared with adult cells. This enhanced endurance during cryopreservation, however, seems to be tissue specific. For instance, data from primates and humans have shown that fetal hematopoietic stem cells, as well as fetal lung, kidney, intestine, thyroid and brain tissues can

be well preserved at low temperatures, whereas non-hematopoietic liver and spleen tissues can also be cryopreserved, but not as easily.

In vivo

The expression of major histocompatibility complex (H-2) antigens in the fetus and, hence, fetal allograft survival in immunocompetent recipients is age and tissue specific. The same applies to fetal allograft growth, maturation, and function. At least in fetal mice, the precise gestational time of detection of H-2 antigen expression and the proportion of cells expressing these determinants depend on inbred strain, specific haplotype, tissue of origin and antiserum batch employed. Nevertheless, the precise factors governing the timing and tissue-specificity of H-2 antigen expression are yet to be determined in most species, including humans.

Other mechanisms, in addition to H-2 antigen expression, also seem to govern fetal immunogenicity. For example, at least in certain mammalian species, by catabolizing tryptophan the conceptus suppresses T cell activity and defends itself against rejection by the mother. In humans, fetal cells can be found in the maternal circulation in most pregnancies, and fetal progenitor cells have been found to persist in the circulation of women decades after child birth [10,11]. Interestingly, a certain population of fetal cells, the so-called pregnancy-associated progenitor cells (PAPCs), appears to differentiate in diseased/injured maternal tissue. The precise original phenotypical identity of these cells remains unknown. They are thought to be a hematopoietic stem cell, a MSC, or possibly a novel cell type. What is known is that pregnancy results in the acquisition of cells with stem-cell-like properties that may influence maternal health post-partum, by triggering disease and/or avoiding/combating it.

All these data allow us to suppose that engineered constructs made with fetal cells should be less susceptible to rejection in allologous applications. Xenograft implantations may also become viable, as studies suggest that fetal cells are also better tolerated in cross-species transplantations, including in humans [12–14].

On the other hand, while less immunogenic, some fetal cells may be too immature and functionally limited, if harvested too early. Yet, experimental models of fetal islet pancreatic cell transplantation have shown that, with time, the initially immature and functionally limited cells will grow, develop and eventually function normally. Conversely, however, certain cells, such as those from the rat's striatum, actually function better after implantation if harvested early, as opposed to late in gestation.

Fetal cells may produce high levels of angiogenic and trophic factors, which enhance their ability to grow once grafted. By the same token, those factors may also facilitate regeneration of surrounding host tissues. Interestingly, significant clinical and hematological improvement has been described following fetal liver stem cell transplantation in humans, even when there is no evidence of engraftment. These improvements have been attributed to regeneration of autologous hematopoiesis and inhibition of tumor cell growth promoted by the infused cells, through mechanisms yet to be determined. The under-differentiated state of fetal cells also optimize engraftment, by allowing them to grow, elongate, migrate and establish functional connections with other cells [13].

Applications

Because of all the general benefits derived from the use of fetal cells, along with others specific to each cell line, several types of fetal cellular transplantation have been investigated experimentally or employed in humans for decades now. Clinically, fetal cells have been (mostly anecdotally) useful in a number of different conditions, including: Parkinson's and Huntington's disease; *Diabetes mellitus*; aplastic anemia; Wiskott-Aldrich syndrome; thymic aplasia (DiGeorge syndrome) and thymic hypoplasia with abnormal immunoglobulin syndrome (Nezelof syndrome); thalassemia; Fanconi anemia; acute myelogenous and

lymphoblastic leukemia; Philadelphia chromosome-positive chronic myeloid leukemia; X-linked lymphoproliferative syndrome; neuroblastoma; severe combined immunodeficiency disease; hemophilia; osteogenesis imperfecta; skin reconstruction; acute fatty liver of pregnancy; neurosensory hypoacusis; malaria; and HIV. They have also been applied to treat inborn errors of metabolism, including Gaucher's disease, Fabry's disease, fucosidosis, Hurler's syndrome, metachromatic leucodystrophy, Hunter's syndrome, glycogenosis, Sanfilippo's syndrome, Morquio syndrome type B and Niemann-Pick disease. Experimentally, fetal cell and organ transplantation have been studied in an ever-expanding array of diseases. *In utero* hematopoietic stem cell transplantation is a promising approach for the treatment of a variety of genetic disorders. The rationale is to take advantage of prenatal hematopoietic and immunological ontogeny to facilitate allogeneic hematopoietic engraftment [15–19]. It is an entirely non-myeloablative approach to achieve mixed hematopoietic chimerism and associated donor-specific tolerance. Nonetheless, actual fetal tissue engineering as a therapeutic means has barely begun to be explored, with comparatively few studies being undertaken thus far [8,9,20–44].

FETAL TISSUE ENGINEERING

Vacanti and coworkers were the first to make use of fetal cells in engineered constructs, in the late 1980s [6]. The experiment, in rats, used fetal cells from the liver, intestine and pancreas, which were cultured, seeded on bioabsorbable matrices and later implanted. The fetal constructs were implanted in heterologous fashion and heterotopically, namely in the interscapular fat, omentum, and mesentery, with no structural replacement. They were removed for histological analysis no later than two weeks after implantation. Successful engraftment was observed in some animals that received hepatic and intestinal constructs, but in none that received pancreatic ones.

Only in 1995 was a second study performed, by the same group, involving fetal liver constructs, also implanted in heterologous and heterotopic fashion in rats [7]. Then, fetal hepatocytes were shown to proliferate to a greater extent than adult ones in culture and to yield higher cross-sectional cell area at the implant. As in the first experiment, neither structural replacement, nor functional studies were included.

Fetal constructs as a means of structural and functional replacement, in autologous fashion, in large animal models, were first reported experimentally in 1997 [8,9]. Those studies introduced a novel concept in perinatal surgery, involving the minimally invasive harvest of fetal cells, which are then used to engineer tissue *in vitro* in parallel to the remainder of gestation, so that an infant, or a fetus, with a prenatally diagnosed birth defect can benefit from having autologous, expanded tissue readily available for surgical implantation, in the neonatal period or before birth.

Congenital anomalies

Congenital anomalies are present in 3–4% of all newborns. Those diseases are responsible for nearly 20% of deaths occurring in the neonatal period and even higher morbidity rates during childhood. By definition, birth defects entail loss and/or malformation of tissues or organs. Treatment of many of those congenital anomalies is often hindered by the scarce availability of normal tissues or organs, either in autologous or allologous fashion, mainly at birth. Autologous grafting is frequently not an option in newborns due to donor site size limitations, and the well known severe donor shortage observed in practically all areas of transplantation is even more critical during the neonatal period.

Although yet to be fully explored, several studies utilizing fetal tissue engineering as a means to treat congenital anomalies have already been reported experimentally [8,9,20–44]. So far, these studies have involved different models of congenital anomalies/structural replacements,

involving the skin, bladder, trachea, diaphragm, myocardium, heart valves, blood vessels, chest wall, and craniofacial structures. However, many other anomalies are likely to benefit from this therapeutic principle. The first clinical application of fetal tissue engineering is expected in the near future, pending arguably more stringent regulatory clearances, as perhaps expected for a concept that involves both the mother and the infant, as well as unique immature cells.

Alternative sources of fetal cells

Fetal cells amenable to processing for tissue engineering can be obtained from a variety of sources besides the fetus. These include the amniotic fluid and membrane, placenta, Wharton's jelly, and umbilical cord blood. Although peripheral maternal blood can be a source of fetal cells, their consistent isolation in numbers and phenotypes compatible with tissue engineering remains to be demonstrated. So far, of all these sources, the amniotic fluid and the placenta have been the most appealing clinically, to a large extent because they are the least invasive ones for both the mother and the fetus, also because amniocentesis and chorionic villus sampling are widely accepted forms of prenatal diagnostic screening. This is particularly true for the amniotic fluid, in that a diagnostic amniocentesis is routinely offered to any mother with a fetus in whom a structural anomaly has been diagnosed by prenatal imaging (Fig. 26.1).

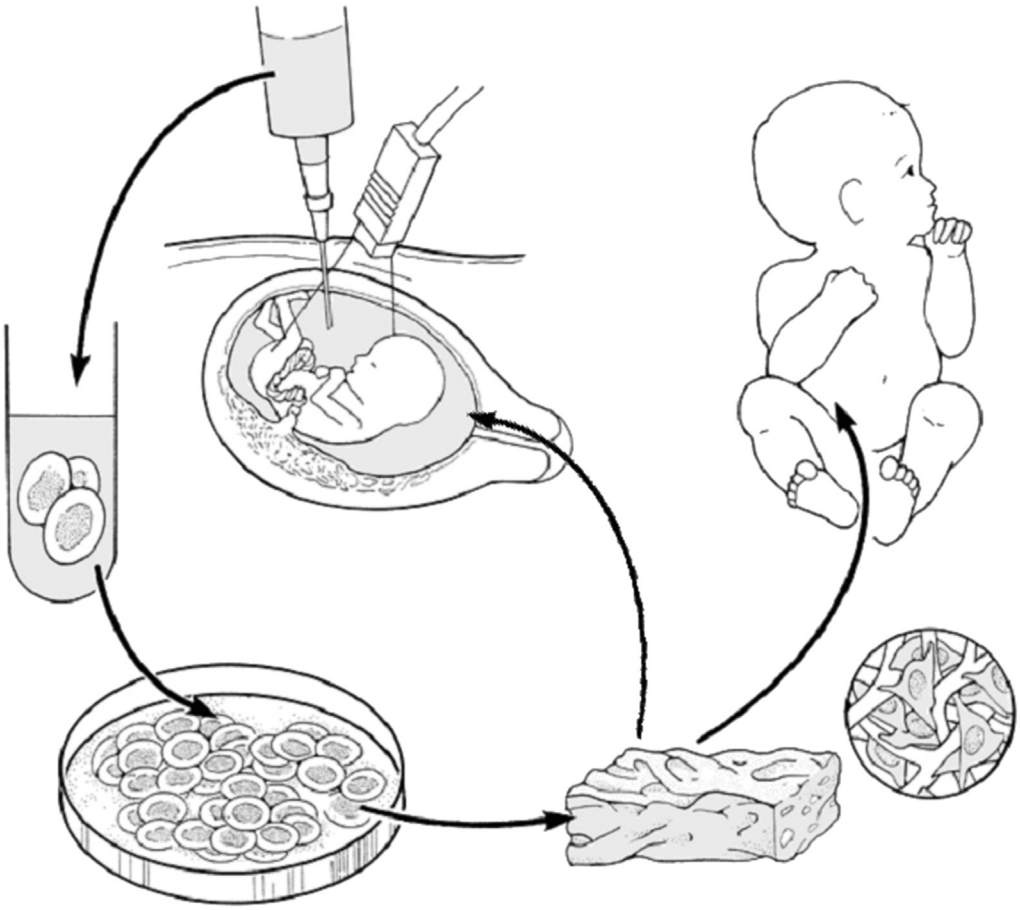

FIGURE 26.1
Diagram representing the concept of fetal tissue engineering from amniotic fluid cells for the treatment of birth defects. A small aliquot of amniotic fluid is obtained from a routine amniocentesis, typically performed when a structural anomaly is diagnosed by routine prenatal imaging screening. Fetal tissue is then engineered *in vitro* from amniotic progenitor cells while pregnancy continues, so that the newborn, or a fetus, can benefit from having autologous, expanded tissue promptly available for surgical reconstruction at birth or *in utero*.

This eliminates any additional risk to the mother and thus expands the ethically sensible range of fetal tissue engineering as a form of perinatal therapy beyond life-threatening structural anomalies.

Many amniotic and placental cells share a common origin: the inner cell mass of the morula, which gives rise to the embryo itself, the yolk sac, the mesenchymal core of the chorionic villi, the chorion, and the amnion. Most, if not all types of progenitor cells that can be isolated from the amniotic fluid and the placenta seem to share many characteristics [45]. However, a common origin of amniotic and placental progenitor cells remains to be unequivocally demonstrated. The full spectrum of cell types that can be obtained from amniotic and placental progenitor cells still has yet to be defined [38]. Moreover, certain fetal pathologic states, such as neural tube and body wall defects, among others, may lead to the availability of cells not normally found in healthy pregnancies, which could become clinically useful [46].

AMNIOTIC FLUID

Embryonic and fetal cells from all three germ layers have long been identified in the amniotic fluid [47–50]. However, the specific origins of many subsets of these cell populations still remain to be determined. The cellular profile of the amniotic fluid varies with gestational age [51]. In addition to the common origin with the mesenchymal portion of the placenta, as mentioned above, the amniotic cavity/fluid receives cells shed from the fetus and, quite possibly, from the placenta as well (although the latter is yet to be definitely confirmed).

The mechanisms responsible for the production and turnover of the amniotic fluid are thought to also determine the cell types present in the amniotic cavity. In the first half of gestation, most of the amniotic fluid derives from active sodium and chloride transport across the amniotic membrane and fetal skin, with concomitant passive movement of water. In the second half, most of the fluid comes from fetal micturition. An additional substantial source of amniotic fluid is secretion from the respiratory tract. Fetal swallowing and gastrointestinal tract excretions, while not voluminous, also play a role in the composition of the amniotic fluid. As a result of such fluid dynamics, cells present in the urinary, respiratory, and gastrointestinal tracts are shed into the amniotic cavity.

Overall, amniotic fluid composition changes predictably throughout gestation. In humans, it is isotonic with fetal plasma in early pregnancy, due to transudation of fetal plasma through the maternal deciduas, or through the fetal skin prior to keratinization, which occurs at approximately 24 weeks. Afterwards and until term, it becomes increasingly hypotonic relative to maternal or fetal plasma. All the variables that play a role in amniotic fluid composition seem to contribute to the changeable profile of its cellular component [51]. Still, much remains to be clarified about the ontogeny of many subsets of amniocytes at any gestational age [45]. This is particularly true before the 12^{th} week of gestation due, to a large extent, to the limitations of performing amniocentesis before that time.

The fact that certain progenitor cells can be found in the amniotic fluid was apparently first reported in 1993, when small, nucleated, round cells identified as hematopoietic progenitor cells were found therein (only) before the 12^{th} week of gestation, possibly coming from the yolk sac [52]. A study from 1996 was the first to suggest the possibility of mutilineage potential of non-hematopoietic cells present in the amniotic fluid, by demonstrating myogenic conversion of amniocytes [53]. That study did not specify the identity of the cells that responded to the myogenic culture conditions, in that case the supernatant of a rhabdomyosarcoma cell line. The presence of mesenchymal cells in the amniotic fluid has been proposed for decades [54,55]. However, the differentiation potential of aMSCs started to be determined only fairly recently [34,37,56–60]. Likewise, the presence of possibly more primitive, embryonic-like stem cells in the amniotic fluid was suggested only in the last few years [59,61–64].

Human amniotic epithelial cells have shown pluripotency, being able to differentiate at least into neural and glial cells, and into hepatocyte precursors. These cells have been employed therapeutically in animal models of cerebral ischemia and spinal cord injury, and as experimental transgene carriers into the liver. However, they are not yet universally considered amniotic fluid cells and will not be further discussed here. Also, given the other, more practical sources of fetal hematopoietic stem cells, other than the difficult pre-12th week amniocentesis, it is unlikely that the amniotic fluid will be a useful option for a source of these cells in clinical practice. Hence, this overview will focus on the mesenchymal and embryonic-like stem cells.

aMSCs

The amniotic fluid is rich in MSCs. We have described a very simple protocol for isolation of these aMSCs, based on mechanical separation and natural selection by the culture medium [28,30,65]. Other protocols for isolating aMSCs have also been described [55,58]. Previous data on amniotic cell culture, without description of the specific nature of the cells grown (possibly predominantly mesenchymal), show that low oxygen tension in the gas phase can be an effective means of enhancing clonal cell expansion. Although not routinely employed, if necessary, molecular HLA typing can be performed on DNA obtained from expanded MSCs, as well as from fetal and maternal blood cells, by polymerase chain reaction/sequence-specific oligonucleotide using a reverse dot blot method, in order to confirm the fetal origin of the cultured cells.

The precise origin(s) of the MSCs found in the amniotic fluid remains to be determined. At first, these cells were thought to be simply shed by the fetus at the end of their life cycle. However, they may actually come from the fetus itself, and/or the placenta, and/or the inner cell mass of the morula, staying viable within the fluid. In no way are aMSCs at the end of their life cycle. Ovine data have shown that amniotic fluid-derived MSCs (aMSCs) proliferate significantly faster in culture than immunocytochemically comparable cells derived from fetal or adult subcutaneous connective tissue, neonatal bone marrow, and umbilical cord blood [28,66]. In humans, the expansion potential of aMSCs exceeds that of bone marrow MSCs [30,57,65]. The phenotype of human aMSCs expanded *in vitro* is similar to that reported for MSCs derived from second-trimester fetal tissue and adult bone marrow [57,66–68].

Human aMSCs have shown not only mesodermal differentiation potential at least into fibroblasts, adipocytes, chondrocytes, osteocytes, and myogenic lineages, after exposure to specific culture conditions (Figs. 26.2 and 26.3) [30,34,57,68,69]. The progenitor nature of these cells imparts the possibility that they could be used to engineer constructs to correct a wide variety of defects. For example, recent large animal studies have shown that the repair of congenital diaphragmatic hernia, tracheal, chest wall and craniofacial defects can be enhanced by the respective use of tendon, cartilaginous/airway, or bone grafts engineered from aMSCs during surgical reconstruction (Figs. 26.4 and 26.5) [24,32,33,37,39,40,43,44]. Another

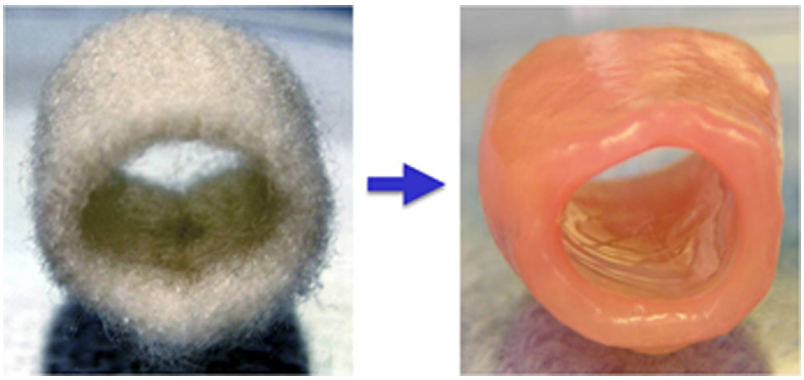

FIGURE 26.2
Representative, cross-sectional view of a 3D cartilaginous tube engineered from aMSCs (right) seeded onto a poly(lactic-co-glycolic acid) scaffold (left), previously maintained in a bioreactor under chondrogenic conditions.

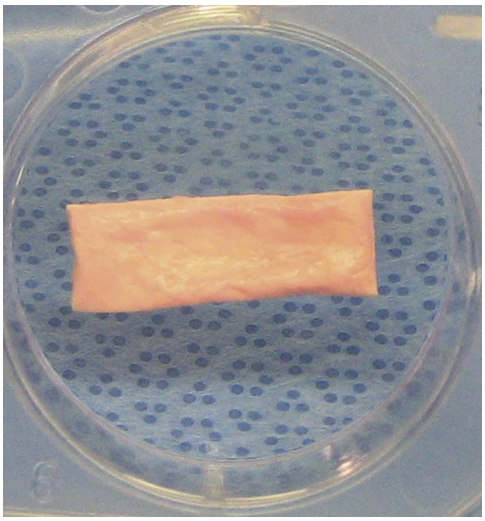

FIGURE 26.3
Representative gross view of a 3D osseous construct engineered from aMSCs seeded onto poly-L-lactic acid electrospun nanofibrous scaffolds, previously maintained in a bioreactor under osteogenic conditions.

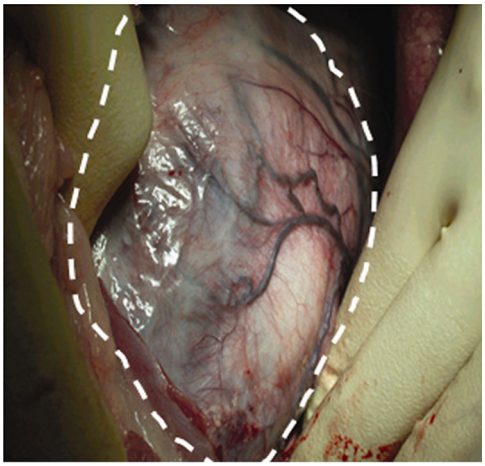

FIGURE 26.4
An intact ovine diaphragmatic tendon seen from the chest, 12 months after autologous repair with an engineered, mesenchymal amniocyte-based construct. The dotted line encircles the area of the graft. *Reproduced, with permission, from 'Kunisaki, S. M., J. R. Fuchs, A. Kaviani, J. T. Oh, D. A. LaVan, J. P. Vacanti, J. M. Wilson and D. O. Fauza (2006). 'Diaphragmatic repair through fetal tissue engineering: a comparison between mesenchymal amniocyte- and myoblast-based constructs.'* J Pediatr Surg **41***(1): 34–9; discussion 34–9'.*

proposed use of an engineered construct based on amniotic mesenchymal (and epithelial) cells is for the repair of premature rupture of membranes during pregnancy, but this has yet to be consistently achieved *in vivo* [70].

An intriguing characteristic of aMSCs is what seems to be unique immunogenic and immunomodulatory properties [71,72]. These cells appear to be immunologically privileged when compared to mesenchymal cells derived from fetal or adult tissue. Their depressed immunogenicity is stable or even further down regulated after multiple passages in culture [71,73]. It has been shown that MSCs enhance the engraftment of umbilical cord blood-derived CD34+ hematopoietic cells [68]. The amniotic fluid has been proposed as a useful source of MSCs to be cotransplanted with hematopoietic stem cells from the same donor [58]. This could be particularly useful in the setting of umbilical cord blood transplantation between siblings.

In utero, transamniotic gene transfer has shown some promise in animal models [74,75]. Young, highly proliferative, less differentiated fetal cells are natural targets for the optimization of gene therapy. Thus, amniotic and placental stem cells could certainly make useful targets for genetic manipulation. This is a potentially exciting development for the foreseeable future.

More recently, an actual biological role for aMSCs has been uncovered. It has been demonstrated that these cells actually enhance normal fetal wound healing [76]. This germane finding was apparently the first demonstration of a biological role for any amniotic cell. It also provided biological validation for the use of aMSCs in regenerative strategies. Within the

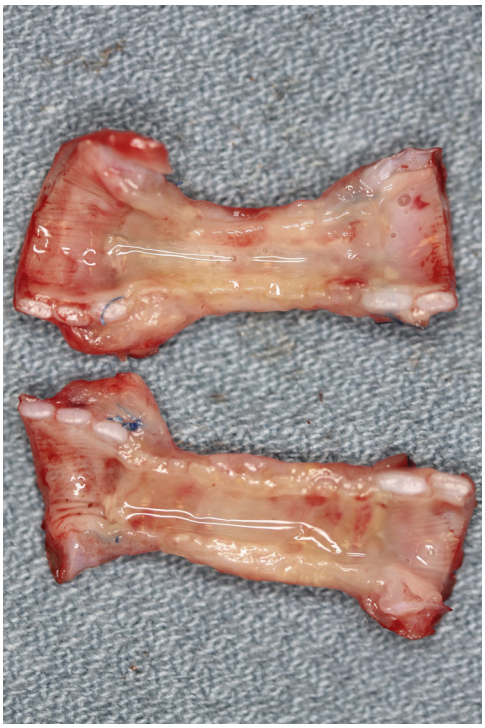

FIGURE 26.5
Representative gross view of a longitudinally opened aMSC-engineered tracheal implant at necropsy showing the intraluminal glistening typical of epithelialization, confirmed histologically.

spectrum of reparative and regenerative processes, fetal wound healing is closer to the latter than to the former. When compared with healing at any stage of postnatal life, wound healing in the fetus involves significantly less inflammation and can be almost scarless, particularly early in gestation. That study has added a new facet to fetal and general wound healing, and has also lent biological support to the use of these cells in a spectrum of conceivable regenerative strategies.

Finally, the translational relevance of aMSCs in fetal tissue engineering has been underlined by the viability of scaled up manufacturing of human aMSCs in compliance with regulatory guidelines and by the establishment of amniotic cell banks [77,78].

Embryonic-like stem cells

From the very little that is currently known about what could be more primitive, embryonic-like stem cells (ESCs) present in the human amniotic fluid, these cells are very scarce, representing zero (i.e., they cannot always be isolated) to less than 1% of the cells present in amniocentesis samples [59,61,62,64]. In addition, these cells have been identified mostly through markers commonly present in ESCs, such as (putatively – see next paragraph) Oct-4, stem cell factor, vimentin, alkaline phosphatase, CD34, CD105, and cKit, but these were not necessarily concomitantly expressed, nor are they exclusive of ESCs proper. Specifically, these markers can also be expressed, alone or in various combinations, in embryonic germ cells; embryonic fibroblasts; embryonal carcinoma cells; MSCs; hematopoietic stem cells; ectodermal, neural, and pancreatic progenitor cells; and fetal and adult nerve tissue; among others [50,67,79–81]. Telomerase activity, another marker for stem cell pluripotency, has also been detected in amniotic fluid cell samples.

As to Oct-4 expression, a central component of the characterization of pluripotency and supposedly expressed in these cells; it must be noted that a recent review study has revealed that the previous reports documenting its expression in these cells, and also in aMSCs, warrant careful re-examination with regard to vital technical pitfalls such as gene and protein isoforms, pseudogenes, and antibody choice, as well as primer design [82]. Although specific detection

of Oct-4A mRNA is achievable, an antibody that can reliably distinguish between Oct-4A (the correct isoform of Oct-4 associated with pluripotency and thus tumorigenesis) and the pseudogene Oct-4B actually remains to be described. Indeed, one could say that true pluripotency based on Oct-4A expression in these cells actually remains to be definitely established [82]. This is further underscored by the fact that these cells do not seem to be tumorigenic *in vivo*.

Still, it is well established that markers alone are not considered enough to characterize human ESCs. A uniform and universal differentiation potential needs to be demonstrated, which remains to be verified in these so-called amniotic ESCs. So far, some of the amniotic cells that express the markers mentioned above have been shown to differentiate into muscle, adipogenic, osteogenic, nephrogenic, neural and endothelial cells, but not necessarily from a uniform population of undifferentiated cells. On the other hand, in accordance with an ESC profile, these pluripotent cells seem to be clonogenic, as at least some of them express cyclin A, a cell cycle regulator. Therefore, although the data currently available can indeed be considered promising, final proof that true ESCs can be consistently isolated from the amniotic fluid, and at which gestational ages, remains to be established. Furthermore, multiple evidences of correspondence between what are considered amniotically-derived ESC-like cells and aMSCs point to the likelihood of basic nomenclature overlaps of what could be just one cell type receiving different names. Regardless, in light of the known presence of other, non-mesenchymal and non-ESC-like stem cells in the amniotic fluid, the simple term 'amniotic stem cell' is thus too vague.

PLACENTA

Different cell types are found in the placenta at different gestational ages, as a result of the mechanisms behind placental development. In humans, placental villous development starts between 12 and 18 days post-conception (p.c.), when the trophoblastic trabeculae of the placental anlage proliferate and form trophoblastic protrusions, the primary villi, into the maternal blood surrounding the trabeculae. Two days later, embryonic connective tissue from the extra-embryonic mesenchyme invades these villi, which then become secondary villi. Between days 18 and 20 p.c., the first fetal capillaries begin to appear in the now abundant mesenchyme of the villous stroma, marking the development of the tertiary villi, the first generation of which are the mesenchymal villi. Mesenchymal villi are the first structures providing the morphological requisites for materno-fetal exchange of gases, nutrients, and waste. They are also the precursors of all other villous types, namely immature intermediate villi, stem villi, mature intermediate villi, and terminal villi. At approximately the fifth week p.c., all placental villi are of the mesenchymal type. From about the 23rd week p.c. until term, the pattern of villous growth changes, with the mesenchymal villi transforming into mature intermediate villi, rather than into immature ones. During that phase, few mesenchymal and immature intermediate villi remain, in the centers of the villous trees, where they comprise a poorly differentiated growth reserve.

As shown above, placental villous sprouting entail active growth of both trophoblastic and mesenchymal tissue components in a coordinated fashion. Further differentiation of the mesenchymal villi into immature or mature intermediate villi is a determining factor in the balance between growth and maturation of the placenta, which, in turn, has a direct impact on the cell types that can be isolated from the placenta at different gestational ages.

The genetic and molecular mechanisms behind placental development have hardly begun to be clarified and should also have a bearing on the pluripotency of placental cells. Interestingly, against the conventional wisdom of searching for placental-specific genes that would control this process, most of the genes that have been shown to be essential for placental development are also involved in the development of other organs [83]. No more than a very limited set of genes is expressed exclusively in the placenta [83].

Since much of the placenta comes from the inner cell mass of the morula, the presence of embryonic progenitor cells in the placenta has long been proposed. More specific types of stem cells, such as trophoblastic, hematopoietic, and MSCs have also been identified in the placenta. Trophoblast stem cells, also known as trophoendoderm stem cells, are defined as cells with the potential to give rise to all differentiated trophoblast cell subtypes, as well as to yolk sac phenotypes. Interestingly, one trophoblast subpopulation, the cytotrophoblasts of the basal decidua, may undergo a full transition to mesenchymal phenotype when they infiltrate maternal mesenchymally-derived uterine stroma and arterial walls. At present, trophoblast stem cells have limited, if any, foreseeable potential for clinical application and will not be reviewed here. Placental/umbilical hematopoietic stem cells have already shown high clinical relevance, but can also be isolated from umbilical cord blood and will be reviewed in another chapter. Placental mesenchymal stem cells (pMSCs) and more primitive, ESCs will be further discussed below.

pMSCs

As described above, the placenta has a large mesenchymal component. While the role of this component in placental development has yet to be better understood, we already know that it relates, in part, to the pluripotent potential of pMSCs. For example, recruitment of these cells supports the so-called vasculogenesis that occur during vascularization of the villous sprouts, in addition to the angiogenesis based on the proliferation of endothelial precursors. These mesenchymal cells also play other roles in placental development, such as the paracrine signals that they send to control the stability of the cytotrophoblast column, which in turn determines the degree of trophoblast invasiveness. Yet, much about the mesenchymal core of the placenta remains to be elucidated.

pMSCs can be isolated by a number of different protocols. We have described an easily reproducible method, analogous to the one described above for the separation of aMSCs (i.e., based on mechanical separation and natural selection by the culture medium), which can be employed in both 'full thickness' and chorionic villus sampling placental specimens [29,65]. These cells have unique characteristics when compared with other mesenchymal cells. They proliferate more quickly in culture than comparable cells harvested from fetal or adult tissue, at a similar rate to that of aMSCs [29,65]. Also like aMSCs, they are often stained by monoclonal cytokeratin antibodies, which is a rare finding in mature mesenchymal cells, but common in fetal and umbilical stroma, smooth muscle tumors, and stromal cells associated with reactive processes. It is as yet unclear whether this immunoreactivity is a result of cross-reactivity between a common epitope found in smooth muscle cells, myofibroblasts, and intermediate-sized filaments of cytokeratin, or whether it actually indicates cytokeratin expression in non-epithelial cells.

In addition to their natural differentiation into smooth muscle cells during placental development, pMSCs have been shown to be able to differentiate into all mesodermal, as well as some neural cells lineages, *in vitro* [84,85]. Given the many similarities between pMSCs and aMSCs, as shown above, it is reasonable to speculate that these two cell subsets are actually the same. This, however, remains to be unequivocally demonstrated.

Embryonic-like stem cells

The embryonary origin of the placenta renders it a natural candidate for a reservoir of ESCs. From the few reported attempts to isolate ESCs from the placenta, like their amniotic counterparts, what could be ESCs are present in zero to less than 1% of the cells present in placental samples. These cells have also been identified mostly through certain markers, including CD34, CD105, and cKit, that, again, were not necessarily concomitantly expressed and are not exclusive of ESCs.

These pluripotent cells have shown potential for self-renewal, but a uniform and universal differentiation potential of these cells also remains to be verified. Like their amniotic counterparts, thus far some of these cells have been shown to differentiate into a variety of cell types, but not necessarily from a uniform population of undifferentiated cells. Another limitation of the existing data is the fact that the placenta is rich in hematopoietic stem cells, known not to be committed solely to the hematopoietic lineage. In addition to blood cells, they can also give rise, at least, to neurons, hepatocytes, and muscle cells, and might have contributed to the differentiation findings reported to date. Again, although the existing data is promising and in accordance with the origins of the placenta, final proof that true ESCs can be consistently isolated from that organ, and at which gestational ages, remains to be verified.

Another interesting potential development of the study of placental cells is the establishment of 3D models of the placenta and of maternal-fetal circulation through the maintenance of live engineered placental models in bioreactors. This could lead to a better understanding of normal and pathological placental physiology, possibly with indirect therapeutic benefits.

MATERNAL BLOOD

Fetal cells can be documented in the maternal circulation in the majority of human pregnancies. Fetal progenitor cells have been found to persist in the circulation of women for up to decades after childbirth [10]. Of particular interest, a novel population of fetal cells, often termed pregnancy-associated progenitor cells (PAPCs), appears to differentiate in diseased/injured maternal tissue, apparently providing some degree of local repair. The precise phenotypical identity of these cells, however, remains controversial. They have been considered to be a hematopoietic stem cell, a MSC, or possibly a novel cell type. At this time, peripheral maternal blood has yet to be proven as a viable source of fetal cells in consistently high enough numbers for tissue-engineering applications. This appealing perspective, however, surely deserves continued scrutiny.

ETHICAL CONSIDERATIONS

The use of fetal tissue has always been object of intense ethical debate. The main reason for the ethical controversies comes from the fact that the primary source of fetal tissue is induced abortion. Spontaneous abortion usually does not raise many moral issues. The National Institutes of Health, the American Obstetrical and Gynecological Society and the American Fertility Society, in accordance with the provisions that control the use of adult human tissue, have been regulating the use of fetal specimens from this latter source for decades. However, spontaneous abortion generally yields unsuitable fetal tissue, as it is frequently compromised by pathology such as chromosomal abnormalities, infections and/or anoxia. Arguments over the use of fetal tissue from induced abortion are largely based on somewhat limited scientific evidence, along with clashing religious and customary beliefs about the beginning of life. Not surprisingly, despite the efforts of numerous national and international ethical committees and governmental bodies, a consensus has not yet been reached. In the US, in spite (or perhaps because) of an ongoing moratorium on federal funding for fetal tissue transplantation research, an agreement on this issue may be slowly forming, albeit a stable solution should still be years away.

This polemic notwithstanding, tissue engineering, as a relatively novel development in fetal tissue processing, adds a new dimension to the discussion concerning the use of fetal tissue for therapeutic or research purposes. If specimens from a live, diseased fetus, or cells from a routine prenatal diagnostic procedure such as an amniocentesis or chorionic villus sampling, are to be used for the engineering of tissue, which in turn is to be implanted in autologous fashion, no ethical objections should be anticipated, as long as the procedure is a valid therapeutic choice for a given perinatal condition. In that scenario, ethical considerations are the very same that apply to any fetal intervention. On the other hand, if fetal-engineered tissue

is to be implanted in heterologous fashion, ethical issues are analog to the ones involving fetal tissue/organ transplantation, regardless of whether the original specimen comes from a live or deceased fetus, or from banked fetal cells obtained from the amniotic, placenta, or umbilical cord blood.

The distinction between autologous and heterologous implantation of engineered fetal tissue is a critical one in that, again, no condemnation of autologous use could be ethically justified. At the same time, regardless of whether an autologous or heterologous application is being considered, should the amniotic fluid or fetal annexes be definitely confirmed as dependable sources of ESCs, the ethical objections to embryo disposal now plaguing the progress of ESC research could possibly be avoided.

THE FETUS AS A TRANSPLANTATION HOST

One could envision a number of advantages of a fetus receiving an engineered construct *in utero*, not only from a theoretical perspective, but also from clinical and experimental evidence derived from intra-uterine cellular transplantation studies already reported. Those potential advantages encompass: induction of graft tolerance in the fetus, due to its immunologic immaturity; induction of donor-specific tolerance in the fetus by concurrent or previous intra-uterine transplantation of hematopoietic progenitor cells; a completely sterile environment; the presence of hormones, cytokines and other intercellular signaling factors that may enhance graft survival and development; the unique wound healing properties of the fetus; and early prevention of clinical manifestations of disease, before they can cause irreversible damage. Most of those advantages should be more or less evident, depending on the gestational timing of transplantation.

Fetal immune development

Among the potential benefits of *in utero* transplantation, the singularity of the fetal immune system deserves special attention. In that respect, basic research on fetal development, as well as studies involving pre and postnatal transplantation of lymphohematopoietic fetal cells have contributed to a better understanding of the fetal immune response.

Fetal tolerance resulting in permanent chimerism has long been shown to occur in nature in non-identical twins with shared placental circulation [86,87]. Little is known, however, about precisely when and by what mechanism this tolerance is lost. During fetal development, the precursors of the hematopoietic stem cells arise in the yolk sac, migrate to the fetal liver and then to the thymus, spleen and bone marrow. The fetal liver has its highest concentration of hematopoietic stem cells between the 4th and the 20th week of gestation. Because of their cellular immunologic 'immaturity', the fetal liver and, to a lesser extent, the fetal thymus have been studied as potential sources of hematopoietic stem cells for major histocompatibility complex/incompatible bone marrow transplantation for more than half a century now. Umbilical cord blood has been increasingly employed as a source of hematopoietic stem cells, in both autologous and heterologous applications [88,89].

Lymphocytes capable of eliciting graft versus host disease (GVHD) are found in the thymus by the 14th week of gestation, but not detectable in the liver until the 18th week. Despite considerable numbers of granulocyte-macrophage colony-forming cells, there is an almost complete absence of mature T cells up to the 14th week in human fetal livers. During gestation, while B cell development takes place mostly in the liver, T cell development occurs predominantly in the thymus. This fact is probably the reason why fetal liver cells are immunoincompetent for cell-mediated and T cell-supported humoral reactions, such as graft rejections and GVHD. Thus, in principle, tissue matching is not necessary in fetal liver transplantation, if this is harvested up to a certain point in gestation. In a number of animal models and small clinical series, fetal liver cells have induced no or merely moderate GVHD in

histoincompatible donor/recipient pairs. More recently, unique sub-populations of innate lymphoid cells (ILCs), a family of effectors and regulators of innate immunity and tissue remodeling, have been isolated from fetal lung and intestine [90]. The significance of this finding and its potential translational implications has only started to be explored.

Although fetal liver stem cells should not cause GVHD, they could still be subject to rejection. Because of this, fetal liver stem cell transplantation has been attempted in the clinical setting preferably in patients with depressed immune function, such as in immunodeficiencies, replacement therapy during bone marrow compromise and during fetal life (*in utero* transplantation). The same principle applies to the use of fetal thymus. Fatal cases of GVHD are considered much less likely in patients who receive fetal liver stem cells harvested before the 14th week of gestation. This complication, however, has been reported in a patient who received liver cells from a 16-week-old fetus [91]. With umbilical cord blood stem cell transplantation, the incidence of GVHD has been minimal [88,89]. Umbilical cord blood-derived MSCs have also been shown to help prevent (but not treat) GVHD in a rodent model [92].

On the other hand, umbilical cord and placental blood, while rich in hematopoietic progenitor cells, contain alloreactive lymphocytes. Although those lymphocytes are also immature, it is unclear, however, whether they are more or less reactive than adult ones. Compared with those from adult blood, the proportions of activated T cells and helper-inducer subsets (CD4/29) are significantly reduced, while the helper-suppressor (CD4/45A) subset is significantly increased [93]. Cord blood natural killer cell activity is low or similar to that in adult blood, but lymphokine-activated killer cell activity may be higher.

Another germane aspect of intra-uterine transplantation is the fact that maternal cells trafficking into the fetus may pose as the chief barrier to effective engraftment of allogenenic cells or tissues delivered prenatally. It has been recently demonstrated experimentally that there may be macrochimerism of maternal leukocytes in the fetal blood of, with substantial increases in T cell trafficking after intra-uterine transplantation [94,95]. This suggests that the clinical success of intra-uterine transplantation, at least of hematopoietic stem cells, may be improved by transplanting cells also matched to the mother.

APPLICATIONS OF *IN UTERO* TRANSPLANTATION

Cellular intra-uterine transplantation has been employed clinically for decades now, to treat a variety of diseases, including lymphohematopoietic diseases, beta-thalassemia, inborn errors of metabolism and genetic disorders, with some success. Knowledge of the optimal gestational age for transplantation, along with cell selection, route of cell administration and post-interventional tocolysis is still evolving. *In utero* hematopoietic stem cell transplantation is a non-myeloablative approach to achieving mixed hematopoietic chimerism and associated donor-specific tolerance, improving survival of other grafts later in life. Through prenatal transplantation of hematopoietic progenitor cells, both allogeneic and xenogeneic chimerisms have been induced in animal models and allogeneic chimerism has been achieved in humans [15,96–98]. A recent development in prenatal cell transplantation is neural stem cell delivery to the fetal spinal cord as a means of reversing at least some of the local damage associated with neural tube defects, i.e., different variations of spina bifida [13].

Tolerance of allogeneic intra-uterine implantation of an engineered construct has recently been first demonstrated in an ovine model of fetal tracheal reconstruction with heterologous cartilage engineered from aMSCs [33]. In like manner, tolerance to an airway construct made with autologous aMSCs and a xenologous decellularized matrix has also been shown in a fetal ovine model [43]. Clinically, structural airway anomalies could be justifiably repaired prenatally in select patients with defects so severe that breathing would otherwise be simply impossible at birth. Further potential benefits of prenatal implantation of engineered tissue,

such as for example advantages stemming from the unique wound healing capabilities of the fetus, among other potentially unsuspected ones, are yet to be fully explored.

CONCLUSIONS

Fetal tissue engineering may become a preferred perinatal alternative for the treatment of a number of birth defects. Given the recently proven viability of minimally invasive fetal cell sources, such as amniotic fluid, placenta, and umbilical cord blood, the promise of fetal tissue engineering applies to both life-threatening and non-life-threatening anomalies. Fetal progenitor cells from various sources are progressively becoming relevant, if not indispensable tools in research related to stem cells, tissue engineering, gene therapy, and maternal-fetal medicine.

Still, much remains to be learned and a variety of evolutionary paths, including unsuspected ones, have yet to be pursued in this relatively new branch of tissue engineering. The whole subfield of *in utero* implantation of engineered tissue also waits to be fully explored. Fetal tissue engineering shall also benefit from the progress expected for the different aspects of tissue engineering in general. Fertile experimental work from an increasing number of groups has introduced promising novel therapeutic concepts utilizing fetal cells. As long as progress is made in the ethical debate over the use of these cells and their banking, the reach of fetal tissue engineering, nonetheless, will likely go beyond the perinatal period, offering unique therapeutic perspectives for different age groups.

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CHAPTER 27

Immunomodulation

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INTRODUCTION

A long-standing goal of the transplant community has been to overcome the immunological rejection of transplanted tissues and organs. Although much of the research has been devoted to identifying new immunosuppressive agents and new combinations of existing agents for allotransplantation, immunosuppression still carries significant long-term side effects, especially enhanced susceptibility to infection. For xenografts, even stronger immunosuppression is necessary. One solution to allograft or xenograft acceptance may reside in novel therapeutic strategies that strive to prevent the need for long-term and high-dose immunosuppression.

Our laboratory has attempted to avert transplant rejection by immunologically modifying the foreign proteins on cells and tissues from the donor, instead of treating the host. Tissues and cells treated before transplantation allow the concealment or elimination of the antigens that summon immune rejection. This technology is sometimes referred to as 'donor antigen modification' or, more stylistically, 'designer tissue and organs' [23]. The goal is to avoid or reduce the need for immunosuppression by directly modifying the graft at the molecular or protein level. Modification at the *cellular* level, by eliminating donor lymphoid cell populations, had been previously successful in murine allografts. These cellular modifications were effective for whole organs and cell transplants in lower species, even for the avoidance of xenograft rejection. Designer tissues and organs modified at the *molecular* or *protein* level offer an alternative approach with greater selectivity and latitude in tailoring the graft to escape immune detection by the host.

Without the risks of massive doses of systemic immunosuppression, designer tissues and organs could be therapeutic for a broad range of chronic conditions that are not life threatening. They also could be offered to conventional organ transplant patients at earlier stages of their disease, when patients are healthier and better able to withstand surgical intervention. The main risk with designer tissues and organs is that the modified donor tissue could be rejected, a risk commonly encountered with any transplant. Because designer tissue can be potentially rendered safer and may ultimately avoid host intervention with toxic drugs, a broader spectrum of diseases could be treated and at earlier time points.

The purpose of this chapter is to describe the evolution of research into designer tissues, and to trace its contribution to a growing body of transplant research. The first success was with murine hosts given transplants of xenogeneic cells and tissues that had been modified at the *protein* level. This success allowed extension of the concept to the molecular level in the form of DNA modifications. This ability to modify proteins at the DNA, RNA, or protein level offers tremendous versatility in developing new cells or organs as biological therapies. Variations on the designer tissue concept are being probed in animal models of diabetes, solid organ failure, and neurological diseases. A concept launched in the laboratory in the early 1990s has already

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culminated in landmark human clinical trials for Parkinson's and Huntington's disease using donor-modified pig neurons in humans.

The wide range of applications of designer tissues and organs for allo- and xenotransplantation is readily apparent; modified donor cells, tissues, and organs can be considered potential therapies for an almost limitless number of conditions as long as the dominant antigens are identified and then effectively shielded or eliminated prior to implantation.

ORIGIN OF THE DESIGNER TISSUE CONCEPT

The idea of targeting donor antigens instead of modifying the host immune system was stimulated by research on the molecular events surrounding the killing of cancer cells by cytotoxic T lymphocytes (CTLs; hereinafter referred to as T cells). In an elegant study, Spits and co-workers [1] identified the sequential stages of T cell destruction of the cancer cell. In contrast to earlier research, which focused primarily on the T cell, they examined the roles of cell surface markers on both the T cell and the target cell. By using antibodies against different cell surface markers on the cancer cells they were able to block distinct obligatory stages of T cell activation and destruction. They were able to tease apart *in vitro* the interactions between molecules on the T cell and the target cell. Spits and colleagues were among the first to identify three stages in T cell cytotoxicity:

1) Adhesion between T cell and target cell through two adhesion proteins,
2) T cell receptor activation through class I, and
3) T cell lysis of the tumor target cell through persistent class I and T cell receptor binding.

A critical finding was that one of the major classes of histocompatibility antigens on the surface of the cancer cell — the major histocompatibility complex (MHC) class I molecules — was involved in both adhesion to, and activation of, the T cell. Class I antigens and other classes of antigens encoded by the MHC complex serve to distinguish 'self' from 'non-self' because they differ across species and between members of the same species. Class I antigens had long been suspected of eliciting T cell cytotoxicity, but this study offered more detailed insight into their pivotal role. What this study also demonstrated was that antibodies to class I antigens on the cancer cell could block T cell adhesion and activation, thereby preventing lysis of the target cell. Indeed, further studies in the cancer field over the years have endeavored to dissect how this escape mechanism of down regulation of MHC class I is used by tumor cells naturally as a selection process to avoid immune detection [2].

Spits and co-workers crystallized the importance of class I antigens in immune rejection of cancer cells. While this study's goal was to dissect and promote CTL killing of the target, our goal was the opposite: to prevent tissue rejection at the same T cell target interface. We chose to 'mask' class I antigens on donor cells by using antibody fragments to class I, a system that prevented death of the target, and then transplant the modified donor cells into non-immunosuppressed hosts.

FIRST DEMONSTRATION OF THE CONCEPT

The first successful demonstration of the designer tissue concept used a xenogeneic model [23]. The cellular graft in the form of insulin secreting islets was coated with antibody fragments to conceal class I antigens, the grafted cells (of human origin) indefinitely eluded the immune system of the murine host. The grafted cells also functioned normally. The host even developed tolerance to the treated graft, as secondary transplants of untreated tissue were later accepted. The mechanism of donor-specific tolerance is still not fully defined, but may involve induction of T cell anergy through altered donor class I density. Altered class I density may be pivotal in T cell shaping in both the periphery as well as the thymus, as defined by Pestano et al. [3].

The graft consisted of purified human cadaveric islets that had been incubated with antibody fragments before being implanted into non-immunosuppressed mice. Pure antibody fragments that lack the portion of the antibody molecule that binds complement, the Fc fragment, were obligatory to prevent lysis of the target. When the Fc fragment is enzymatically cleaved from the $F(ab')_2$ fragment, the purified $F(ab')_2$ fragment binds to the graft for several days without fixing complement; this prevents the graft from being destroyed. Grafts survived for 200 days and functioned appropriately, as determined by assays for human C′-peptide, a proinsulin processing product. Finally, human liver cells similarly treated with antibody fragments also survived for an extended period.

Treatment with whole antibodies to class I antigens failed to prolong graft survival. Whole antibody class I proteins coated the donor cell, but also, upon transplantation, killed the cell due to host derived complement. Treatment with antibodies and antibody fragments to CD29 was effective in an allogeneic transplant barrier using cells, but not in a xenogeneic barrier. CD29 is an antigen with restricted expression on the passenger lymphocytes that accompany the graft. Passenger lymphocyte elimination can be important for allografts but less important for cross-species transplants. Treatment of cellular grafts with polyclonal antibody fragments to all antigenic determinants prior to transplantation did prolong allograft, and xenograft survival. Polyclonal antibody fragments with class I antibody fragment removal had minimal effects at facilitating cellular transplants.

For the sake of rigor, it is generally accepted that xenografts represent a more challenging transplant barrier than allografts. Furthermore, if experimental test concepts are applied to xenografts with success, similarly enhanced allograft survival is likely to ensue. If designer tissues could succeed in a difficult xenogeneic case, then these same procedures could be considered for simpler allogeneic transplant models. Additionally, cellular transplant models represent the intermediate model of simplicity. In the setting of cells, the graft tissue contains one dominant antigen that needs to be masked. For instance, human islet tissue stongly expresses class I antigens, while displaying only minimal expression of two adhesion molecules, intercellular adhesion molecule-I (ICAM-I), and lymphocyte function-associated antigen-3 (LFA-3) (which, in other tissues, are thought to stabilize binding and to contribute to T cell activation). Second, xenogeneic cellular transplants lack the vasculature that can be separated from the tissue to avoid hyperacute rejection, the earliest and most formidable barrier to discordant xenograft acceptance. Third, targeted antigens at the surface protein level, rather than at the genetic level, allow greater flexibility and less expense than the creation of genetically engineered pigs. Our goal was to conceal protein antigens that already appeared on the surface of graft cells. Other approaches, discussed later in this chapter, target antigens not at the protein level, but at the DNA and RNA level. The use of more sophisticated transgenic and anti-sense technology, respectively, can similarly prevent antigen expression. They also can validate the donor antigens as a target for the immune response.

In summary, this tough xenogeneic model establishes the paramount role of class I antigens on cellular transplants. By providing a challenging test of the designer tissue concept, it also helped to launch a novel therapeutic strategy.

EXPANSION OF RESEARCH ON DESIGNER TISSUES

The creation of designer tissues to produce tissues and organs with reduced surface proteins can be achieved using a variety of donor antigen modification techniques in various transplantation settings. This body of early research is, in part, summarized in Table 27.1 and can be classified by the method that interferes with the expression of the surface protein of interest. The methods to remove or disguise surface proteins in the donor cells can include antibody 'masking', gene ablation, anti-sense, and enzymatic treatments, and are the topic of this review.

TABLE 27.1 Designer donor tissues

Technique/Tissues	Allo/xeno	Donor/recipient	Target	Reference
Masking antibody				
Islets	Xeno	Human/mouse	Class I	[23]
Islets	Xeno	Human/mouse	All surface antigens	[23]
Islets	Allo/xeno	Human or monkey/monkey	Class I	[8]
Islets	Allo	Mouse/mouse	Class I	[4–6]
Neurons	Xeno	Pig/rat	Class I	[4–6], [9]
Neurons	Xeno	Pig/monkey	Class I	[10]
Liver cells	Xeno	Human/mouse	Class I	[23]
Gene Ablation				
Islets	Allo	Mouse/mouse	Class I	[12]
Islets	Allo	Mouse/mouse	Class I	[13]
Islets	Allo	Mouse/mouse	Class I	[4–6]
Liver cells	Allo/xeno	Mouse/mouse/ guinea pig/frog	Class I	[14]
Kidneys	Allo	Mouse/mouse	Class I	[11]

ANTIBODY MASKING

Antibody masking of xenogeneic tissue is the first of the donor antigen modification techniques to progress to primate and human trials. Pancreatic islet cells and neurons are the most common types of donor cells to be camouflaged with antibody masking, although any type of tissue can theoretically be treated once the dominant antigens have been identified. All of the studies cited below targeted class I antigens using antibody fragments and relate to cellular transplants.

Islet cell masking for the treatment of diabetes was early on pursued with mixed results. Osorio and colleagues [4–7] targeted class I antigens in a mouse allograft model. To approximate a diabetic state, the mice first were treated with a drug that chemically induced hyperglycemia. Then they received islet allografts that had been pretreated with antibody fragments. Graft survival was prolonged relative to controls, but within one month the grafts were eventually rejected. Investigators attributed the eventual allograft rejection to a variety of possibilities, including the absence of sufficient quantities of $F(ab')_2$ fragments, antiidiotypic antibodies against the $F(ab')_2$ fragment, and an immune pathway independent of class I activation of T cells. Steele and colleagues [8] investigated islet cell transplants in a primate model. Cynomolgus monkeys received either allogeneic or xenogeneic (human) islets. The grafts were pretreated with antibody fragments to class I antigens. Histologic evidence revealed that donor islets were present months after transplantation into non-immunosuppressed monkeys.

Neuronal xenografts with antibody masking have been investigated for Huntington's disease [9] and Parkinson's disease [10]. In the first study, fetal pig striatal cells were implanted into rats whose striatum had been lesioned one week earlier with injections of quinolinic acid. These injections destroy striatal neurons in an attempt to simulate the dysfunction present in Huntington's disease. Rats received either untreated tissue or tissue pretreated with $F(ab')_2$ fragments against porcine class I antigens. Control rats receiving untreated tissue were either immunosuppressed with the immunosuppressant cyclosporin A (CsA) or left untreated. Three to four months later, graft survival was found to be prolonged in animals given $F(ab')_2$-treated grafts and in the CsA-treated control animals given unaltered grafts. Grafts did not survive in non-immunosuppressed controls. Graft volume, determined histologically with the aid of computer image analysis, was significantly larger in the CsA group compared with the $F(ab')_2$ group. Yet, in both of these groups, immunohistochemistry revealed the graft cytoarchitecture to be well organized, and graft axons to have grown correctly in the direction of their target

nuclei. It was encouraging that pig neurons appeared to be capable of locating their murine target.

In a similar study design, Burns and co-workers [10] applied antibody masking to a primate model of Parkinson's disease. Porcine mesencephalic neuroblasts were implanted into monkeys with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism. Measures of locomotor activity and dopamine fiber density in the host striatum confirmed that pre-treatment of donor tissue with $F(ab')_2$ fragments succeeded in restoring motor function and replenishing dopamine fibers at the site of implantation. A control animal, maintained on CsA after receiving untreated cells, showed similar improvement. A non-immunosuppressed control showed no improvement after transplantation, suggesting graft rejection.

The studies described in this section highlight the range of potential applications of antibody masking in both allo- and xenotransplants. Neuronal cell masking has been sufficiently effective to usher in human clinical trials.

GENE ABLATION

Gene ablation, or gene 'knockout' technology, offers another vehicle to modify donor tissue and organs. With gene ablation, the gene or genes encoding protein antigens can be permanently deleted, thereby eliminating antigen expression in all cells. In one common method of gene ablation, the target gene is inactivated in cultured embryonic stem cells via homologous recombination; a target vector containing an inactive version of the gene recombines with, and thereby replaces, the wild-type gene. Through reintroduction of the embryonic cells into a foster mother and through selective breeding, progeny can be produced that are homozygous for the mutation. Rejection by the host immune system is expected to be avoided when the targeted gene encodes a protein antigen slated for expression on the surface of donor cells.

The major advantage of gene ablation over protein modification of donor tissue is that the antigen is permanently eliminated in all cells, tissues, and organs in which it is expressed. The major limitation of this technology is that it is restricted to potential xenografts from pigs and requires pig 'farming', an expensive, albeit potentially abundant, source of donor tissue. The permanent nature of the modification also can sometimes be a limitation, especially when the protein encoded by the gene has additional functions. Inactivation of the gene might lead to physiological changes that compromise the utility of the donor tissue. Gene ablation can also target genes that encode proteins essential for the target protein expression.

Several transplantation laboratories have exploited the availability of knockout mice deficient in $ß_2$-microglobulin. $ß_2$-microglobulin is a peptide that performs a chaperone function as part of the class I molecule and is necessary for its assembly and expression. Mice homozygous for the $ß_2$-microglobulin mutation fail to display class I antigens on the cell surface, a feature that makes them highly desirable for transplantation studies. Class I ablation through the mutation is not necessarily a permanent depletion of class I due to host $ß_2$-microglobulin reconstituting the graft. Although much of the research described below has focused on islet cell and liver cell xenotransplants, one project examined whole organ allografts. Coffman and co-workers [11] found that kidneys from $ß_2$-microglobulin-deficient mice functioned significantly better in allogeneic recipients than did kidneys from normal mice.

The fate of pancreatic islet transplants from $ß_2$-microglobulin-deficient mice has been explored in several studies. The islets showed prolonged survival when implanted into a normal mouse strain [12,13]. Most grafts survived indefinitely (>80% beyond 100 days) and were capable of reversing hyperglycemia that had been chemically induced prior to transplantation. Investigators attributed the few instances of graft rejection to three possibilities. First, some surface expression of class I antigens can occur in the absence of $ß_2$-microglobulin. The presence of even a fraction of the total number of class I antigens may be sufficient for T cell recognition and lysis. Second,

ß$_2$-microglobulin circulating in the serum of the recipient can be used to reconstitute class I antigens on the donor tissue (because ß$_2$-microglobulin is a highly conserved protein not encoded by genes at the MHC locus). Third, rejection of the tissue from ß$_2$-microglobulin-deficient donors may be mediated by other immune pathways, such as by natural killer cells. Support for the second and third possibilities was presented by Li and Faustman [14] in their study of liver cell allo- and xenografts. Other studies also confirmed that masking of human leukocyte antigen (HLA) also enhanced susceptibility of cells to natural killer cells [15].

For whole organ xenografts, it has long been recognized that certain sugars on donor tissues especially the vascular endothelium, such as gal α(1,3) galactosyltransferase have a central role in hyperacute rejection. This hyperacute rejection is mediated by preformed antibodies in disparate species, often referred to as natural antibodies [16,17]. If hyperacute rejection of whole organ xenografts can be prevented, the other cellular barriers, such as disparate class I expression, can be addressed.

Given the important role of donor sugar epitopes in eliciting rapid organ rejection, knockout pigs were produced with the first strategy applied to donor antigen modification for this epitope. The pigs had complete elimination of the gene that encoded α1,3galactosyl-transferase. To the surprise of the scientific community, the knockout pigs continued to express considerable levels of the carbohydrate sugars.

Therefore, in the pig, more than one genetically encoded enzyme is able to successfully synthesize Gal α(1,3) gal surface sugars [18]. These studies demonstrate that although three prior methods can change donor antigen expression in cells, i.e., gene ablation, anti-sense for RNA, or 'masking' antibodies, some methods may have unexpected shortcomings specific to the interruption method.

RNA ABLATION

RNA ablation is another strategy that strives to prevent antigen expression by blocking gene transcription or translation. RNA ablation can be achieved through the creation of oligo-deoxynucleotides that are complementary to, and hybridize with, DNA or RNA sequences to inhibit transcription or translation, respectively.

To date, RNA ablation in the transplant field has been less frequently pursued. Ramanathan and co-workers identified an oligodeoxynucleotide that inhibited induction of class I and ICAM expression by interferon-γ [19]. The studies were performed *in vitro* in a cell line, K562, that normally has low-level expression of class I antigens. They first postulated that the oligodeoxynucleotide acted in the early stages of interferon-γ induction rather than post-translationally. In their follow-up study, they showed that the oligodeoxynucleotide acted even earlier via a novel mechanism: it inhibited binding of interferon-γ to the cell surface [19,20]. This may be an unusual mechanism for an oligodeoxynucleotide,, but it only enhances the possibilities for xenotransplant research. RNA ablation, unlike protein modifications with masking or DNA modifications in transgenic donors, offers an additional challenge. For RNA ablation to work, all cells must be equally treated with the interfering RNA. Anti-sense technology or even newer methods such as siRNA often result in uneven distributions of interruptions in the cells or organs. What these studies provide is yet another means of blocking expression of class I antigens or other transplantation antigens.

ENZYME ABLATION

Although MHC class I surface structures are extremely polymorphic, the polymorphisms are predominately confined to the exterior region of the protein and the regions of the protein that are exterior facing. This feature allows the host's immune system, i.e. T cells, the early opportunity to reject tissues transplanted from unrelated individuals.

As the genome effort has advanced and hundreds of MHC class I alleles of the gene have been sequenced, it has also become apparent that certain regions of the MHC structure are highly polymorphic in contrast to other regions of the protein that are highly conserved. Even across species, the conserved regions of the MHC allele compose the protein portions that are near and within the cell surface membrane. This conservation of structure is maintained across species as diverse as mice and humans.

The conserved regions of MHC class I within and across species have afforded, in solubilized cells, the opportunity to specifically purify these proteins by enzymatic cleavage. Most typically, cell lysates have been treated with papain to solubilize all MHC structures from the cell surface, usually at very acid pHs [21]. Papain has the remarkable ability to cleave the conserved hydrophilic regions of the class I protein without cleaving other cellular proteins [22]. Papain's conserved cleavage feature is preserved for mouse, human, and primate class I alleles.

We have gradually worked with this system in mouse transplant model to allow papain to cleave class I from all cells and have the cells remain fully viable at the end of the brief treatment. This MHC class I modification method, comprising pre-treatment with papain with modified buffers at physiological pH, now allows complete class I depletion and subsequent transplantation of cell transplants with prolonged survival. Survival of the transplanted cells in the form of insulin secreting islets or liver cells is comparable to either methods of genetically ablated class I deficient tissues or 'masked' class I deficient tissues.

Each method of modifying donor cell surface proteins, such as MHC class I, prior to transplantation has features that are unique to the translation of these methods to clinical trials (Table 27.2). The production of genetically-engineered donor pigs permits the generation of abundant tissue, but the donors' tissue will be xenografts, and will thus encounter tough

TABLE 27.2 Positive and negative features of different methods of donor antigen modification compared to systemic immunosuppressants

	Host treatment	Donor tissue modification*		
	Immunosuppression	Donor modification		
	Chemicals/biologics	Transgenic pigs	Masking abs	Enzymatic
Experimental Time for Efficacy Testing				
Animals	+++	+++	++	++(limited)
Humans	+++	N/A	+/−	N/A
Safety				
Side Effects Profiles	+++	None	None +/−	None
Applicability/Market Size				
Allografts	+++	0	+++	+++
Xenografts	0	+++	+++	+++
Development Costs and Time				
Basic Research	Large	Very Large	Average	Inexpensive
Clinical Testing	Large	Very Large	Average	Moderate
Manufacturing				
Feasibility	Moderate	Difficult	Moderate	Easy
Costs	Moderate	High	Moderate	Low
Regulatory/FDA				
Frequency	Proven	No Prior Approvals; Xenosis	No Prior Approvals	+/−
Time-line	Average	Very Long	Long	Short

**Anti-sense or siRNA technology has had limited success in settings of cell or tissue transplantation and thus is not represented in the table as a method for donor antigen modification*

transplant barriers and high xenosis risk. Also, the cost of developing the herds of animals is very high. The generation of masking antibodies allows the donor tissue modifications to be applied to allografts or xenografts. The masking antibodies are protein fragments and can be designed to different donor HLA types. The production of masking antibodies is easier than the production of genetically engineered pigs, but requires manufacturing of a novel protein fragment and standardization for Food and Drug Administration (FDA) approval. Also the pre-clinical efficacy studies using masking antibodies would be specific for the species chosen, thus the translation to human studies would not use an identical antibody fragment. Finally, the use of specific enzymes to cleave off donor antigens such as MHC class I is inexpensive to develop and the same enzyme product could be applied to pre-clinical murine studies, baboon studies, and human indications. All three donor-tissue modifications could be combined with reduced or eliminated immunosupression, thus increasing the safety margins for cellular transplants for non-lethal diseases.

MECHANISMS OF GRAFT SURVIVAL AFTER CLASS I DONOR ABLATION OR ANTIBODY MASKING

The ability of class I modified tissues or cells to survive long term without host immuosuppression has been closely studied. Indeed, it was appreciated that although the method for altering donor class I expression prior to transplantation could be diverse, i.e., 'masking' antibodies, gene ablation of chaperone proteins or enzymes, a common theme in these models was the long-term stable survival of the functioning tissue. The transplants were viable and certainly re-expressed donor class I with the passage of time. Insight into the role of systemic tolerance in these transplants is important for future refinements of cellular transplant methods to optimize success.

Systemic tolerance of the graft was initially addressed for donor tissues with modified class I due to ablation of the class I chaperone protein, i.e., ß$_2$-microglobulin. First, as the donor and recipient diverge phylogenetically, so does the homology of the ß$_2$-microglobulin proteins. Donor grafts from highly divergent species have slower reconstitution of surface class I with ß$_2$-microglobulin from the host serum, and also shorter survival times [24]. The shortened survival times compared to less divergent cross-species transplants suggests some degree of donor class I antigen expression is beneficial after the transplant is established. Indeed, many have proposed the intact natural killer cells of the host, a natural defense against class I deficient tumors, as the reason for more brisk rejection, with more permanent class I depletion.

The reconstitution of donor surface class I with host ß$_2$-microglobulin in the gene ablation model also explains why transient ways of interruption class I allow systemic tolerance. The primary cellular grafts with transient class I interruption permit secondary, non-genetically manipulated or non-antibody-masked grafts to survive — if from the same donor [23]. The data using the 'masking' method to conceal class I, taken together, suggest donor-specific tolerance occurs in these transplant models.

Insights into the mechanisms of T cell tolerance in hosts receiving transient class I-ablated grafts may have been clarified. In a publication by the Cantor laboratory, peripheral CD8 T cell tolerance was mechanistically characterized in terms of maintenance of peripheral tolerance [3]. It has been recognized in some experimental settings that the persistence of peripheral class I-expressing cells is necessary for peripheral CD8+ T cell tolerance [25]. Using ß$_2$-microglobulin-deficient mice, potentially cytotoxic and mature CD8 T cells from a normal donor were transferred into the mutant host. The transferred T cells then failed to engage their T cell receptors. The CD8 cells downregulated their CD8 gene expression and underwent apoptosis. Thus, inhibition of, or interference with, the T cell receptor and CD8 binding to host class I triggered these CD8 cells into a pathway of cell death. This is an important observation, and lack of class I expression immediately after transplantation eliminated

potential direct and immediate T cell killing. As the genetically modified graft gradually re-expresses class I, it is protected from the next layer of the immune response, natural killer cell lysis of totally class I-negative transplants, and active host tolerance is additionally achieved.

Elimination of class I antigens also may be insufficient for select tissues and select combinations of donor and host species. Liver cells from ß$_2$-microglobulin-deficient donors were implanted into allogeneic recipients and two different xenogeneic recipients [14]. The allotransplants and the xenotransplants into guinea pigs were not effective. In contrast, xenotransplants of mouse cells into frog recipients survived, and when liver cells were transplanted from humans to mice in antibody-masking experiments, they were accepted [23]. These studies show that results of class I antigen removal vary according to the species combination of donor and host.

Gene ablation experiments have demonstrated the advantages and limitations of eliminating class I antigens. There are clearly dominant antigens on islet cells and neurons in some allogeneic and xenogeneic combinations. However, class I antigens may play a secondary role depending on the type of donor tissue, the species combination, and/or the disease state of the recipient. In most cases, re-expression of class I antigens on the donor cells was beneficial for long-term survival. Indeed with antibody 'masking' of class I, secondary transplants from the same donor were possible if the primary transplant was still in the host [23].

Rare cases of stable allograft acceptance in humans who discontinue their immunosuppressive regiments have been documented in the setting of whole organs such as kidney transplants. A study of a mouse model found that pre-engraftment of donor cells bearing a single foreign MHC class I allele resulted in lifelong donor cell acceptance and an immune system that both *in vivo* and *in vtiro* was unresponsive. Similar to the data presented above for temporary class I ablation, removal of the primary transplant reversed the systemic tolerance in a time dependent fashion. This suggests that the transplants can actively maintain host unresponsiveness towards a single MHC class I allele by continuously inactivating a reactive T cell [26].

ROLE OF CLASS I MODIFICATIONS IN RESISTANCE TO RECURRENT AUTOIMMUNITY

The fate of pancreatic islet transplants from ß$_2$-microglobulin-deficient mice has been explored in several studies using chemically induced mice as well as spontaneously diabetic mice. Non-obese diabetic (NOD) mice are a well recognized model of spontaneous type 1 diabetes and have a long prodrome of pre-diabetes from 6–8 weeks of age prior, progressing to spontaneous diabetes at approximately 18–24 weeks of age, the stage where islet destruction sufficient for hyperglycemia occurs. Although clinically asymptomatic, this stage of pre-diabetes is the most active phase of disease.

Class I depleted islets, either with masking antibodies or from donors with ablation of the ß$_2$-microglobulin gene, show prolonged survival when implanted into allogeneic mouse strains without autoimmunity [12,13]. Additional experiments also tested the transplantation of cells from ß$_2$-microglobulin hosts into the autoimmune prone NOD mouse strain, both in pre-diabetic mice and fully diabetic mice. When genetically modified islets were transplanted into young NOD (but not yet diabetic) mice, graft rejection due to ongoing autoimmunity almost invariably occurred [12]. Although at this stage NOD mice are clinically asymptomatic due to survival of some islets, this is the most active phase of disease. Soon after implantation into pre-diabetic NOD mice, but at a slightly delayed rate, islet allografts from ß$_2$-microglobulin-deficient mice or class I-masked xenogeneic islets are rejected after a two-fold increased survival beyond control islets. This modest prolongation is in contrast to almost complete success with recipients whose diabetes is chemically induced, or in NOD mice that are already hyperglycemic. Fully diabetic NOD mice receiving MHC class I deficient islets demonstrate indefinite survival [27]. In conclusion, class I deficient islets, rendered deficient

by a number of methods, can show modest to dramatic prolongations in survival in murine hosts with different stages of diabetic autoimmunity.

THE LAUNCHING OF XENOGENEIC HUMAN CLINICAL TRIALS IN THE UNITED STATES USING IMMUNOMODULATION

Few cross-species transplantation technologies have progressed to human clinical trials. In part, this has been due to primate models showing minimal efficacy. Also, early whole organ human clinical trials in the 1970s demonstrated minimal success, even with massive levels of immunosuppression. Additionally, some segments of the medical community have been concerned about cross-species infections, therefore necessitating new technologies to avoid the use of donor species closely related to humans, i.e., baboons. The testing of novel transplantation approaches needs to avoid the limitation of a severely compromised host immune system with decreased ability to fight infections.

Clinically close primate and rat models are available for neurological diseases such as Parkinson's disease. The approach of masking class I antibody fragments shows promise using porcine neurons for treating spinal cord injuries in rat models, and porcine liver cells for treating transient liver failure from hypotension or infection. Fetal neuronal xenografts with antibody masking have been investigated for Huntington's disease and Parkinson's disease [28]. In the first study, fetal pig striatal cells were implanted into rats whose striatum had been lesioned one week earlier with injections of quinolinic acid. These injections destroy striatal neurons, and attempt to simulate the dysfunction present in Huntington's disease. Rats received either untreated tissue or tissue pretreated with $F(ab')_2$ fragments against porcine class I antigens. Control rats received untreated tissue and were immunosuppressed with CsA; the others were not immunosuppressed. In treated animals, graft volume, determined histologically with the aid of computer image analysis, was significantly larger and better organized, and graft axons had correctly grown in the direction of their target nuclei. It was encouraging that the pig neurons appeared to be capable of locating their target. Also, because of the use of fetal tissue, the transplanted mass had become significantly enlarged at the time of autopsy, demonstrating post-transplantation survival as well as growth of the transplant. Six Parkinson's patients have now been treated with fetal pig neurons masked with class I antibody fragments and their long-term survival (>2 years) has been reported, with the patients demonstrating mild to marked functional improvements. Six more patients similarly treated with fetal pig neurons and CsA showed less clinical improvement, but still had function exceeding base line. At eight months, one of these patients died of a thromboembolic event and an autopsy was performed. As reported by Deacon et al. [28], similar to the primate studies performed before clinical trials, the fetal pig neurons survived and correctly sent out axons over long distances in the brain toward their target nuclei. This confirmed the suitability of using pig tissue in this transplant setting, and of using the masking approach to decrease tissue immunogenicity.

Additional clinical trials of masked neuron transplants have continued in the United States, including a blinded phase II/III trial using pig cells for treating Parkinson's disease has been conducted to evaluate safety and efficacy. The advantages of the donor antigen modifications methods include a lack of host interventions, thus allowing a broader audience for applications of cellular transplants for disease treatments.

COMMENT

Designer tissues and organs, created through donor antigen modification, hold tremendous promise for xenotransplantation and allotransplantation. Research in animal models has already demonstrated that long-term xenograft survival can be achieved without immunosuppression. This achievement has galvanized the transplantation community, since it shows

that an overwhelming obstacle to graft acceptance can be alleviated in select settings. Immune rejection need not occur if graft antigens can be immunologically masked, enzymatically cleaved or genetically eliminated. Researchers now have at their disposal a battery of techniques that operate at the DNA, RNA, or protein level to remove or conceal antigens. Improved xeno or allogeneic transplantation is within reach, not just for patients with life-threatening conditions, but also for patients with chronic conditions.

The successes with cellular and tissue grafts still are not the final solution for the far more difficult task of whole organ xenotransplantation or recurrent autoimmune disease. Solid organs have a multiplicity of antigens, particularly those which elicit hyperacute rejection due to differences in the expression of sugars between species. Once all dominant antigens are identified, the therapeutic strategy for whole organs and tissues is conceptually identical: modify the donor, not the host. The barriers for recurrent autoimmune disease still stand, but it is hoped that donor antigen modification may also be beneficial in this setting during select times in the disease process.

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CHAPTER 28

Challenges in the Development of Immunoisolation Devices

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INTRODUCTION

Immunoisolation devices can be used to transplant cells to treat a variety of human diseases without the need for immunosuppressive drugs. Transplanted cells are enclosed within a material that provides protection from the immune system while allowing adequate transport of nutrients, oxygen, waste products, and therapeutic products. There are three types of immunobarrier devices: (1) intravascular, (2) extravascular macrocapsules, and (3) microcapsules. Challenges exist which prevent the widespread applications of cell encapsulation therapies: tissue supply, effective immune protection of encapsulated cells, and maintenance of cell viability post-transplantation. This chapter discusses approaches that can be used to overcome these problems and focuses specifically on the maintenance of cell viability and function in the treatment of type 1 diabetes, including use of vascularizing membranes, *in situ* oxygen supply, exogenous oxygen supply, reduction of islet cell aggregate size, and manipulation of encapsulant properties. One method to enhance cell viability by increasing oxygen permeability of the encapsulating material with perfluorocarbons (PFCs) is discussed in detail, and a mathematical model is used to predict the extent of enhancement of islet viability and function.

Cell therapies have the potential to treat a large range of diseases by providing *in vivo* delivery of a required protein. A problem that complicates and restricts the use of such therapies is the need for transplanted cells to be protected from the immune system by immunosuppressive drugs. One engineering approach that addresses this problem is the use of immunobarrier devices which provide a physical barrier to protect transplanted cells from the recipient's immune system through use of a semipermeable membrane. Some diseases that have been investigated for treatment with immunobarrier devices are diabetes [1], hemophilia [2], anemia [3], parathyroid disease [4], chronic pain [5], Parkinson's disease [6], Huntington's disease [7], and amyotrophic lateral sclerosis [8]. An immunobarrier device can contain cells that constantly produce a therapeutic protein, as is required for the treatment of most of the diseases listed above, or, for the case of diabetes treatment, the cells secrete insulin in response to changes in the blood glucose level in a feedback-controlled manner.

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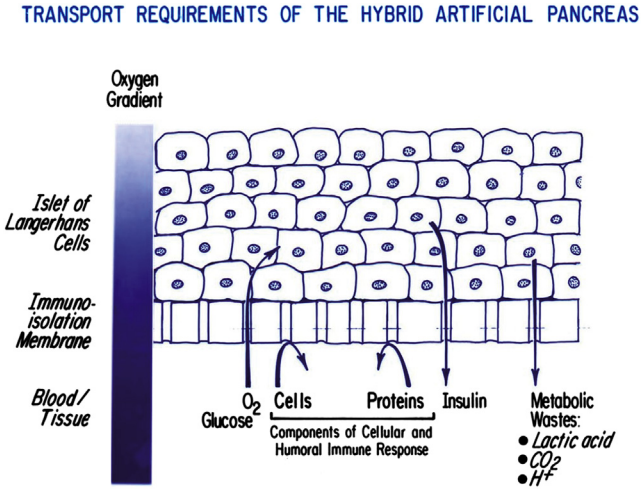

FIGURE 28.1
Essential elements of an implanted device that incorporates encapsulated cells illustrating the case of a biohybrid artificial pancreas. *Reproduced, with permission from Colton (1995) [10].*

The essential elements of an implanted device incorporating encapsulated cells are shown in Fig. 28.1, which is a conceptual illustration of a biohybrid artificial pancreas. The implanted tissues are separated from the host by an immunoisolation membrane (sometimes referred to as an immunobarrier membrane). Cells can be encapsulated at a high, tissue-like density, as illustrated in Fig. 28.1, or dispersed in an extracellular gel matrix, such as agar, alginate, or chitosan. The membrane prevents access of immune cells and prevents or minimizes access of humoral immune components, but permits passage of the secreted product (insulin). At the same time there must be sufficient access to nutrients, such as glucose and oxygen, and removal of secreted metabolic waste products, such as lactic acid, carbon dioxide, and hydrogen ions as well as nitrogenous products of metabolism. Transplanted cells must be supplied with nutrients by diffusion from the nearest blood supply, through surrounding tissue, the immunobarrier membrane, and the graft tissue itself.

This chapter focuses on the engineering challenges associated with developing immunobarrier devices and the methods that are under study to circumvent the problems of supply of tissue, protection from immune rejection, and maintenance of cell viability and function. The emphasis of the chapter is on immunobarrier devices used in transplanting islets for type 1 diabetes treatment with a focus on the challenge of maintaining cell viability and function. After describing the challenges, approaches are discussed to improve immunobarrier device designs to overcome some of these difficulties, including use of vascularizing membranes, *in situ* oxygen generation, exogenous oxygen supply, reduction of islet cell aggregate size, and manipulation of encapsulated properties. One particular approach is described in detail: this is the use of encapsulating materials that contain perfluorocarbons to enhance oxygen delivery and increase islet survival and function. A mathematical analysis is presented to demonstrate the benefits of this approach.

The material presented here builds on previous reviews of the field published since the early 1990s [9–13]. Consequently, in this chapter we focus on recently reported work.

ENGINEERING CHALLENGES

Device designs

Immunobarrier device designs typically fall into three categories: intravascular devices, extravascular macrocapsule devices, and microcapsules [9,10]. Intravascular devices create a vascular shunt between an artery and a vein. The device is typically made of a hollow fiber, where blood flows through the lumen and the transplanted cells are separated from the blood by an immunoisolating membrane. This device design is appealing because it

brings blood into very close proximity with the transplanted tissue, which will aid in nutrient, waste, and therapeutic protein transport. However, the implantation of this device is associated with greater risks because it disrupts the patient's vascular system, thereby leading to a greater risk of complications. Human clinical trials of this type of device for islet transplantation were being planned when they were stopped by the FDA because of a mechanical failure of the cannula in dogs and they have never been resumed. At this point in time this type of device is not under study.

The second device design type is the extravascular macrocapsule, which is typically a planar diffusion chamber or a hollow fiber. This type of device is typically implanted within a cavity of the body and contains tissue within the device lumen surrounded by an immunoisolating membrane. Delivery of oxygen and other nutrients requires diffusion from the surrounding tissue to the device, across the device membrane, and then through the interior of the device itself to the tissue. This type of device can be limited in the amount of tissue that can be included within the device due to oxygen supply limitations. Oxygen supply limitations can be even further aggravated by overgrowth of fibrotic tissue, which is another transport barrier, and which also consumes some of the oxygen (if there are cells present) that would normally be delivered to transplanted tissue [14]. Benefits of using macroencapsulation devices are that many tend to be made of materials that are very stable on implantation in the body, and if needed these types of devices can easily be retrieved because of their larger size. This type of device is under study for several applications, including diabetes [15] and diseases of the central nervous system [16]. Examples of materials used for macroencapsulation are polytetrafluoroethylene [17], which can be used to form an immunoisolating membrane or an exterior vascularizing membrane, poly-ether-sulfone [8], which is used for devices that have entered into clinical trials, alumina [18], and nanoporous micromachined silicon-based membranes [15], which can result in a more stringent control over the exact pore size of the material.

The final type of immunobarrier device is the microcapsule. These are small spherical gels ranging in size from 200 μm for islet conformal coatings to 2 mm for macrobeads. Microcapsules are currently being studied the most extensively for islet transplantation to treat diabetes. Each microcapsule typically contains 1 to 2 islets, and the microcapsules are most commonly transplanted into the peritoneal cavity. The peritoneal cavity is the implantation site of choice, because there is ample space for the implant, and immune responses are not as high as at other implantation sites, such as the subcutaneous space. Capsules in this location may be located far away from the blood supply and therefore have very limited oxygen, which can have detrimental effects on tissue survival. The feasibility of microcapsule implantation in the liver via intraportal injection (the location and method for naked human islet transplantations) is being studied in order to enhance microcapsule proximity to the blood supply [19]. A drawback of the liver as a transplantation site is that there is an increased immune response, which is found to be reduced by short-term immunosuppression with gadolinium chloride, rapamycin, or tacrolimus [19].

The most common choice of materials for microcapsules is alginate, a polysaccharide derived from seaweed. Alginate can be dissolved in water to form a viscous solution that, upon exposure to a divalent (e.g., calcium or barium) or a trivalent (e.g., gadolinium) ion, is transformed into a hydrogel. This very gentle gelation process is the reason for widespread use of alginate, because tissue can be encapsulated without causing damage to the cells. However, because it is a naturally derived product, its properties are batch and source dependent, and the impurities in the alginate itself can have detrimental effects on the success of immunobarrier devices. Alginate microcapsules usually come in one of two forms: alginate alone cross-linked with barium ions [1,20] or alginate cross linked with calcium and then coated with poly-L-lysine [21] or poly-L-ornithine [22] to form a perm-selective barrier and enhance capsule stability. Alternative materials to alginate are

being developed. One promising example is synthetic Tetronic polymers that thermally gel and chemically cross-link to form more stable gels while still needing gentle processing steps [23]. The capsule formation process for the Tetronic polymers is adaptable to the machinery developed for making alginate capsules [23]. A recent review of the field of micro-encapsulation [24] provides additional information.

Supply of tissue

Tissue for cell therapy applications can be derived from three main types of sources:

1) Human primary tissue,
2) Primary xenogeneic tissue, and
3) Cell lines.

Each type of tissue has reasons why it is an attractive and an unattractive source. Allotransplantation, or use of primary human tissue, is desirable because this tissue, were it to be transplanted alone, would illicit less of an immune response than tissue derived from another animal (xenotransplantation). Therefore it should be easier to provide immunoprotection to allotransplanted tissue compared to xenotransplanted tissue. However, the main drawback of primary human tissue is that it requires a cadavaric donor, and therefore the supply is extremely limited. Additionally, a procedure is required to isolate the tissue prior to transplantation, which can be costly and time consuming.

Some researchers are looking to use tissue derived from animal sources in immunobarrier devices. The main advantage of xenogeneic tissue is that its supply is not as limited. However, there is a risk of retroviral disease transmission, immunobarrier requirements are more stringent, and tissue isolation procedures still need to be developed.

The final type of tissue that is under study for transplantation is cell lines, of which the supply of a particular cell type is infinite and there are no isolation requirements. Cell lines can be allogeneic or xenogeneic in nature. When immunobarrier devices are used to deliver a desired product at a continuous rate, as is typically desired for gene therapy strategies, the use of cell lines has proven to be an adequate cell source for this application, as is discussed in a review of encapsulation of genetically modified cells and their clinical applications [16]. The main drawback of using cell lines for diabetes treatment is that the cell line must secrete insulin under the same feedback-control mechanisms as the islet and at the same rate. Also, in order to prevent hypoglycemia in diabetes treatments, the growth of the cell line should be arrested prior to transplantation. To date, this type of cell line has yet to be developed, although work is being done to address these issues in the hope that a fully functional beta cell line will solve the tissue-shortage problem associated with islet transplantation, and make the therapy available to all type 1 diabetic patients [25,26]. Stem cells also offer great promise as an unlimited source of cells for immunobarrier devices, but a greater understanding and control of the differentiation process is required in order to generate cells with the desired phenotype for a particular application.

Preventing immune rejection

Possible rejection pathways elicited by tissue in immunobarrier devices are shown in Fig. 28.2. The process may begin with diffusion across the immunobarrier of immunogenic tissue antigens that have been shed from the cell surface, secreted by live cells, or liberated from dead cells. Recognition and display of these antigens by host antigen-presenting cells initiate the cellular and humoral immune responses. The former response leads to the activation of cytotoxic T cells, macrophages, and other immune cells. Preventing cells from entering the tissue compartment is easily achieved using microporous membranes. This may be the only requirement for immunoisolation of allogeneic tissue, at least for periods up to several months [27]. With xenografts it is also necessary to keep out components of the humoral

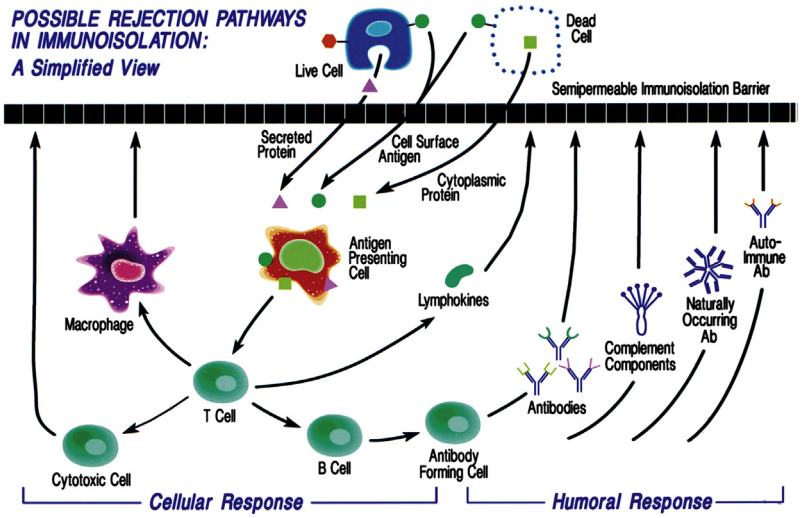

FIGURE 28.2
Possible pathways for immune rejection mechanisms with encapsulated cells. *Reproduced, with permission, from Colton (1995) [10].*

immune response, which is more difficult to accomplish. Such components include cytokines and lymphokines (e.g., interleukin-1), which can have deleterious effects on β-cells, as well as newly formed antibodies to immunogenic antigens that have leaked across the barrier. In addition, there may be naturally occurring antibodies, most likely IgM, to cell surface antigens on xenografts. Antibodies produced during pre-existing autoimmune disease, such as type 1 diabetes, might also bind to cell surface antigens. Lastly, macrophages and certain other immune cells can secrete low molecular weight reactive metabolites of oxygen and nitrogen, including free radicals, hydrogen peroxide, superoxide, and nitric oxide, which are toxic to cells in a non-specific fashion. The extent to which these agents may play a role in causing rejection of immunoisolated tissue depends on how far they can diffuse before they are inactivated by chemical reactions. Based on diffusion and reaction parameters for nitric oxide and superoxide, the reaction-length scales for nitric oxide and superoxide in water are estimated to be on the order of 1 mm and 1 μm, respectively [10]. Therefore a macrophage that is at the surface of an immunoisolating device secreting these molecules will be capable of delivering nitric oxide to the encapsulated tissue but not superoxide, for it will react away before it can diffuse far enough into the capsule to damage the tissue.

Cytotoxic events occur if antibodies and complement components pass through the membrane. Binding of the first component (C1q) to IgM or two or more IgG molecules initiates a cascade that culminates in the formation of the membrane attack complex, which can lyse a single cell. IgM (910 kDa) and C1q (410 kDa) are both larger than IgG (see Fig. 28.3), so if host IgM and C1q can be prevented from crossing the barrier, then a specific,

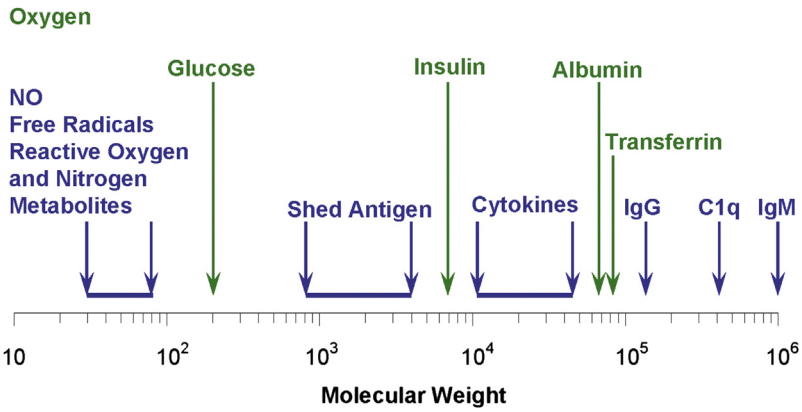

FIGURE 28.3
Comparison of molecular weights of immune system molecules and molecules required by cells for survival. *Modified from Colton (1995, Fig. 19) [10].*

antibody-mediated attack on the islet cell should be averted. If the alternative complement pathway is activated and not inhibited by the implanted tissue, then passage of C3 (200 kDa) across the membrane must be prevented.

The precise mechanism(s) that may play a role in rejection of encapsulated tissue are, in general, incompletely understood, and they probably depend on the specific types of cells present, the species and its phlyogenetic distance from humans, and the concentration of humoral immune molecules to which the implanted tissue is exposed. The latter depends on the transport properties of the immunobarrier membrane as well as on the total mass of cells implanted (which affects the magnitude of the immune response), the tissue density, and the diffusion distances between tissue and immunobarrier membrane (which affect access of the generated humoral components to the graft tissue). If complete retention of IgG (or even C1q and IgM) coupled with passage of albumin and iron-carrying transferrin (81 kDa) is required, there is a problem because of the size discrimination properties of membranes, in which there is invariably a wide distribution of pore sizes. If cytokine transport must also be prevented, the problem is even more difficult. A comparison of molecule sizes required for cell survival, the insulin that is required to be secreted so that the device is functional, and the immune system components are shown in Fig. 28.3. This figure demonstrates that if the cells require albumin and transferrin, then the transport of cytokines to the tissue cannot be blocked by an immunobarrier alone. The membrane would have to allow for cytokine transportation but could potentially contain components that could deactivate the cytokines before they reach the encapsulated tissue. If free radicals and other reactive oxygen and nitrogen species pose a significant problem, no passive membrane barrier will be able to provide immunoprotection, and some other approach (e.g., scavenging of free radicals, immunomodulation of transplanted tissue, local suppression of host immune response) will be necessary. The portion of overgrown allotransplanted alginate-PLL microcapsules containing rat islets explanted four weeks post-transplantation were shown to have mostly macrophages, with some fibroblasts on the capsule surface, without any NK cells, granulocytes, T cells, or B cells [21]. These results indicate that in an allotransplantation model it may be most important to block macrophage activation and neutralize the effects of nitric oxide and superoxide to prevent immune rejection.

Maintenance of cell viability

Maintenance of cell viability and function is essential and is limited by the supply of nutrients and oxygen. Diffusion limitations of oxygen in tissue *in vivo* are far more severe than those of glucose because the concentration of glucose in tissue is many times higher [28]. The requirements of specific tissues for other small molecules and for large macromolecules are poorly understood or have not yet been quantified; the possible existence of transport limitations for large molecules is highly dependent on immunobarrier membrane properties, whereas oxygen limitations are always serious. Oxygen partial pressure (PO_2) at levels high enough to keep cells alive can nonetheless have deleterious effects on cell functions that require high cellular ATP concentrations — for example, ATP-dependent insulin secretion [29]. Supply of oxygen to immunoisolated tissue is therefore the critical component that determines tissue survival and function.

Oxygen supply to encapsulated cells depends in a complicated way on a variety of factors, including:

1) The site of implantation and the local PO_2 in the blood,
2) The spatial distribution of host blood vessels in the vicinity of the implant surface,
3) The oxygen permeability of the membrane or encapsulant,
4) The oxygen consumption rate of the encapsulated tissue,
5) The geometric characteristics of the implant device, and
6) The tissue density and spatial arrangement of the encapsulated cells or tissues.

Despite encouraging results with various tissues and applications [16], the problem of oxygen transport limitations is one of the major hurdles that remain. The maximum PO_2 (about 40 mmHg for the microvasculature) available for extravascular devices limits the steady-state thickness of viable tissue that can be supported.

Islets are particularly prone to oxygen supply limitations because they have a relatively high oxygen consumption rate [29]. In the normal physiologic state they are highly vascularized and are supplied with blood at arterial PO_2. When cultured *in vitro* under ambient normoxic conditions, islets develop a necrotic core, the size of which increases with increasing islet size, as is to be expected as a result of oxygen diffusion and consumption within the islet [29]. Central necrosis of the encapsulated islets occurs after islet microcapsule transplantation, resulting in reduction in transplant volume when only a small fraction (~10%) of the capsules has fibrotic overgrowth [30]. The fact that necrotic tissue is at the center instead of the periphery of the islet indicates that it is likely a nutrient or oxygen supply limitation causing the necrosis and not a mechanism of the immune system. Hypoxia causes encapsulated islets in culture to become necrotic and to upregulate inducible nitric oxide synthase (iNOS), which indicates that islets are producing NO that can cause damage to themselves. Hypoxia also causes islets to upregulate monocyte chemoattractant protein 1 (MCP-1), which can attract macrophages and hence also induce islet damage post-implantation [31]. The results of all of these studies indicate that oxygen transport limitations exist within transplanted islet microcapsules and can have serious effects on islet survival.

Previous modeling studies have investigated how device designs affect oxygen transport limitations. Amongst devices that incorporate tissue in the form of slabs, cylinders, or spheres, the spherical geometry is the most beneficial when comparing the volume fraction of non-anoxic tissue in each geometry [11]. When small amounts of tissue are required for transplantation, the size of the device that is required is feasible in all geometries, but as the amount of tissue increases, especially for the large amount required for islet transplantation, the surface area of the slab, the length of the cylinder, or the number of microcapsules increases significantly [11].

The size of a planar diffusion device and the volume of microcapsules needed were estimated by assuming that 500,000 islets may be required to treat type 1 diabetes [32]. In a planar diffusion device with 120 μm islets sandwiched between 100 μm thick membranes and device surface oxygen partial pressure of 40 mmHg, the maximum islet density is 1100 islets/cm [2] for fully functional tissue, which corresponds to a total device surface area of 450 cm^2 [33]. If it is assumed that there is 1 islet/500 μm diameter microcapsule, then 500,000 capsules would need to be transplanted, equaling a total volume of 33 mL.

STRATEGIES FOR IMPROVING IMMUNOBARRIER DEVICES

Enhancement of immunoprotection capacity of devices

It will never be possible to create a device that provides complete immunoprotection. Therefore it is most likely necessary to augment the immunoprotection by short-term administration of immunosuppressive drugs, trapping or neutralizing small toxic molecules released during an immune response, or enhancing the resistance of the islet to the stresses experienced during an immune response. Administration of immunosuppressive drugs defeats the purpose of an immunobarrier device, but tissue survival can be enhanced if immunosuppressive drugs are administered to inhibit the immune response, when it is most severe, for a short time period post-transplantation. Administration of antibody molecules that block T cell costimulation pathways (CTLA4-Ig, anti-CD154, and anti-LFA-1) enhances the length of graft function for neonatal porcine cell clusters or adult porcine islets in alginate microcapsules [34,35]. Alternatively, in order to decrease the immune response post-transplantation, macrophage activation can be prevented by systemic administration of

gadolinium chloride [19] or local administration of clodronate liposomes to reduce cell overgrowth of alginate microcapsules [36]. These studies demonstrate that short-term immunosuppression, which is far less risky than lifetime immunosuppression, can be beneficial in promoting graft survival and enhancing the efficacy of immunobarrier devices.

An alternative approach to suppressing the immune system to enhance graft survival is to neutralize or trap the toxic molecules of the immune system that are released to destroy the transplanted tissue. Macrophages play a role in immune responses to immunoisolated tissues, and it has been demonstrated that the release of nitric oxide from activated macrophages, and not cytokines, is responsible for islet destruction, in a dose-dependent manner [37]. Therefore, including a nitric oxide scavenger such as hemoglobin within the microcapsules can inhibit islet cell death through nitric oxide-mediated mechanisms. Results have shown that the inclusion of live erythrocytes, fixed erythrocytes, or cross-linked hemoglobin in alginate microcapsules enhances islet survival with exposure to activated macrophages or nitric oxide [37,38].

Enhancement of oxygen transport to encapsulated tissue

In order for immunobarrier devices to be successful, the transplanted cells must remain viable and functional post-transplantation. Methods have been developed to enhance mass transfer to immunoisolated tissue, most specifically transport of oxygen, by using vascularizing membranes, *in situ* oxygen generation, exogeneous oxygen supply, thinner microcapsules, smaller islet cell aggregation, and enhancement of the oxygen-carrying capacity of encapsulating materials in order to ensure its permeability. Mass transfer of oxygen to an immunoisolated device can be enhanced if the vasculature is brought in very close contact with the device. The vasculature cannot come into direct contact with the tissue itself, which would be a breach of the immunobarrier, but vessels in close proximity to the device are beneficial. The Theracyte™ planar diffusion chamber (originally developed at Baxter Healthcare, later marketed by Theracyte, Inc) has two membranes:

1) An exterior vascularizing membrane that has an optimal pore size (5 μm) so that cells can penetrate the layer and vascularization is induced, and
2) An immunoisolating membrane, pore size 0.45 μm.

Both of these are made from PTFE [17]. The timecourse of membrane microarchitecture-driven neovascularization involves migration of vessels towards the membrane surface over a 10 day period [39], after which the microvascular structure stabilizes for a long period of time [40]. The Theracyte™ device has been effective in maintaining cell viability and protecting from allograft rejection in rodent models [41,42]. The device can be preimplanted in order to induce vascularization of the device prior to transplantation, which aids in islet survival post-transplantation [43]. In addition, infusion of the device with vascular endothelial growth factor (VEGF) improves the density of blood vessels that form around the device [44]. Other materials have been examined for use in promoting neovascularization, including a dual porosity electrospun nylon membrane [45] and a stainless steel mesh [46].

An alternative approach to overcoming oxygen limitations is to supply implanted tissue with oxygen generated *in situ* adjacent to one side of the immunobarrier device [47]. On the other side, the exterior of the device is exposed to either culture medium for *in vitro* studies or the host tissue for *in vivo* conditions. *In situ* oxygen generation can occur by the electrolytic decomposition of water in an electrolyzer [47]. The electrolyzer takes the form of a thin, multilayer sheet, within which electrolysis reactions take place on the anode and the cathode to form oxygen and hydrogen, respectively [47]. *In vitro* studies with βTC3 cells in the *in situ* oxygen generation device show that the thickness of viable tissue increases with oxygen generation [47]. Furthermore, insulin secretion rate from islets contained in such devices is higher with than without oxygen generation. However, control of the

maximum PO_2 to which islet cells are exposed is relatively complicated with this approach [12]. Another method described for *in situ* oxygenation is light illumination of encapsulated algae to produce oxygen by photosynthesis [48]. However, this method is far less efficient, and therefore more costly, than direct electricity for water hydrolysis.

An alternative to *in situ* generation is periodic supply of oxygen in gaseous form to an implanted device. One novel approach, termed the βAir® device, contains islets immobilized within a flat alginate slab, supplied with oxygen by diffusion through a gas permeable membrane from an adjacent inner gas chamber that is replenished daily through an external port and protected from the host immune cells by a microporous membrane, the pores of which are filled with alginate. Initial studies with the βAir® device achieved normoglycemia in diabetic rats with isogenic and allogenic islet implants [49,50] and demonstrated the benefit of supplying oxygen at increased PO_2 levels from one side of the alginate slab.

Oxygen transport to encapsulated islets can be enhanced by reducing the diffusion distance through the use of smaller capsules or thinner membranes. There are drawbacks to reducing the diffusion distance, because free radicals released from immune system effector cells may not become inactivated prior to reaching the encapsulated tissue, and the amount of shed antigens from the encapsulated tissue can be increased, thereby enhancing the recipient's immune response to the transplant. Alginate capsules made using an electronic droplet generator, as opposed to an air-driven droplet generator, can be made to be <500 μm instead of ~ 800 μm. There are also techniques in which only a thin coherent membrane is used to coat the islets. Examples are the use of an emulsion procedure to form calcium alginate PLO microcapsules [51] or by centrifuging an islet alginate cell suspension in a discontinuous gradient that contains a barium chloride layer [52]. Polyethylene glycol derivatives have also been employed to prepare extremely thin coatings with a layer-by-layer encapsulation technique [52a].

Two other approaches can be employed to enhance oxygen delivery to encapsulated tissue:

1) Enhance the dissolved oxygen-carrying capacity of the material and thus increase the oxygen permeability and the rate at which oxygen can be delivered to the tissue, for example, by combination of a highly concentrated perfluorocarbon (PFC) emulsion with alginate (PFC alginate);
2) Reduce islet tissue size so as to decrease the diffusion distance, for example, by dispersing the islets into single cells followed by reaggregation into cell clusters smaller than the original islet.

In a recent study [53] a theoretical reaction-diffusion model was used to predict the three-dimensional distribution of oxygen partial pressure in a spherical microcapsule and a planar slab containing islet tissue, from which the loss of cell viability and the reduction in insulin secretion rate was eliminated. Numerical simulations were carried out for normal alginate and PFC alginate to examine the effect of surface oxygen partial pressure, capsule diameter, slab thickness, and the size and density of dispersed islet tissue on viability and function. Results showed that hypoxic conditions can be reduced, thereby enhancing islet viability and substantially maintaining insulin secretion rate when PFC emulsion is incorporated in the encapsulation material or when smaller islet cell aggregates are used in both types of geometries. Components that can be used for enhancing oxygen permeability are organic compounds with high oxygen solubility, such as perfluorocarbons, silicone oils, or soybean oils. Perfluorocarbon emulsions have been developed as blood substitutes and could be incorporated into the encapsulation material to increase its oxygen permeability. Perfluorocarbons and silicone oils have been used to enhance oxygen transfer in bioreactors [53,54]. There are reports in the literature that including a perfluorocarbon emulsion in islet culture medium enhances islet function [55]. Perfluorocarbons have also been used in the storage of the pancreas prior to islet isolation to increase islet yield and storage time [56]. Inclusion

of hemoglobin in alginate microcapsules increases the length of islet survival after transplantation [57], but this enhancement likely results from a mechanism that does not involve enhanced oxygen permeability such as trapping of nitric oxide.

The effect of enhancing encapsulant oxygen permeability was examined experimentally [58] using two model systems: barium alginate and barium alginate containing a 70% (w/v) perfluorocarbon (PFC) emulsion. Mitochondrial function was assessed by oxygen consumption rate measurements. After low-oxygen culture for 2 days, islets in normal alginate lost substantial viable tissue and displayed necrotic cores, whereas most of the original oxygen consumption rate was recovered with PFC alginate, and little necrosis was observed. These findings suggested that enhancement of oxygen permeability of the encapsulating material with a concentrated PFC emulsion improved survival of encapsulated islets under hypoxic conditions.

In another recent report, small islet cell aggregates were studied to determine whether their survival and function were superior to intact islets within microcapsules because of reduced oxygen transport limitation and inflammatory mediators. Islet cell aggregates were generated by dispersing rat islets into single cells and allowing them to re-aggregate in culture. Rat islets and islet cell aggregates were encapsulated in barium alginate capsules and studied when cultured in low or normal oxygen or transplanted into mice. Encapsulated islet cell aggregates were able to survive and function better than intact islets in terms of oxygen consumption rate, nuclei counts, insulin-to-DNA ratio and glucose-stimulated insulin secretion. They also showed reduced expression of pro-inflammatory genes. Islet cell aggregates showed reduced tissue necrosis in an immunodeficient transplant model, and a much greater proportion of diabetic xenogeneic transplant recipients receiving islet cell aggregates had reversal of hyperglycemia than recipients receiving intact islets. These aggregates were superior to intact islets in terms of survival and function in low-oxygen culture and during transplantation and are likely to provide more efficient utilization of islet tissue.

We envision that the type of microcapsule that results in successful islet transplantation will provide some immunoprotection by preventing contact between islets and activated immune cells, complement components, and antibodies. However, this is likely not enough to provide complete immune protection, and inclusion of a costimulatory blocker such as

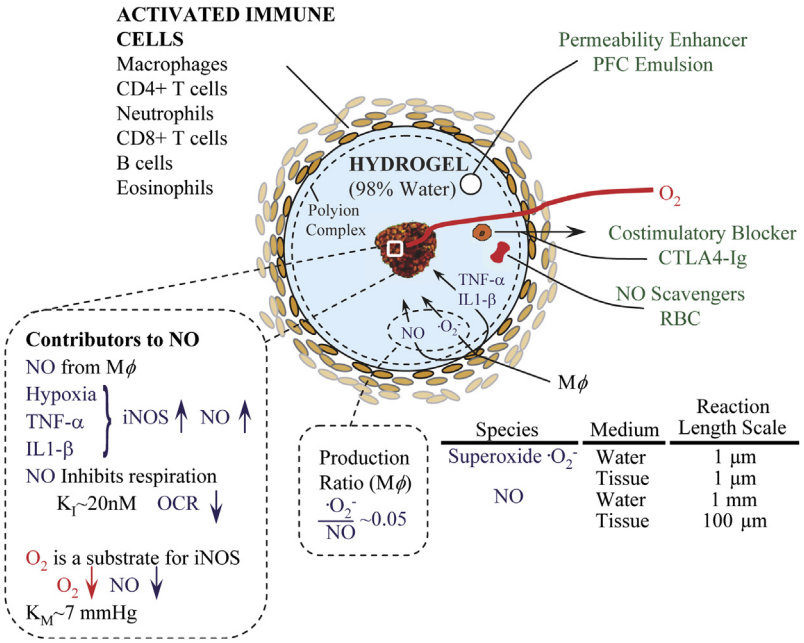

FIGURE 28.4

Activated immune cells surround the microcapsule, causing islet damage. Immune cell types are listed roughly in decreasing order of involvement in inflammatory and immune reactions to implanted immunobarrier devices. Macrophages damage islets through the release of nitric oxide, super oxide, and cytokines (labeled in blue). Oxygen supply (labeled in red) to encapsulated islets is limited, causing hypoxic conditions that are detrimental to islet survival. Potential methods to protect tissue from immune reactions or enhance oxygen delivery to improve encapsulated islet survival are labeled in green.

CTLA4-Ig and an NO scavenger such as red blood cells will greatly enhance the immunoprotective properties of the microcapsules. These modifications should help in preventing immune destruction of the islets, but increased delivery of oxygen is still required to maintain islet survival and function. Increased oxygen delivery can be accomplished by including a permeability enhancer, such as a PFC emulsion, in the alginate matrix. A picture of this ideal microcapsule that is likely to overcome many of the challenges that hamper immunobarrier devices is depicted in Fig. 28.4. The following section examines the oxygen levels within microcapsules that contain islets and a PFC emulsion for the purpose of improving islet survival and function.

THEORETICAL ANALYSIS OF PFC-CONTAINING MICROCAPSULES

One way to enhance oxygen delivery to immunoisolated tissue is to increase the permeability of the immunobarrier material. One method to enhance the permeability of alginate microcapsules for islet transplantation is to make the capsules from an alginate solution that contains a perfluorocarbon (PFC) emulsion. Perfluorocarbons are highly desirable materials for enhancing oxygen delivery, due to their very high oxygen solubility, approximately 25 times that of water on a volumetric basis. Enhanced solubility will lead to enhanced permeability because the permeability is the product of the gas solubility and diffusivity in the material of interest. We have currently been studying a perfluorocarbon emulsion made from perfluorodecalin and 20 wt% (w/v) Intralipid® (Baxter), a soybean oil emulsion [59]. To assess the benefits of PFC-containing microcapsules, a theoretical model has been developed to predict the local partial pressure of oxygen, which, in turn, is used to assess tissue viability and the fraction of normal insulin secretion for encapsulated islets and single cells. Predictions of the model will be presented for a 500 μm capsule that contains a 150 μm diameter islet or single cells that have a total tissue volume equal to that of the 150 μm diameter islet. A diagram of the two geometries is presented in Fig. 28.5. The islets are assumed to have the properties of rodent islets, in which the beta cells (Layer 1), comprising about 75% of the islet volume, are at the center of the islet and the non-beta cells form an outer shell (Layer 2) that is then surrounded by the alginate microcapsule (Layer 3). It should be noted that human islet beta cells are distributed throughout the entire islet; consequently, predicted beta cell viability and insulin secretion predictions will be slightly higher than for rodent islets.

Problem formulation

We used the one-dimensional species conservation equation for reaction and diffusion in spherical coordinates to predict the oxygen profile within the microcapsule:

$$D_i \frac{1}{r^2}\frac{d}{dr}\left(r^2 \frac{dC_i}{dr}\right) = V_i \tag{28.1}$$

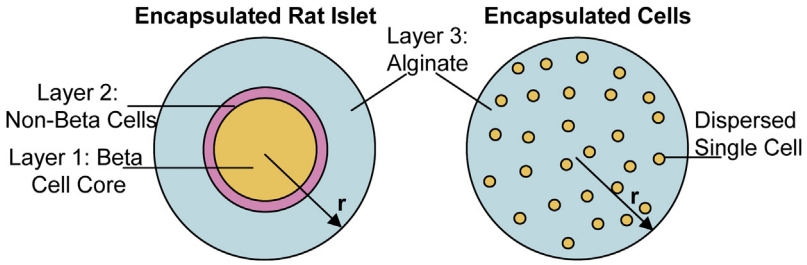

FIGURE 28.5
Capsule geometry (drawing not to scale).

where D_i (cm^2/s) is the effective diffusivity of oxygen in layer, C_i (mol/cm^3) is the concentration of oxygen in layer *i*, and V_i ($mol/cm^3/s$) is the local oxygen consumption rate per unit volume in layer i. For convenience, since partial pressures are equal across interfaces of different materials, we make use of oxygen partial pressure instead of concentration, which are related by:

$$C = \alpha P \tag{28.2}$$

where α ($mol/cm^3/mmHg$) is the effective Bunsen solubility coefficient in layer *i*, and *P* is the partial pressure of oxygen. Combining Eqs. (28.1) and (28.2) gives:

$$(\alpha D)_i \frac{1}{r^2}\frac{d}{dr}\left(r^2\frac{dP_i}{dr}\right) = V_i \tag{28.3}$$

The oxygen consumption rate is assumed to follow Michaelis-Menten kinetics for all tissue,

$$V_i = \frac{V_{\max}(1-\varepsilon_i)P_i}{K_m + P_i} \tag{28.4}$$

where V_{max} is the maximum oxygen consumption rate for the tissue, ε_i is the tissue volume fraction in layer *i*, and K_m is the Michaelis-Menten constant. For the capsule that contains an islet, no oxygen is consumed in the alginate layer; therefore V_3 is equal to zero, and the tissue volume fraction in the islet Layers 1 and 2 is equal to 1.

Equation 28.3 is solved simultaneously for the three layers of the islet microcapsule, subject to the following boundary conditions. The islet is assumed to be centrally located. At the capsule center a symmetry boundary condition is used:

$$\left.\frac{dP_1}{dr}\right|_{r=0} = 0 \tag{28.5}$$

Transport and reaction parameters in Layers 1 and 2 are identical. There is no need for a separate solution in regions 1 and 2 or for the associated boundary conditions. At the interface between Layers 2 and 3 (the interface between the non-beta-cell portion of the islet and alginate), where $r = R_2$:

$$r = R_2 \quad P_2 = P_3 \tag{28.6}$$

$$r = R_2 \quad (\alpha D)_2\frac{dP_2}{dr} = (\alpha D)_3\frac{dP_3}{dr} \tag{28.7}$$

The final boundary condition required to solve the equations is the assumption that the external partial pressure of oxygen is specified at the capsule surface (P_s):

$$r = R_3 \quad P_3 = P_s \tag{28.8}$$

For the case of a capsule containing dispersed single cells that have a total volume equal to a 150 μm islet, the model consists only of one layer, $\varepsilon = 0.027$, the boundary conditions are represented by Eqs. (28.5) and (28.8), and all subscripts referring to layers can be dropped.

Material and tissue properties

Several material properties for each layer were determined from theoretical relationships. The ratio of the effective permeability $(\alpha D)_{eff,i}$ for layer i, consisting of a dispersed (*d*) phase and a continuous (*c*) phase (as occurs in the alginate layers of the model system), to the permeability of the continuous phase $(\alpha D)_c$ was calculated from Maxwell's relationship:

$$\frac{(\alpha D)_{eff,i}}{(\alpha D)_c} = \frac{2 - 2\phi + \rho(1+2\phi)}{2 + \phi + \rho(1-\phi)} \tag{28.9}$$

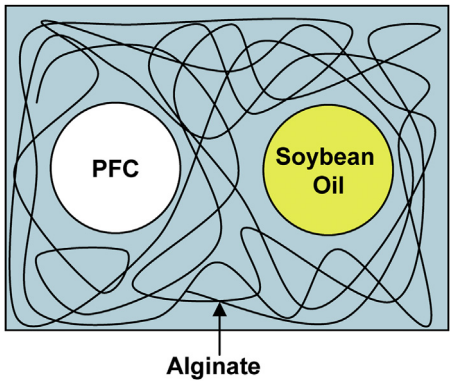

FIGURE 28.6
PFC alginate consists of PFC and soybean oil droplets surrounded by an alginate matrix with a continuous water phase.

where αD is the effective permeability of the material (mol/cm/mmHg/s), $\rho = (\alpha D)_d/(\alpha D)_c$ is the ratio of the permeability of the dispersed phase to the permeability of the continuous phase, and ϕ is the volume fraction of the dispersed phase. For the multiple dispersed phases employed in our model system, Maxwell's relationship was used sequentially, starting with the phase with the smallest particle size and ending with the phase with the largest particle size. For particle types of the same size, Maxwell's relationship was used for one particle and then the other (and in the reverse order), and the two results were averaged.

In the final PFC alginate composite phase, there were perfluorodecalin and soybean oil droplets of approximately the same size; the alginate polymer itself was treated as an impermeable phase dispersed in water (Fig. 28.6). The effective permeability of PFC emulsion was estimated by first using Maxwell's relationship for PFC droplets in water and then for soybean oil droplets in the PFC and water emulsion. Secondly, Maxwell's relationship was used for soybean oil droplets in water and then for PFC droplets in the soybean oil and water emulsion. The two effective permeabilities as calculated were averaged (Fig. 28.7). Finally, the effective permeability of PFC alginate was estimated with alginate as the dispersed phase and PFC emulsion as the continuous phase. The pure component material transport

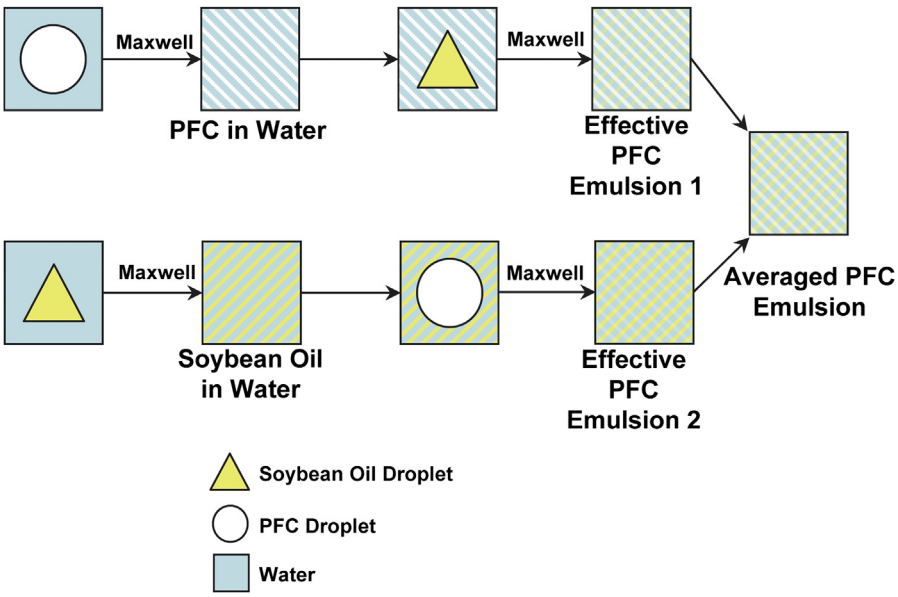

FIGURE 28.7
Sequence of calculations performed using Maxwell's relationship to calculate the effective permeability of PFC emulsion that consists of three phases: PFC droplets (○), soybean oil droplets (△), and water (□) as the continuous phase.

TABLE 28.1 Transport properties of materials in system

	D (cm^2/s)	α (mol/mmHg/mL)	αD (mol/cm/mmHg/s)	Source
H_2O	2.78×10^{-5}	1.27×10^{-9}	3.53×10^{-14}	Avgoustiniatos and Colton (1997b)
PFC	5.61×10^{-5}	2.54×10^{-8}	1.42×10^{-12}	Tham *et al.* (1973)
Soybean Oil	2.13×10^{-5}	6.84×10^{-9}	1.46×10^{-13}	Bailey (1979) Fillion and Morsi (2000)
Tissue	1.24×10^{-5}	1.00×10^{-9}	1.24×10^{-14}	Avgoustiniatos and Colton (1997b)
Alginate	0	0	0	Avgoustiniatos and Colton (1997b)

TABLE 28.2 Effective permeability calculated using Maxwell's relationship

	αD (mol/cm/mmHg/s)
70 wt% PFC emulsion	9.92×10^{-14}
70 wt% PFC alginate	9.63×10^{-14}
Dispersed cells, 70 wt% PFC alginate	9.31×10^{-14}
Pure alginate	3.43×10^{-14}
Dispersed cells, pure alginate	3.35×10^{-14}

All capsules are composed of 2 vol% alginate.

properties are given in Table 28.1, and the effective properties for different alginate types are given in Table 28.2.

The enhancement of PFC emulsion permeability relative to that of water or to the Intralipid® emulsion used as the starting material is shown in Fig. 28.8. A 70 wt% PFC emulsion has approximately 2.8 times the permeability of water and 2.1 times the permeability of Intralipid®. There is a smaller relative enhancement in the permeability of the emulsion when compared to Intralipid® because Intralipid® contains soybean oil, which has enhanced permeability to oxygen compared to water. In comparison, a 90 wt% PFC

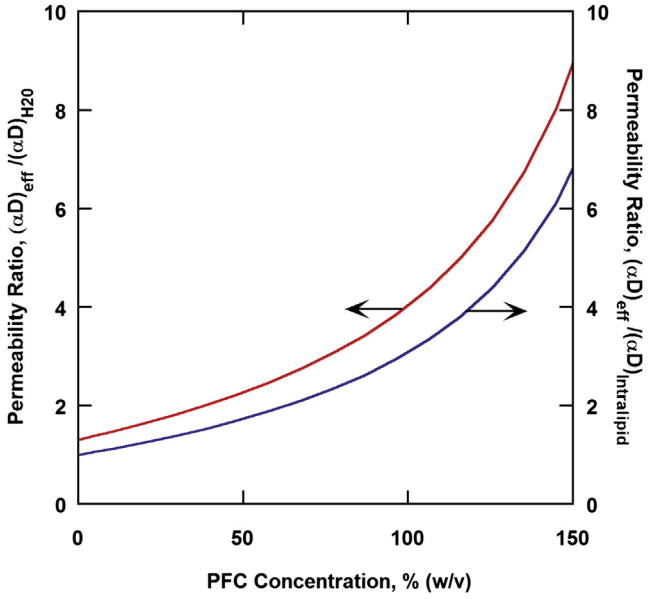

FIGURE 28.8
Permeability enhancement of PFC emulsion relative to water (left axis) or Intralipid® (right axis). PFC wt% = ρPFC × PFC vol%.

emulsion (maximum PFC loading in our experimental system) has 3.6 times the permeability of water, and a 110 wt% PFC emulsion (maximum reported loading for a PFC emulsion in the literature) has 4.6 times the permeability of water.

The relationship used to predict the local fraction (FS) of normal insulin secretion rate as a function of local oxygen partial pressure within the islet was developed from data on the effect of hypoxia on islet insulin secretion, estimating the oxygen partial pressure profile within the islet in the experimental system, and then determining the simplest model type and parameters(s) that best predict the insulin secretion level [29,33]. The result for a value of $V_{max} = 4 \times 10^{-8}$ mol/cm^3/s is:

$$\begin{aligned} P < 5.1 \text{ mmHg} \quad F_s &= \frac{P}{5.1} \\ P \geq \text{mmHg} \quad F_s &= 1.0 \end{aligned} \tag{28.10}$$

The fraction of normal insulin secretion averaged over the whole islet was determined by evaluating the volume integral of F_s in Layer 1. The fractional viability of the beta cell core (Layer 1) was estimated by determining the volume of tissue where $P_1 > P_c - P_c$ is the critical oxygen partial pressure below which tissue dies, and the value of P_c is assumed to be 0.1 mmHg.

Comparison of O_2 profiles in microcapsules containing single cells or an islet

The model equations were solved using the parameters given in Tables 28.1, 28.2, and 28.3. The resulting oxygen profiles for a surface oxygen partial pressure (PS) of 36 mmHg are given in Fig. 28.9. The results demonstrate that for the same amount of total tissue, its distribution within the capsule can greatly affect tissue survival and the minimum oxygen level that the tissue experiences. In a pure alginate capsule, the oxygen partial pressure drop from the capsule surface to its center is only 3 mmHg for dispersed cells, whereas P decreases to values equal to PC for a capsule containing one islet in the center, thereby indicating that there will be some loss of islet tissue in the pure alginate capsule. When the alginate microcapsule is made from a perfluorocarbon emulsion, the drop in the partial pressure of oxygen is smaller in both examples. The enhanced permeability of the PFC-alginate composite prevents oxygen partial pressure from decreasing to PC in the case of an encapsulated islet.

Predicted fractional viability and insulin secretion rate

By using the oxygen profiles shown in Fig. 28.9, the overall tissue fractional viability and insulin secretion rate were calculated for the capsules containing an islet. Comparison of beta cell fractional viability and relative insulin secretion for an islet centrally located in microcapsules containing 0, 30, 70, and 110 wt% PFC-alginate microcapsules are shown in Figs. 28.10 and 28.11, respectively. The range in capsule surface oxygen partial pressure (PS)

TABLE 28.3 Other model parameters

	Parameter value	Units
R_1	68	μm
R_2	75	μm
R_3	250	μm
K_m	0.44	mmHg
V_{max}	4×10^{-8}	mol/cm^3/s
P_C	0.1	mmHg
ρ_{PFC}	1.93	g/mL
$\rho_{soybean\ oil}$	0.92	g/mL

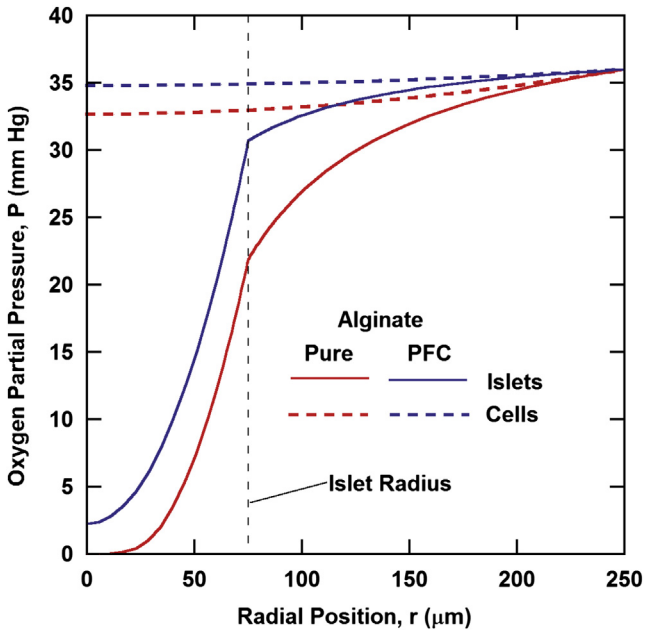

FIGURE 28.9
Oxygen profiles for microencapsulated islets and single cells in pure alginate and alginate containing 70 wt% PFC emulsion. PFC wt% = ρPFC × PFC vol%.

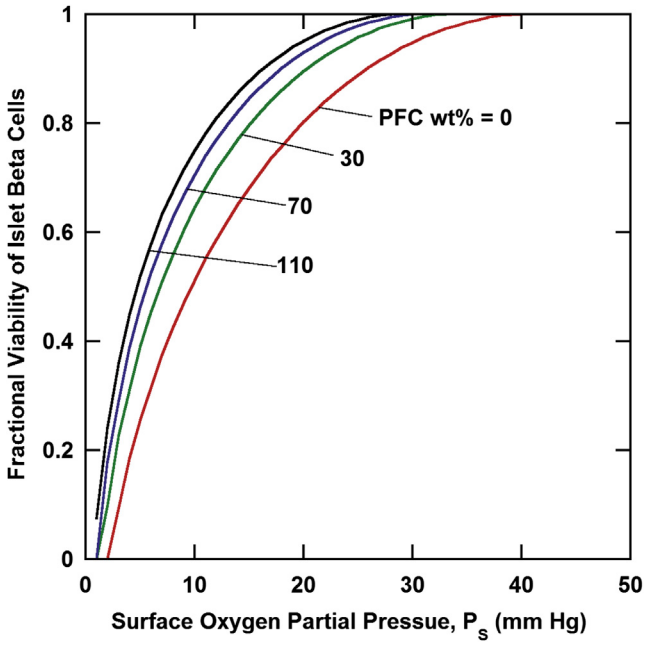

FIGURE 28.10
Predicted beta cell fractional viability for a centrally located islet in pure- and PFC-containing alginate microcapsules with variable PFC loading. PFC wt% = ρPFC × PFC vol%.

studied corresponds to the ranges that could occur in the peritoneal cavity, the most common implantation site of microcapsules. Experimental measurements of PO_2 in empty perfluorocarbon-loaded capsules have resulted in measurements ranging from 38 to 0 mmHg [60]. Over a range of PS there is an enhancement of beta cell survival for islets encapsulated in PFC-containing microcapsules, and the degree of enhancement is a function of PFC loading. Maintenance of normal insulin secretion of islet beta cells is substantially enhanced by including PFC in the microcapsules in a dose-dependent fashion (Fig. 28.11). The results in Figs. 28.10 and 28.11 demonstrate that including PFCs in alginate microcapsules can increase islet survival and even further enhance islet function after capsule transplantation.

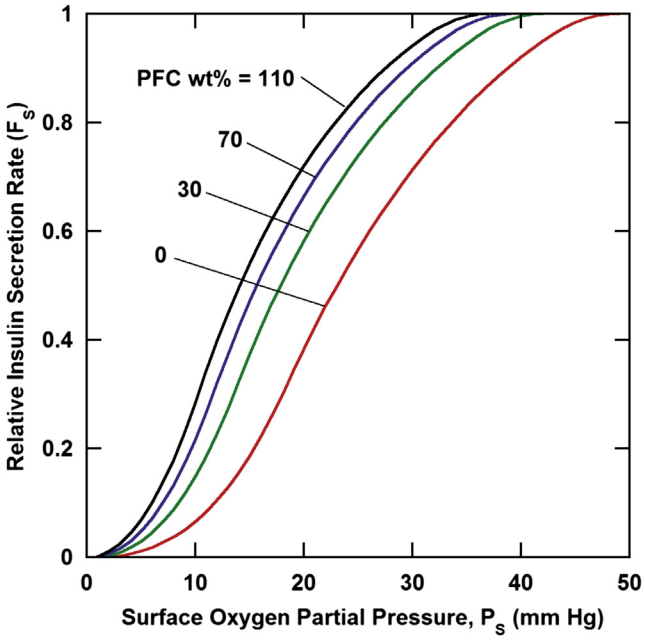

FIGURE 28.11
Predicted fraction of normal insulin secretion for a centrally located islet in pure- and PFC-containing alginate microcapsules with variable PFC loading.
PFC wt% = ρPFC × PFC vol%.

FUTURE DIRECTIONS

Immunobarrier devices have the potential to provide treatments for many human diseases. Immunobarrier devices have entered into clinical trials in which transplantation of smaller numbers of cells or placement in less immune responsive sites is possible or even desirable, and recently a clinical trial with islet containing alginate microcapsules has begun [16,61]. No encapsulation method has yet come into widespread clinical use. Through studies of new immunobarrier materials, methods to enhance the immunoprotection properties of the materials used as immunobarriers, and methods to increase the oxygen supply to the encapsulated tissue, many human diseases, including type 1 diabetes, may one day be treated by cell therapies using immunobarrier devices.

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PART 6

Stem Cells

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CHAPTER 29

Embryonic Stem Cells

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INTRODUCTION

Tissue engineering relies on the availability of cells capable of repairing or replacing damaged tissues and organs. Although multiple sources of cells from cadavers to animal tissues have been considered, stem cells are of intense interest because they can be expanded indefinitely in culture, and can also be directed to differentiate into a particular cell type of interest. Adult tissue-derived stem cells, such as those from bone marrow, fat, and skin are among the most studied and while some are currently being used in the clinic, these sources are donor-dependent, have limited expansion potential, and a highly restricted differentiation capacity. Ideally, having a single inexhaustible source of pluripotent cells that can generate large quantities of therapeutic cells would greatly facilitate regenerative medicine endeavors while simplifying quality control and safety testing. Human embryonic stem cells (hESCs) offer the unique possibility of meeting these criteria and thus may be able to circumvent many of the problems associated with variations in donor-dependent cell sources.

Derivatives of hESCs have been regarded by many as a panacea for the treatment of various ailments, ranging from osteoarthritis to blindness, heart disease and spinal cord injuries, among others. The list is long, and numerous promising studies in animal models indicate that the question now is not whether hESC-based therapies could be safe and effective but what indications are they best suited for and how soon can they become a standard of care. Before this happens however, several unique challenges will need to be overcome. For example, the generation of hESCs is wrought with controversy, since the standard derivation process involves destruction of a developing human embryo. Similar to adult tissue-derived cell sources, histo-incompatibility may also limit the widespread use of hESC-based therapies, since careful HLA matching will be needed to avoid immune rejection. Of note, the advent of induced pluripotent stem cells (iPSCs) could one day alleviate both the ethical and histo-incompatibility issues surrounding hESC use [1,2]. iPSCs can be generated from a person's own mature somatic cells and through the process of reprogramming, be turned back into a pluripotent state for use as a patient-specific renewable cell source with broad differentiation capabilities. Currently, iPSC technology is still being optimized for efficiency and safety, yet one day could rival hESC as the future of regenerative medicine. This chapter will focus on hESC and their derivatives, but much of the knowledge that has been gained through this research is applicable or even transferable to the iPSC field.

APPROACHES TO hESC Derivation

hESCs were first isolated in 1998 from the inner cell mass (ICM) of a pre-implantation human blastocyst in a landmark study by Thompson et al. [3]. As research with hESC gained interest,

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great promise as well as great controversy was brewing in the press. hESCs theoretically have the capability to give rise to all tissues of the human body and may provide crucial therapeutic treatment for a wide variety of diseases. However, the only known hESC derivation process back in the late 1990s/early 2000s involved destruction of a human embryo, and this was seen by some as the equivalent of killing a human being. Acknowledging the enormous potential of hESC research while also trying to quell the ethical firestorm that hESC derivation had garnered, in 2001, the Bush administration made the decision to allow federal funding for hESC research on the condition that only hESC lines already in existence could be used. The NIH hESC registry list [http://stemcells.nih.gov/research/registry/ (Accessed May 21, 2013)] was established to keep track of the approved lines. The original hESC lines derived by Thompson et al. at the University of Wisconsin [3] were among the first hESC lines registered, and have become the most widely used and published hESC lines available. Since then however, many other hESC lines have been derived using private funds, and other stem cell banks and registries have been created. Examples include the University of Massachusetts (UMass) International Stem Cell Registry http://www.umassmed.edu/iscr/index.aspx (Accessed May 21, 2013), European Human Embryonic Stem Cell Registry http://www.hescreg.eu/ (Accessed May 21, 2013), and UK Stem Cell Bank http://www.ukstemcellbank.org.uk/ (Accessed May 21, 2013).

hESCs retain a fundamental property of ICM cells — the ability to give rise to all tissues of the human body. The same markers of pluripotency are found in both ICMs and hESCs — the transcription factors Oct-4, NANOG, Rex-1, cell surface antigens SSEA-3, SSEA-4, TRA-1—60, TRA-1—81 (Fig. 29.1) and high alkaline phosphatase activity [4,5]. Cells of the ICM only exist for a few days in the natural course of embryo development; they arise during blastocyst formation from a morula on day 4 and persist throughout the first days or hours post-implantation before

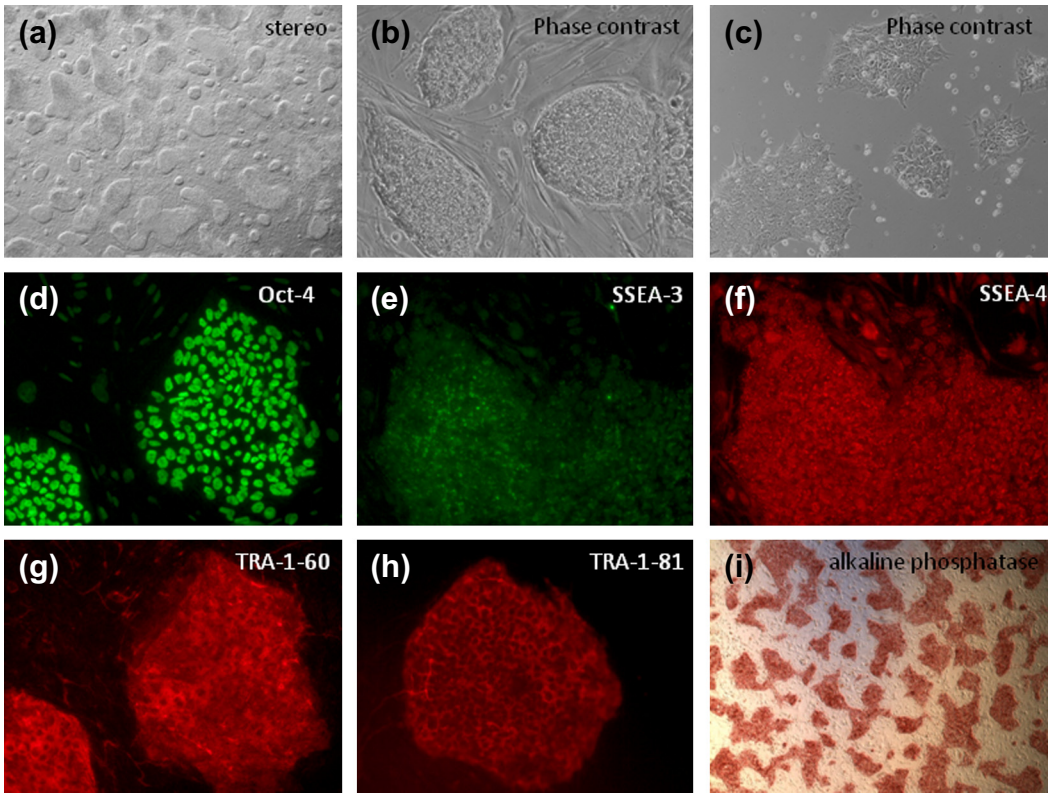

FIGURE 29.1

Morphology and markers of hESC. a, b — hESC grown on feeder cells, c — hESC grown on Matrigel, d—i — markers of pluripotency in hESC. a — stereo microscope image (as seen during mechanical dissesction), b, c — phase contrast, d — H immunofluorescence, i — bright field. d — Oct-4, e — SSEA-3, f — SSEA-4, g — TRA-1—60, h — TRA-1—81, i — alkaline phosphatase. Magnification: a, ×10, b, d—h, ×200, c, ×100, i, ×4.

beginning the highly orchestrated process of differentiation. Embryonic stem (ES) cells may be seen as an immortal extension of the ICM in culture; they retain high telomerase activity and can continue to self-renew indefinitely under appropriate culture conditions. When injected into immune-deficient mice, hESC form teratomas — tumors that contain derivatives of all three germ layers, the most typical ones being bone, cartilage, neural rosettes and epithelium of the airways and gut. Like the ICM of a developing blastocyst, pluripotent embryonic stem cells (ESCs) in culture are primed to differentiate, and as we will discuss later in the chapter, this makes working with them both easy and difficult at the same time.

Traditionally, ES cells have been derived from day 5–7 blastocysts with or without immunosurgery (removal of the trophoblast) by plating a blastocyst or an isolated ICM (Fig. 29.2) on a feeder layer of mitotically inactivated mouse embryonic fibroblasts [3,6]. However, new lines have now been derived using alternative approaches in an attempt to overcome the ethical issues surrounding embryo destruction during hESC derivation [7,8]. Moreover, the use of mouse feeder cells and animal products during hESC derivation and maintenance has been eliminated in newer methods because of safety concerns that they could harbor unknown xeno viruses. Producing cells that are compatible with a patient's own immune system is also an important consideration for preventing rejection. Thus at present, hESCs have been derived on human feeders [9–11] and even on feeder-free matrices [12]; they have been derived from growth-arrested IVF embryos that cannot be used for implantation [13], from unfertilized oocytes that are incapable of forming a developmentally competent embryo [14–17], and from a single blastomere of a morula stage embryo — a technology that allows the generation of hESCs while leaving the embryo unharmed [11,18–20].

Derivation of hESCs from chemically activated unfertilized oocytes (parthenote hESCs) was reported by several groups [14–17]. These studies showed that parthenote hESCs are very similar to ICM-derived hES cells: they have the same colony morphology and growth behavior, maintain a normal karyotype, express the same markers of pluripotency, and differentiate into derivatives of all three germ layers. These cells could become a solution to both the ethical and immune compatibility issues that plague traditional hESC lines, as a limited number of parthenogenetic hESC lines from carefully selected donors could match almost everyone on the planet because they have a reduced number of HLA. However, more recent studies showed

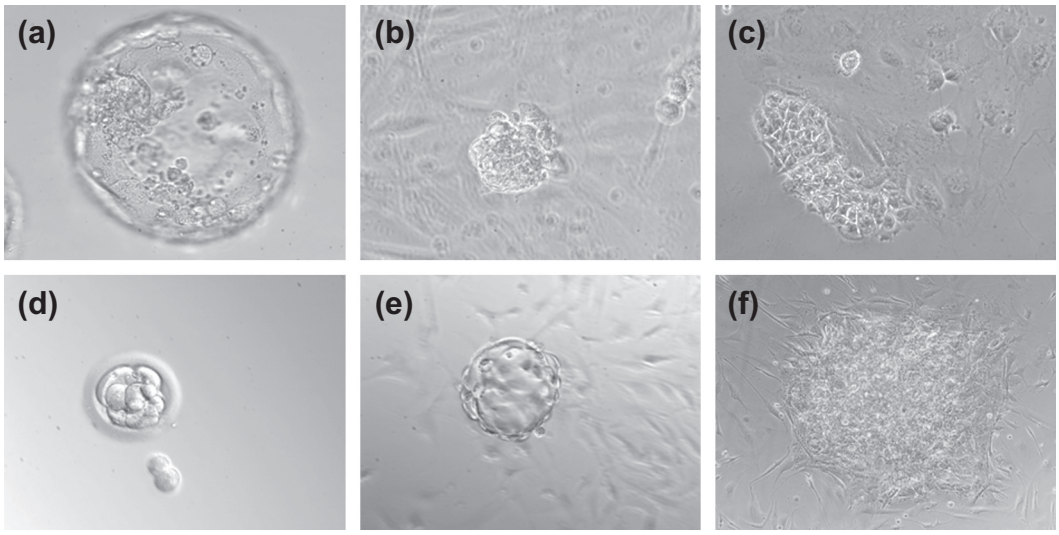

FIGURE 29.2
Early stages of hESC derivation from a blastocyst (a–c) and a single blastomere (d–f). a — expanded blastocyst, b — ICM after immunosurgery plated on feeder cells, c — ICM outgrowth, d — biopsied embryo and a developing blastomere, e — developing blastomere before plating on feeder cells, f — blastomere outgrowth.

that a large percentage of parthenogenetic blastomeres were affected by an excessive number of centrioles, a high aneuploidy rate [21], as well as genetic and epigenetic instability [22]. At this point in time, there are only limited data on the differentiation of parthenote hESCs [23–26] and properties of their derivatives, such as safety and functionality *in vivo*. While promising, it is probably too early to consider parthenogenetic hESC a safe alternative to 'conventional' hESC for generation of cells for regenerative medicine.

Another approach which has proven to be successful for generating hESCs without embryo destruction is the use of embryo biopsy. This procedure, commonly used in pre-implantation genetic diagnostics (PGD) in the course of IVF, involves removal of a single blastomere from a morula stage embryo and has resulted in hundreds of healthy babies being born. Using this procedure, we established several hESC lines from single blastomeres while the donor embryos were allowed to develop to the blastocyst stage and be cryopreserved [20]. Single blastomeres were first co-cultured with the biopsied embryos and then plated on feeder cells in microdrops [18–20]. Outgrowing colonies were treated the same way as ICM outgrowths (Fig. 29.2). In early experiments, blastomere outgrowths were co-cultured with GFP-expressing hESCs, which presumably helped to condition the microenvironment and help boost the outgrowth of hESC from blastomeres. After initial outgrowth was observed and the colony of blastomere-derived cells became large enough for passaging, GFP-negative colonies were marked under the fluorescence microscope and then mechanically dissected and re-plated; this procedure was repeated for several passages to ensure that the new cell lines were free of any contaminating GFP-expressing hESC. Additionally, after the blastomere-derived hESC lines were established, they were extensively tested for the absence of GFP-positive hESC used for co-culture and shown to be free of any GFP-expressing hESC [18,19]. Two hESC lines were generated that had similar properties to ICM-derived hESC: they stained positively for Oct-4, SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, alkaline phosphatase, had a normal karyotype, and differentiated into derivatives of all three germ layers both in teratoma assays in NOD-SCID mice and *in vitro*. In the first proof of principle study, the derivation yield was low and the embryos were not preserved after the biopsy, but later this study was repeated using an optimized approach which allowed the derivation of new lines from single blastomeres with higher efficiency and still without embryo destruction. Biopsies that were performed to isolate a single blastomere did not significantly affect the embryos; they developed to the blastocyst stage and were then frozen as expanded and/or hatched blastocysts [20]. Five hESC lines were produced this way from biopsied embryos and all of the embryos developed into healthy-looking blastocysts prior to cryopreservation [20]. This technique was subsequently used by several other groups to derive hESC lines with high efficiency and/or without destroying the embryos [11,27,28]. Single blastomere-derived hESCs showed a similar transcriptional profile to 'conventional' hESCs [29], and several differentiation derivatives of single blastomere-hESCs appeared functional *in vitro* and *in vivo* [30,31]. By avoiding the destruction of human embryos, this technique has addressed the ethical concerns surrounding hESC derivation, thereby offering a way to overcome a major hurdle in developing hESC-based therapies.

MAINTENANCE OF hESCs

Currently there are several popular hESC culture systems in existence, and with a little optimization, all of them can be used to grow pluripotent cells with relative ease. Mouse embryonic fibroblasts or human fibroblasts derived from fetuses, or placenta, or foreskin have been used as feeders for many years, and the corresponding medium for this culture system is based on Knockout-DMEM (KO-DMEM) and Knockout Serum Replacement (KSR, both components from Invitrogen), supplemented with at least 8 ng/ml human bFGF [12,18–20]. Variations of this system include addition of Plasmanate (Talecris) [12,18–20] or using a 1:1 mix of Knockout-DMEM and F12 medium, or feeder-produced extracellular matrix instead of live feeder cells. On feeders, cultured hESCs have an entirely unique morphology and are best

described as well-defined colonies of small tightly packed epithelial-looking cells with a high ratio of nuclei to cytoplasm and visible nucleoli (Fig. 29.1). When grown in KO-DMEM:F12 media mix or in the absence of feeders (i.e., 'feeder-free'), hESCs can deviate from this phenotype and look more spread-out and even grow as a sheet of cells: however, such cells still retain their pluripotency and are able to both self-renew and differentiate (Fig. 29.1).

Recently, a robust and user-friendly feeder-free system that employs a very dilute Matrigel solution (extracellular matrix produced by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells) and matching defined medium was introduced by Stem Cell Technologies. Another feeder-free system, made by Invitrogen utilizes CellStart, a human placenta-derived extracellular matrix. Feeder-free protocols are usually provided by individual manufacturers together with a recommended medium and should be optimized by the researcher. In our own studies, we used extracellular matrix produced by mouse embryo fibroblasts, which was deposited and assembled by live cells and provided a three-dimensional growth surface similar to that provided by live feeder cells. This matrix was used in combination with KSR/Plasmanate-based culture medium and was successfully used for derivation of hESCs. Derivation of an hESC line when the initial outgrowth consists of only a few cells is more challenging than routine culture of millions of cells, and this culture system proved to be very supportive of such a demanding task [12].

SUBCULTURE OF hESC

To subculture hESC, several methods can be employed. Mechanical colony dispersion and hand picking has been the gold standard in the field as it allows one to select colonies of correct morphology with minimal stress to the cells, and is probably the only method to derive new lines when there are only a few pluripotent cells and the colony needs to be dissected to avoid differentiation. However, the operator should have appropriate experience because it is also possible to select for aneuploid cells that have a growth advantage as they are quicker to form good-sized and 'good-looking' colonies. On the other hand, a skilled operator may be able to rescue an aneuploid culture by carefully picking colonies of the correct morphology [34]. Of note, extra care must be taken to avoid damaging cells when using mechanical dispersion on hESC grown in a feeder-free system, as these colonies are more fragile than those grown in a feeder-based system. Mechanical passaging is very labor-intensive, and for large scale culture, as required for manufacturing of differentiated cells for therapeutic use, enzymatic dissociation of colonies into single cells and small clumps is much more efficient. Collagenase, accutase, trypsin, TrilpLE (Invitrogen) and EDTA are commonly used for dissociation of hESCs, but all of these can contribute to clonal aneuploidy at later passages, as single cells and small cell clumps (potentially containing aneuploid cells) are distributed throughout the cell population. The tendency of hESCs to undergo clonal aneuploidy reinforces the importance of routine karyotyping while subculturing them. G-banding with examination of a minimum of 20 cells or fluorescence *in situ* hybridization with probes to chromosomes 12 and 17 are appropriate karyotyping methods, as the gain of an extra copy of chromosomes 12 and 17 commonly gives aneuploid ES cells a growth advantage [32–34].

NUANCES OF hESC CULTURE

Almost every culture of hESCs will contain some differentiating cells, and if the colonies are allowed to overgrow, become multi-layered and/or touch each other, they will begin to show massive spontaneous differentiation within hours. Fig. 29.1 shows the morphology of hESCs grown on feeder cells and feeder-free on Matrigel in undifferentiated and slightly differentiated states. Once the cells begin to differentiate, it is more difficult to isolate self-renewing cells. Hand picking may be used in an attempt to isolate non-differentiated colonies, yet even normal looking colonies may harbor cells committed to differentiation; they may have already lost Oct-4 expression and may even express early differentiation markers like nestin (Fig. 29.3).

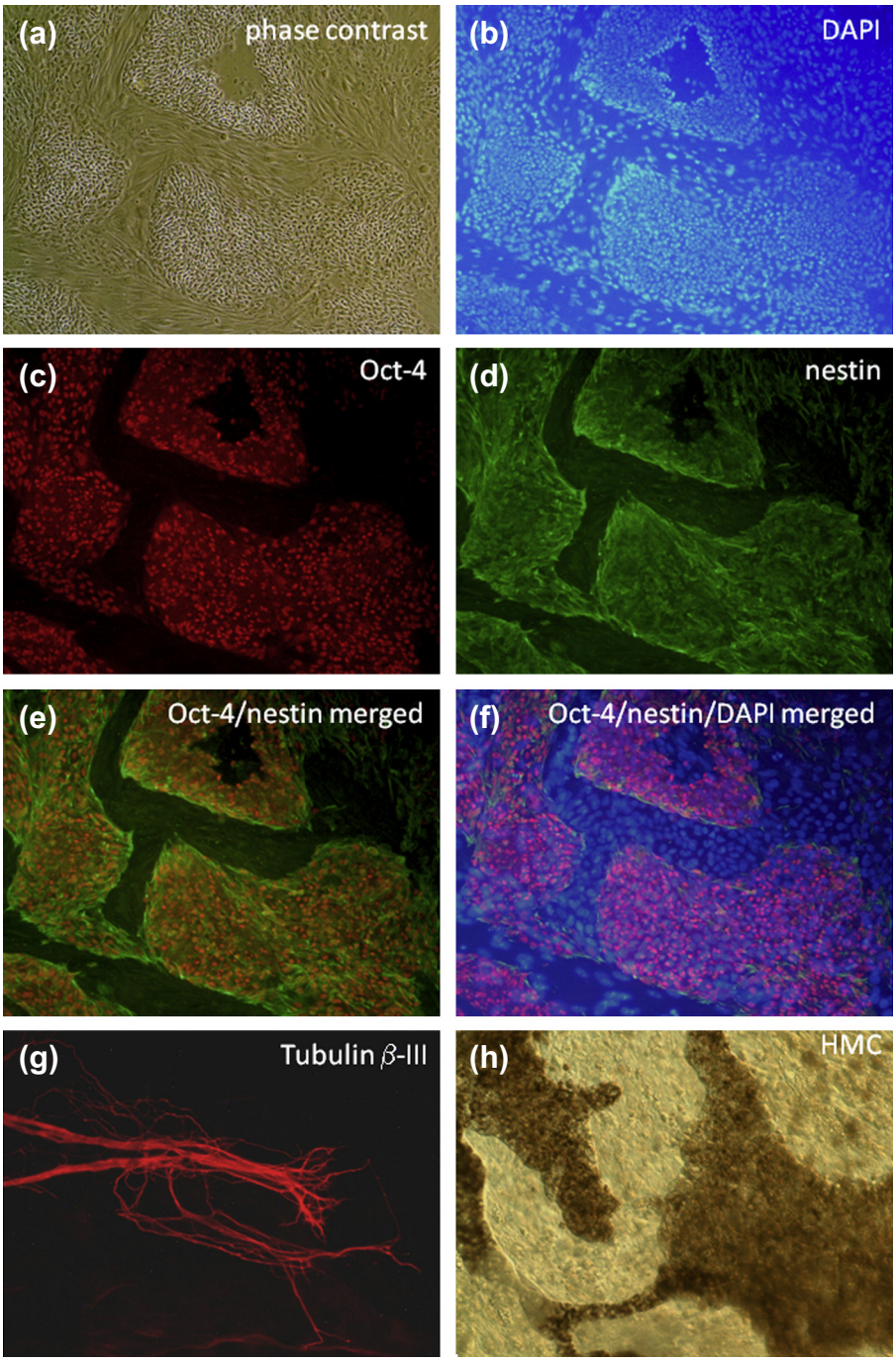

FIGURE 29.3
Spontaneous differentiation of hESC. a—f, nestin-expressing cells are seen within Oct-4 positive colonies. a — phase contrast, b — DAPI co-staining, c — Oct-4, d — nestin, e — Oct-4/nestin merged, f — Oct-4/nestin/DAPI merged. g — tubulin β-III staining showing a differentiated neuron, h — a cluster of RPE. Magnification: a—f, ×100, g, h, ×200. *Images of Oct4/nestin are courtesy of Jennifer Shepard.*

Other nuances of hESC culture come to light with experience and it has been observed many times that different culture conditions may predispose hESC to one or another differentiation route or make a routine differentiation method more difficult to perform. For example, embryoid body (EB) formation is a common first step in many differentiation protocols and it involves plating a suspension of freshly harvested hESC into plates with low adherence properties where they can aggregate into clumps and differentiate into cells of all three germ layers. EB formation occurs quite readily when hESCs have been growing on feeder layers: multitudes of large, round EBs form in suspension with little cell death. Yet, when hESC have been cultured in a feeder-free system, EB formation is problematic; the EBs that form are much smaller and sparser, with a lot of cell death observed in the culture. Additionally, our

experience with multiple hESC lines has shown that some slow growing lines are difficult to maintain due to spontaneous differentiation but they often show very good yields of derivatives when specifically directed to differentiate. On the other hand, other hESC lines are fast-growing and easy to maintain but do not differentiate as robustly. Given these types of peculiarities, differentiation protocols should be tested and modified when different ESC culture conditions or different hESC lines are used.

DIRECTED DIFFERENTIATION

While spontaneous differentiation of hESCs in an adherent state or via EB formation may produce certain types of derivatives (e.g., cardiomyocytes, adipocytes, retinal pigment epithelium, neurons), in many circumstances a higher yield or purity is desirable. Various bioactive factors and/or selective growth conditions can be employed to direct hESC differentiation towards a particular lineage and/or enrich for a desired cell type. While theoretically hESC can give rise to all cell types of the human body, in reality, some derivatives are easier to produce in culture than others, and some have a higher therapeutic potential than others. Some of the highly sought after derivatives with therapeutic value are cardiomyocytes, neural progenitors, hemangioblasts, mesenchymal stem cells, endothelial progenitors, smooth muscle cells, hepatocytes and insulin-producing beta cells. As we discuss below, many different hESC derivatives are being explored and developed for potential clinical use and to date, two derivatives have entered human clinical trials: oligodendrocyte precursors (trial #NCT01217008) and retinal pigment epithelium (trials ## NCT01345006, NCT01469832, NCT01344993; clinicaltrials.gov).

A very interesting hESC derivative with great potential for clinical application is the hemangioblast [35–37] – a multipotent cell which gives rise to both hematopoietic and vascular cells. These blasts can be expanded, cryopreserved (if necessary) and subsequently differentiated into multiple other cell types. Since hemangioblasts can give rise to all types of blood cells, they may one day serve as alternative cell source to primary hematopoietic stem cells for reconstituting the blood system after myeloablation. Or, alternatively, they may be used to generate large scale production of mature blood cells. Erythrocytes and platelets have both been efficiently generated from hemangioblasts [39,40]; the former carried oxygen in a manner similar to primary red blood cells, while the latter formed lamellipodia and filopodia in response to thrombin activation and contributed to thrombi formation at sites of induced vascular injury. Lu et al. have shown that hESC-derived hemangioblasts can also efficiently repair damaged vasculature in rodent models of ischemic reperfusion injury to the limb and eye [37]. In this study, human blast cells integrated into the endothelial network and differentiated into endothelial cells as was shown by double staining with antibodies to CD31 and human nuclear antigen. Another study demonstrated that hemangioblast-produced smooth muscle cells integrated into the vessel wall in nude mice [41]. Other cell types may also be generated from hESC through the use of a hemangioblast intermediate stage. The robust expansion capabilities of this multipotent precursor facilitate large scale production of more mature cell types further downstream.

Other potentially therapeutic cells that have been differentiated from hESCs are mesenchymal stem/stromal cells (MSCs). MSCs are plastic, adherent, fibroblast-like cells that, at a minimum, can differentiate into bone, fat and cartilage. The exact cell surface marker profile that defines true a MSC is still up for debate, yet most MSCs, including those derived from hESCs express CD73, CD90 and CD105 [42–47]. Given their differentiation capabilities, MSCs are being evaluated for treating joint disorders such as osteoarthritis and sports-related injuries. MSCs have also been shown to harbor powerful immunomodulatory properties and this has generated a lot of interest in using them to treat autoimmune disorders. Importantly, MSCs are able to evade immune rejection in non-HLA-matched patients, thereby facilitating allogeneic therapies without the need for immunosuppression. While the exact mechanism responsible

for their immuno-privileged status is unclear, the lack of HLA class II, CD40, and co-stimulatory receptors CD80 and 86, as well as the expression of HLA G, a class I heavy chain paralogue may all be involved [48]. MSCs can be isolated from various adult tissues such as bone marrow, adipose and dental pulp [49] and numerous publications have described the efficacy of these cells in treating animal models of human disease. Over 200 clinical trials are currently underway with adult-derived MSCs for treating anything from autoimmune disorders like lupus, to lung diseases like asthma, to healing wounds and diabetic ulcers. An emerging theory is that younger tissue sources such as cord blood, Wharton's jelly, and hESC may be a better source material for MSC than adult tissues, as their youthful nature facilitates greater *in vitro* expansion and preservation of therapeutic function [43,50–52]. Although hESC-derived MSCs have yet to be tested in clinical trials, they have shown promise in animal studies; they have exerted protective effects in mouse inflammatory bowel disease [43], rat tendon injury [42] and rat limb ischemic injury [46] models. As with tissue-derived MSCs, the possibilities for hESC-MSC clinical use are far-reaching and diverse. Because of their superior *in vitro* expansion capabilities and longer lasting immunomodulatory properties, MSCs derived from hESCs could one day compete with or even replace adult tissue-derived MSCs as an allogeneic off-the-shelf product that can be used with minimal immune suppression.

Cardiomyocytes were one of the first identified differentiated cell types produced from embryonic stem cells [53–60]. Observed in tissue culture dishes as 'beating hearts', patches of cardiomyocytes can easily differentiate from mouse embryoid bodies. Despite the ease of generating patches of cardiomyocytes, they are not easily purified from other cell types that spontaneously arise in differentiating ESC cultures. Multiple growth factors such as activin A and BMP4 and small molecules have been employed to enhance the differentiation of cardiomyocytes with varying success [59]. They can be purified using a Percoll gradient or, because they have a high mitochondria content, a fluorescent dye, tetramethylrhodamine methyl ester perchlorate (TMRM) that labels mitochondria and allows cell sorting can be used [60]. Cell surface markers such as VCAM1 and signal-regulatory protein alpha (SIRPA) have also appeared useful for purification of cardiomyocytes using antibody-based cell sorting [57,58]. Unfortunately, differentiated cardiomyocytes have a limited lifespan and engraftment potential, and the production scale obtainable from hESC is still far too low to repair an ischemic heart. Cardiac progenitor cells may be a better candidate, as they can proliferate and engraft better, and the search for efficient generation of such cells from hESCs is ongoing.

The treatment of diabetes has perhaps been one of the most highly sought after applications for hESC-based therapies, yet the need to effectively differentiate hESC into functional insulin-producing beta cells remains unmet. Multiple studies have been published which report insulin-producing cells differentiated from stem cells, but the majority of insulin-producing cells in culture also make glucagon, which indicates that they are not fully mature [61]. Promising protocols to differentiate hESCs into beta-like cells attempt to simulate different stages of endoderm development *in vitro* by using small molecules and growth factors, manipulating the substrate composition, and using a 3D microenvironment. Several groups have been able to identify stage-specific cell surface markers and isolate enriched populations of cells using PDX1, a pancreatic progenitor marker [62,63]. A staged suspension-based method allowed the scalable production of pancreatic progenitors which, upon engraftment, produced glucose-responsive, insulin-secreting, islet-like tissue that protected mice from streptozotocin-induced hyperglycemia [64]. Poly-hormonal beta-like cells are not fully functional and resemble poly-hormonal cells of early developmental stages, but they can mature into functional cells upon transplantation [65], and the main challenge now is to accomplish the second stage of differentiation of pancreatic progenitors *in vitro*.

Some of the very first and best-studied derivatives of hESC were cells of the neuronal lineage – perhaps, because they are the easiest to obtain in culture [Fig. 29.3] due to a widely recognized ability of hESC to assume a neuronal fate in the absence of other differentiating

signals [66–68]. Many mature and progenitor-like neuronal cell types have been isolated from hESCs [69–73] and shown to be functional in animal models. Such are dopamine-secreting neurons, which improve the behavior of Parkinsonian mice after their transplantation into the brain [71,72]; motor neurons, oliogodendrocytes and motor neuron progenitors [73–78] capable of improving function in a spinal cord injury model; and neural progenitors showing functional engraftment in the brain in experimental ischemia and stroke [79–84]. Oligodendrocyte precursor cells were the first hESC-derived product to be tested in a clinical trial, for the treatment of subacute thoracic spinal cord injury [www.geron.com/grnopc1 (Accessed May 21, 2013)]. While preliminary results from the trial did not show any deleterious effects due to injection of the hESC-derived product [http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1635760&highlight=spinal cord (Accessed May 21, 2013)], its manufacturer Geron surprisingly shut down its entire ESC research division a year after the trial began, citing the need for financial restructuring and the desire to focus on oncology drug-based therapies [www.businesswire.com/news/home/20111114006308/en/Geron-Focus-Cancer-Programs (Accessed May 21, 2013)].

Besides Geron's oligodendrocyte precursors, the only other derivative of hESCs that has entered human clinical trials to date is Advanced Cell technology's (ACT) retinal pigment epithelium, or RPE. hESC-derived RPE [31,85–87] is being tested in Phase I/II clinical trials in both the US and Europe for two diseases that involve retinal degeneration, namely dry age-related macular degeneration and the heritable juvenile Stargardt's disease [www.advancedcell.com (Accessed May 21, 2013)]. RPE provides 'life-support' to the photoreceptor cells by supplying nutrients and removing shed photoreceptor fragments by phagocytosis, and its malfunction leads to photoreceptor degeneration and eventual blindness. RPE has several unique features that allow this cell type to be an attractive hESC derivative for regenerative medicine purposes:

1. It is easy to detect in culture and isolate with a high degree of purity. Differentiated RPE cell appear as pigmented clusters visible by the naked eye among spontaneously differentiating hESC after several weeks. Their morphology is very similar to that of cultured human RPE from *in vivo* sources – polygonal cells with different degrees of pigmentation. When RPE originating from hESC was first discovered in long term differentiating cultures of hESC, it was due to its unique morphology. Clusters of pigmented epithelial cells were observed and mechanically isolated, expanded and then confirmed to be of this cell type by the molecular marker analysis [85,86]. This 'mechanistic' approach is still being successfully used for isolation of RPE from differentiating cultures of hESC containing multiple layers of multiple cell types, RPE being a minor fraction among other derivatives. When such differentiated cultures are dissociated, it is possible to establish pure cultures of hESC-RPE using cell morphology as the only guidance throughout the mechanical hand picking of pigmented clusters because, although these cells are mature and differentiated, they are nevertheless fully capable of multiple divisions. One six-well plate of differentiating hESC can provide enough RPE at passage 2 to treat 10–20 patients (including many more millions of cells for quality testing).
2. The fully differentiated cells retain their proliferative potential for several passages, allowing one to scale up production without any loss of functional properties. This is owed to a distinctive feature of RPE called transdifferentiation. During this process, cells of a differentiated phenotype are capable of partial de-differentiation and re-differentiation for multiple passages. Under proliferation-promoting conditions, mature RPE cells lose their pigmented epithelial morphology and turn into neural progenitor-like cells (as if going back to their neuroectoderm developmental roots). This is accompanied by the down-regulation of mature RPE markers such as bestrophin, CRALBP, RPE65; loss of cell polarity and cuboidal morphology; and upregulation of neural and retinal progenitor markers – for instance, nestin, Pax6, Sox2, tubulin beta III. When the cells reach confluence and their proliferation slows down, they re-differentiate into pigmented epithelial polarized cuboidal cells and up regulate RPE markers, and if passed again, the cycle would continue for several

passages before they senesce and stop dividing or re-differentiating [85,86]. The ability of these fully differentiated cells to proliferate for several passages while maintaining their phenotype and capabilities after they re-differentiate each time is critical for the large scale production that is needed for both animal safety studies and human clinical trials.

3. hESC-RPE cells can be frozen and thawed without any loss of functionality and can be maintained in culture in a fashion that ensures optimal conditions for post-transplantation survival and functional engraftment. Thus, once mature RPE cells are isolated and plated at passage 0, it becomes possible to monitor and control the degree of their differentiation at each passage. Cells with optimal pigmentation and at the optimal differentiation stage can be identified with differentiation markers and produced for the final harvest. Our studies showed that there was a correlation between the adhesion/survival of RPE *in vitro* and the degree of maturity/melanin content [31] as well as between their effectiveness in animal models and their degree of pigmentation [31].

SAFETY CONCERNS

Safety is the number one requirement for the clinical use of cellular products. For hESC derivatives, this means that they have to be non-tumorigenic. They must be free of pluripotent cells, which can form teratomas *in vivo* and show no adverse effects in animal models. They should also be able to survive long enough to be effective after transplantation but not proliferate uncontrollably, although some proliferation followed by a steady quiescent state would be permissible and in certain cases even desirable. This may not be attainable for every derivative of hESCs, because for clinical use, large numbers of cells need to be generated in culture first. This means that cells of a given type should be able to proliferate in culture but nevertheless maintain a differentiated phenotype after transplantation. This requirement would only allow one to use either fully differentiated cells which are still capable of multiple population doublings (like RPE or MSC), or progenitor/stem cells which are already committed to a known lineage and do not 'misbehave' upon transplantation.

All cell products for clinical use need to be manufactured under Good Manufacturing Practices (GMP), pass sterility and micoplasma tests, and be free of known animal and human pathogens. The presence of animal products during derivation and maintenance of hESC and their progeny is currently unavoidable in most cases, but even co-culture with live animal cells is not forbidden by FDA (Food and Drug Administration) regulations. However, the use of animal cells or products adds more stringent testing of the source cells and their differentiated products. Extensive testing for animal viruses typical for the species whose products were used during manufacturing is required. Special guidelines exist for a xeno product — human cells that have been co-cultured with live animal cells — while cells manufactured in the absence of live animal cells and only exposed to animal products are not considered a xeno product. All bovine sera need to come from spongiform-free sources, typically from the US, New Zealand and Australia.

As previously mentioned, known spontaneous aneuploidy of hESCs in culture can occur quite readily and this presents an additional hurdle during manufacturing. Cells need to be frequently monitored for chromosomal aberrations. Additionally, any and all hESC derivatives need to undergo extensive testing in animal models to exclude any probability of tumor formation or other serious adverse effects. Such studies should be performed in immune compromised or immune suppressed animals to allow long-term survival of the donor cells. Ideally, the cells should be present in the host long term, in some cases throughout the lifetime of the animal; if they proliferate soon after transplantation, they should later become quiescent and stay negative for proliferation antigens, such as Ki67. They should maintain a normal phenotype which could be assessed by double staining animal tissues with anti-human antigen (nuclei, mitochondria) antibodies and antibodies to specific cell markers, and cause no adverse effects including neoplasia. The cells should be able to perform their function in

order to justify their transplantation in people. ACT's hESC-derived RPE met all these safety requirements in long-term studies (nine months) in mouse and rat animal models [31,85]: the cells integrated into the host's own RPE layer, provided photoreceptor support in the models of retinal degeneration (Royal College of Surgeons (RCS) rat) and Stargardt's disease (Elov4 mouse), showed no proliferation beyond the initial three months, had normal RPE morphology (cuboidal pigmented polygonal cells) and showed the late RPE marker bestrophin organized in a baso-lateral, and typical for RPE *in vivo* fashion.

CONCLUSIONS

hESCs offer the unique possibility of generating a wide variety of differentiated cells for tissue engineering 'from scratch'. Their immortal nature allows the production of hESCs in virtually unlimited numbers, enabling the creation of a Master Cell Bank, and together with the ability to produce differentiated derivatives, makes it possible to generate the desired cells on a scale large enough for both treating patients and performing all *in vitro* and *in vivo* tests necessary to ensure the safety and functionality of these cells. Such derivatives may include progenitor/tissue specific stem cells capable of differentiating further *in vivo* (which may offer advantages in tissue repair) and also mature differentiated cells which are still capable of proliferation. The derivatives, when cultured *in vitro* from a hESC origin, should be maintained in a manner that ensures their optimal *in vivo* performance. In the coming years, we will hopefully see more hESC-based therapies move from bench to bedside, not only for a range of diseases caused by tissue loss or dysfunction, but as an unlimited source of cells to replace more complex tissues such as skin, bone, and blood vessels, and eventually whole organs such as kidneys and hearts.

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CHAPTER 30

Induced Pluripotent Stem Cells

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INTRODUCTION

Induced pluripotent stem cells (iPSCs) exhibiting high similarity to embryonic stem cells (ESCs) have been derived from diverse somatic cell types and many animal species. Given their easily accessible somatic cell origin, human iPSCs not only eliminate the ethical issues presented by human ESCs due to their embryonic origin, but also enable the easy production of patient-specific pluripotent stem cells. Similar to human ESCs, human iPSCs are capable of robust proliferation *in vitro* while retaining the developmental potential to differentiate into many cell types of the body. Thus, these cells can deliver an essentially unlimited number of patient-specific somatic cells for disease modeling, toxicity testing, drug screening and transplantation therapies. Towards these ends, many improvements have been made in the methods for iPSC derivation with the ultimate goal of generating high quality clinical-grade human iPSCs. This chapter gives a detailed overview of iPSC technology, the similarities and differences between iPSCs and ESCs, the current applications of human iPSCs, and current challenges for these applications. It will give readers a clear understanding of the current status of iPSC research and the impact of iPSCs on the future of regenerative medicine.

METHODOLOGICAL OVERVIEW OF iPSC Derivation

After the initial excitement over the successful derivation of iPSCs [1–3], three obstacles to realizing the full potential of human iPSCs immediately became apparent. First, the reprogramming efficiency was very low (~0.01–0.02%) for the original sets of reprogramming factors, the Yamanaka factors (*OCT4*, *SOX2*, *c-MYC*, *KLF4*) and the Thomson factors (*OCT4*, *SOX2*, *NANOG* and *LIN28*). There also appeared to be iPSC clone-to-clone variations with significant functional consequences [1,3]. Both would undoubtedly impede the industrial application of human iPSCs, which would require a robust and efficient process for the derivation and expansion of high quality cells in order to ensure a high success rate for samples having diverse genetic background and age groups. Second, the initial methods for iPSC derivation employed genome-integrating retroviral/lentiviral vectors, which could produce tumorigenic insertional mutations. Furthermore, residual and/or reactivation of transgene

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expression during iPSC differentiation could affect lineage choice and the functionality of iPSC derivatives [2–3]. To overcome these problems, methods were needed in order to enable the derivation of iPSCs free of exogenous DNA (footprint-free). Third, most of the derivation and culture of human iPSCs employed undefined human ESC culture media and feeder cells. The inherent variations associated with both undefined culture medium and feeder cells greatly affect both the derivation efficiency and the quality of human iPSCs. The preparation of feeder cells (both autologous and non-autologous) can be labor intensive. More importantly, the use of feeder cells can introduce immunogenic antigens into human iPSCs, making them unsuitable for transplantation therapies [4]. Human iPSCs are thus ideally derived and cultured in chemically defined medium and matrix free of feeder cells.

IMPROVING REPROGRAMMING EFFICIENCY AND THE QUALITY OF iPSCs

Improving reprogramming efficiency and the quality of iPSCs has been a significant focus of the research following the initial derivation of iPSCs. Studies show that both reprogramming efficiency and the quality of iPSCs can be greatly affected by donor cell types, reprogramming factor stoichiometry, additional reprogramming factors and chemical compounds. To date, iPSCs have been successfully generated from many different somatic cell types, such as skin cells, neural cells, adipose tissue-derived cells and blood cells. It now appears that iPSCs can be derived from most, if not all, somatic cell types of the body. However, the specific donor cell type has significant impact on both the kinetic and efficiency of reprogramming. For example, compared to skin fibroblasts, a commonly used donor cell type, keratinocytes give over 100-fold higher reprogramming efficiency and 2-fold faster reprogramming kinetics [5]. Among the same somatic cell lineage, cells of early developmental stages and stem/progenitor cells were generally more amenable to reprogramming compared to terminally differentiated cells. An elegant demonstration of this notion is shown in a study carried out by Eminli et al. [6]. The authors generated high-degree chimera mice using iPSCs produced with inducible lentiviral vectors expressing the Yamanaka factors. Upon transgene induction, hematopoietic stem and progenitor cells isolated from the chimera mice gave rise to iPSCs at efficiencies nearly 300-fold higher than the terminally differentiated B and T cells (0.02–0.04%). In addition to reprogramming kinetics and efficiency, different donor cell types showed different minimal requirement for reprogramming factors. For example, although at least three transgenes, including *OCT4*, *SOX2*, *KLF4*, were used to reprogram most donor cell types, iPSCs can be successfully derived from human neural stem cells via the expression of a single *OCT4* gene [7], likely compensated by their endogenous gene expression. Nevertheless, efficient reprogramming is generally accomplished with more than one reprogramming factor. The inherent difference in the epigenetic stability between various donor cell types will be an important factor in the eventual selection of appropriate donor cell types for the derivation of clinical-grade human iPSCs.

A somewhat less studied factor significantly impacting both the reprogramming efficiency and the quality of iPSCs is the balance or stoichiometry between the expression of different reprogramming factors, mostly due to the difficulty in exerting precise control over transgene expression in donor cells. The first set of reprogramming experiments employed transgenes individually packaged into viral vectors. Transduction of differentiated cells was carried out with saturating multiplicity of infection for each transgene [1–3]. It was later found that the expression levels and ratios between different transgenes had great impact on both reprogramming efficiency and the quality of iPSCs. For example, the balanced expression of *OCT4*, *SOX2*, *NANOG* and *LIN28* led to a nearly 10-fold increase in the reprogramming efficiency of human neonatal foreskin fibroblasts [8]. Using fluorescent proteins that track the expression of each reprogramming transgene, it was found that an optimal level of *Oct4* expression relative to *Sox2*, *Klf4* and *c-Myc* was necessary to achieve efficient reprogramming [9]. Another illustration

of the importance of the reprogramming factor stoichiometry was shown by Carey et al. [10]. High expression of *Oct4* and *Klf4* along with lower expression of *Sox2* and *c-Myc* was able to produce iPSCs that efficiently generated 'all-iPSC mice' by tetraploid complementation, an unequivocal demonstration of iPSC pluripotency. Thus, care must be taken when drawing conclusions from current studies on iPSCs generated with different reprogramming factor stoichiometry.

The reprogramming efficiency and quality of iPSCs were also greatly affected by the combination of reprogramming factors used. The successful derivation of iPSC generally requires multiple rounds of cell division and the expression of transgenes for over 10 days [11]. Reprogramming by somatic cell nuclear transfer or cell-cell fusion, however, happens rapidly and efficiently. In nuclear transfer or cloning experiments, the nuclei of differentiated cells are transferred into enucleated eggs, which are then allowed to develop into whole new animals [12]. In cell-cell fusion experiments, the somatic cell nuclei can be reset to a state of pluripotency when fused with pluripotent stem cells such as embryonic carcinoma cells, ESCs, and embryonic germ cells [13]. This pointed to the existence of additional factors that can facilitate reprogramming. Indeed, although both the Yamanaka factors and the Thomson factors were sufficient to restore pluripotency in differentiated human somatic cells, the combination of both sets of reprogramming factors (*OCT4*, *SOX2*, *c-MYC*, *KLF4*, *NANOG* and *LIN28*) led to significantly improved reprogramming pace and efficiency ($\sim$100 fold) [14]. Replacement of the wild-type *c-MYC* with transformation-deficient *N-MYC* or *L-MYC*, which are enriched in human ESCs, resulted in even higher reprogramming efficiency and less tumorigenic iPSCs [15]. Additional reprogramming factors were identified, e.g., *AICDA* [16], *TBX3* [17], *Nr5a2* [18], *SV40LT* [19], and the list is still growing. Among these factors, *Nr5a2* was able to replace *Oct4* in iPSC derivation from mouse somatic cells [18]. The p53 pathway was shown to limit iPSC establishment [20–22]. And its suppression by either short hairpin RNA or protein expression (e.g., SV40LT) significantly increased both the kinetic and efficiency of reprogramming. More interestingly, microRNAs enriched in ESCs were also found to enhance the production of iPSCs. The miRNAs, miR-291-3p, miR-294 and miR-295, appeared to be the downstream effectors of *c-Myc* [23]. Successful production of both mouse and human iPSCs using mature microRNAs only (e.g., mir-200c, mir-302 and mir-369) were also reported [24–25]. Aside from native genes, synthetic transcription factors were shown to dramatically improve reprogramming. For example, fusion between the powerful transactivation domain of VP16 or *MYOD1* and *OCT4* resulted in significantly improved reprogramming efficiency [26–27].

One area of iPSC research that has been rapidly expanding in recent years is the application of small molecules in the derivation, culture and differentiation of iPSCs. Combined genetic and chemical engineering has not only improved our current understanding of the establishment and maintenance of pluripotency, but also greatly facilitated the practical applications of iPSC technology. The list of small molecules implicated in reprogramming are continuously growing, and include chromatin modifiers such as DNA methyltransferase inhibitor 5-aza-cytidine and histone deacetylase inhibitor valproic acid; cell signaling modifiers such as mitogen activated protein kinase/ERK kinase (MEK) inhibitors, glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021, TGF-β/Activin/Nodal receptor inhibitors; and others such as vitamin C [28]. These compounds were not only able to improve both the pace and the efficiency of iPSC derivation, but also could affect the outcome of the pluripotent state of iPSCs obtained. The use of epigenetic modifiers including both DNA methyltransferase inhibitors and histone deacetylase inhibitors, however, requires caution. Their broad and nonspecific effects will cause an overall dysregulation of gene expression, increasing safety concerns for the practical applications of human iPSCs. For example, 5-aza-cytidine is highly mutagenic [29], and mice with global alterations in DNA methylation levels develop tumors at a high frequency [30]. Some of these molecules can replace specific reprogramming transgenes. For example, the inhibition of TGF-β signaling can bypass the requirement for exogenous *c-Myc* or *Sox2* [31–32], and the use of kenpaulane can substitute for *Klf4* [33]. The replacement of

reprogramming genes with small molecules, however, was often accompanied with significant decreases in both the kinetic and efficiency of reprogramming.

Progress made over the last several years has put iPSC technology within reach of many laboratories around the world. However, with the identification of more and more reprogramming factors and chemical compounds, identifying an optimal iPSC reprogramming approach has become increasingly complicated. It remains to be addressed how different combinations of reprogramming factors and their stoichiometry, along with chemical compounds, affect the reprogramming process and the epigenetic status of iPSCs. This is further complicated by the fact that different donor cell types might require a different set of reprogramming factors and stoichoimetry for optimal reprogramming. These are the issues that need to be taken into consideration when interpreting results from current studies on iPSCs produced by different methods.

Generating footprint-free iPSCs

Currently, genome-integrating retroviral/lentiviral vectors are the most used transgene delivery system for iPSC derivation. Compared to most available transgene delivery systems, these vectors enable prolonged expression of reprogramming factors, thus providing an efficient means for iPSC derivation. However, the retention of these viral vectors and transgenes in the genome of iPSCs creates many problems for the applications of iPSCs. Removal of transgenes via Cre/loxP recombination can alleviate problems associated with reprogramming factors [34]. Residual viral vector sequences, however, are still retained in the iPSC genome, causing potentially tumorigenic insertional mutations. For practical applications, ideally iPSCs would be free of vector and transgene sequences. For this purpose, many methods have been developed, including repeated treatments of reprogramming factors (plasmids, minicircle DNA, non-integrating adenoviral vector and proteins) [35–38]. These methods, however, are generally associated with low reprogramming efficiency. *piggyBack* transposon-based vectors were also used to derive footprint-free iPSCs because of their seamless excision property [39–40]. Although good reprogramming efficiency was achieved with this approach, the removal of vector and transgenes from iPSCs can be labor-intensive.

One method allowing efficient production of footprint-free iPSCs employed a vector based on Sendai virus, a single-stranded RNA virus that does not integrate into the host genome [41]. The Sendai viral vectors replicate constitutively in the cytoplasm of infected cells, allowing the generation of iPSCs. The established iPSCs can gradually lose these vectors over extended culture, which occurs at a variable rate among different iPSC clones. This process could be sped up by the introduction of temperature-sensitive mutations in the polymerase-related genes [42]. Nevertheless, stringent screening of iPSC clones completely free of viral vectors will be required. Any residual presence of the Sendai viral vectors in iPSCs carries risks of amplification when iPSCs or their differentiated derivatives are introduced *in vivo*. Additionally, this method requires viral packaging. Although commercially available, packaged reprogramming Senviral vectors are far from cost-effective.

A second method used synthetic modified mRNAs for the derivation of footprint-free human iPSCs from fibroblasts [43]. Compared to viral and DNA-based reprogramming methods, the mRNA-mediated transgene delivery offers a safer approach for the derivation of clinical-grade human iPSCs. The requirement for repeated transfections, however, limits the application of this method to cells types that are easily transfectable. It remains to be seen whether this method is robust enough for processing multiple donor cell samples, and whether it can be readily adapted to cells that are relatively resistant to lipid-mediated transfections, such as cells of hematopoietic lineages. As the selection of appropriate donor cell types likely will be important for the derivation of clinical-grade human iPSCs, a reprogramming method that is readily applicable to different cell types and multiple donor cell samples will be highly desirable.

We have previously developed an oriP/EBNA-1(Epstein-Barr nuclear antien-1)-based episomal reprogramming approach for the derivation of footprint-free human iPSCs [8]. Compared to currently available methods, this approach has several advantages. First, the oriP/EBNA-1-based episomal vectors have wide host cell range, enabling the application of this method to most, if not all, human somatic cell types. Second, it does not require viral packaging. Third, no repeated treatments of reprogramming factors are needed. A single transfection of episomal reprogramming vectors is sufficient for the derivation of human iPSCs. Moreover, higher transfection efficiency of human somatic cells can be achieved with these vectors due to oriP/EBNA-1-mediated nuclear import and retention of vector DNA [44]. Fourth, the oriP/EBNA-1-based episomal vectors replicate once-per-cell cycle and are generally present at a low copy number per cell, thus minimizing DNA rearrangement and genome integration [45]. Last, the removal of episomal vectors from established human iPSCs can be easily accomplished by extended cell culture without any additional manipulation due to the silencing of the viral promoter that drives the expression of EBNA-1 in iPSCs, and the inherent instability of oriP/EBNA-1 episomal state (e.g., stably established episomes are lost from cells at a rate of approximately 5% due to defects in plasmid synthesis and partitioning) [46]. Although the initial report on the oriP/EBNA-1-based episomal reprogramming method yielded low efficiency from fibroblasts, this did not seem to be inherent to the oriP/EBNA-1-based transgene delivery system. Using this system, iPSCs were efficiently produced from cord blood, bone marrow, and to a lesser degree, adult peripheral mononuclear cells [47–48]. Additionally, by modifying reprogramming culture conditions, efficient iPSC derivation was achieved using this method from fibroblasts, adipose tissue-derived cells, and even hard-to-reprogram terminally differentiated B cells [49–51]. Thus the oriP/EBNA-1 based episomal reprogramming approach offers a robust alternative for the routine derivation of footprint-free iPSCs from cell types readily available from living humans.

Developing defined reprogramming conditions

Most methods currently developed for iPSC derivation and culture use undefined culture media, matrix or feeder cells with animal origins. This creates problems for both industrial and clinical applications of human iPSCs. The preparations of both undefined culture media and feeder cells consistently yield products with variable qualities, directly impacting both the derivation and quality of iPSCs. Direct contact of human iPSCs with animal products risks contamination of animal pathogens and immunogenic antigens such as non-human sialic acid [4]. Human skin fibroblasts and adipose tissue-derived cells were successfully used as both the feeder cells (isogenic) and the donor cells for iPSC derivation and culture [52–53]. The use of isogenic feeder cells, however, does not resolve problems associated with quality variations, and does not provide a universal solution for iPSC derivation from different donor cell types, such as cells of hematopoietic lineages. It is highly desirable to develop chemically defined conditions for all steps of iPSC production, including the isolation and culture of human donor cells, iPSC derivation and expansion. Various xeno-free or chemically defined conditions have already been established for the isolation and culture of many human somatic cell types, such as skin fibroblasts, adipose tissue-derived cells and hematopoietic precursors. Studies of human ESC culture in the last decade have also enabled the development of chemically defined culture media such as TeSR from STEMCELL technologies [54] and STEMPRO® hESC SFM from Invitrogen [55]. Defined surfaces have been identified that could support the robust proliferation of human pluripotent stem cells in chemically defined culture media, e.g., recombinant vitronectin [56] and E-cadherin [57]. These defined culture conditions can be readily adapted to the expansion and maintenance of human iPSCs. Conditions that support human ESC and iPSC proliferation were often directly used for the derivation of iPSCs. These conditions, however, are generally not optimal for the iPSC derivation. For example, although TGF-β signaling plays a critical role in the proliferation of human ESCs and iPSCs, and its ligands are often included as an important component in chemically defined

human ESC and iPSC culture media, the inhibitors of the TGF-β receptor were, nevertheless, able to greatly promote the derivation of iPSCs [58]. Thus modifications of the ESC and iPSC culture media are required to establish robust chemically defined conditions for the derivation of iPSCs [49–50].

To summarize, iPSC technology has undergone rapid progress in the last several years. Methods are already available for the efficient derivation of footprint-free human iPSCs under chemically defined conditions. With these technological advancements made, the question now is whether iPSC derivation or the transdifferentiation between different somatic cellular states can be accomplished with pure chemicals without the use of either genetic factors or their RNA and protein products. In this pure-chemical reprogramming, there would be no genetic modifications of the iPSC genome caused by insertion of exogenous DNA or the expression of mutagenic reprogramming factors. However, robust screening of iPSC genome will still be necessary since small molecules can be mutagenic, especially when chromatin modifiers are used.

COMPARISON OF iPSCs and ESCs

Pluripotent stem cells are characterized by their robust, unlimited self-renewal ability and the potential to differentiate into all cell types of the body. Although different pluripotent states have been identified, i.e., naïve pluripotent state (e.g., mouse ESCs originated from the inner cell mass (ICM) of pre-implantation blastocysts), and primed pluripotent state (e.g., mouse epiblast derived stem cells or EpiSCs originated from the epiblast of post-implantation mouse embryos [59–60]), for the purpose of this discussion, we will only focus on mouse ESC-like iPSCs, and human ESC-like iPSCs.

Mouse ESCs have domed colony morphology in culture, and their self-renewal is supported by either leukemia inhibitory factor and BMP4 signaling [61], or dual inhibition of differentiation-inducing signaling from FGF/ERK (e.g., SU5402, PD184352 and PD0325901) and GSK3β (CHIR99021) [62]. Mouse ESCs express cell surface antigen SSEA-1 and key pluripotent marker genes such as *Oct4*, *Sox2*, *Nanog*, *Klf2*, *Klf4*, *Rex1* and *Stella*. They can form embryoid bodies and differentiate into any somatic cell type *in vitro*. They can form teratomas consisting of differentiated cells of all three germ layers when injected into immunodeficient mice. They can contribute to all cell lineages including germ cells in chimera mice when injected into blastocysts, and give rise to all-ESC mice by tetraploid complementation. Mouse iPSCs that share all these key features have been obtained. They showed morphology, growth properties and gene expression pattern similar to mouse ESCs, formed teratomas, gave rise to live-birth chimeras, and more importantly, produced viable mice through tetraploid complementation [63–64], the unequivocal test for authentic pluripotency. Thus the reprogramming technology can produce iPSCs that are molecularly and functionally equivalent to ESCs. However, all iPSCs are not equal. Significant variations between different iPSC clones have been observed. Although they share similar morphology, growth properties, global gene expression pattern and the ability to form teratomas, many iPSC clones failed to efficiently contribute to chimeras, or to produce viable mice through tetraploid complementation [1]. Attempts to identify markers for authentic pluripotency came upon the imprinted Dlk1-Dio3 gene cluster on mouse chromosome 12F1 [65–66]. Loss of imprinting at this locus, however, did not strictly correlate with reduced pluripotency, although it did decrease the efficiency of generating all-iPSC mice [10]. Thus the ability to form all-iPSC mice remains the only reliable test for authentic pluripotency.

Human ESCs, despite their ICM origin, share many features with mouse EpiSCs. They have flat compact colony morphology. Their growth depends on FGF and TGFβ signaling. They express cell surface antigens SSEA-3, SSEA-4, Tra-1–60, Tra-1–81 and pluripotent marker genes *OCT4*, *SOX2*, *NANOG*. Similar to mouse ESCs, these cells can form embryoid bodies and differentiate

into many somatic cell types *in vitro*, and form teratomas consisting of differentiated cells of all three germ layers. Due to both ethical and practical reasons, it is not possible to test whether these cells can contribute efficiently to chimeras when injected into blastocysts, or produce all-ESC humans. Human iPSCs share all key features with human ESCs. Their equivalence, however, has been constantly challenged. For example, human iPSCs were suggested to have a gene expression signature unique from human ESCs, retain residual gene expression of donor cells, harbor residual DNA methylation signatures characteristic of their donor cells, and favor their differentiation along lineages related to the donor cells (epigenetic memory) [67–69]. Prolonged passaging of iPSCs or treatment with chromatin modifiers was shown to reduce these abnormalities, bringing human iPSCs closer to human ESCs. Although these results raised concerns, the question is still open regarding whether human iPSCs are molecularly and functionally equivalent to human ESCs. One factor contributing to this controversy lies in the selection of control human ESC lines used for comparison. Since human ESC lines are known to exhibit significant variations between them, and their derivation and culture history affect both the genetic and epigenetic status of these cells, it is problematic to draw conclusions based on comparisons between a limited number of human iPSC and ESC lines. Considering studies on mouse iPSCs, the epigenetic memory seen with various human iPSC clones most likely reflects incompleteness in reprogramming. With optimized reprogramming conditions, including the combination and stoichiometry of reprogramming factors, chemically defined reprogramming culture (e.g., inclusion of appropriate small molecules), and iPSC expansion conditions for each donor cell type, we will likely be able to obtain high quality human iPSCs molecularly and functionally equivalent to human ESCs and with much reduced heterogeneity between different iPSC clones on a routine basis.

CURRENT APPLICATIONS OF HUMAN iPSCs

Despite the ongoing debate regarding the equivalence of ESC and iPSCs, human iPSCs have nonetheless found their way into many applications previously envisioned for human ESCs. One such application, which received immediate attention, was disease modeling. The ease of generating patient-specific human iPSCs provides a valuable experimental platform for modeling human diseases, and may greatly facilitate the development of novel therapeutic treatments. From diseases characterized by single gene defects and rapid disease progression in early childhood, to disorders with complex genetic and epigenetic etiology [70–71], human iPSCs could provide an unlimited number of previously inaccessible and clinically relevant cell types for mechanistic studies on diseases, examples of which are shown in Table 30.1. Given the heterogeneity in the quality of human iPSCs produced with most currently available methods, and the lack of better markers or functional assays to confirm the authentic pluripotency in these iPSCs, some technical caveats must be considered when interpreting results from these promising studies. For example, the residual expression or reactivation of reprogramming factors using a genome-integrating transgene delivery system could affect the molecular characteristics of human iPSCs, their differentiation into specific lineages of interest and the functionality of differentiated derivatives, complicating the interpretation of phenotypes arising from diseases and imperfect reprogramming.

A second area where human iPSCs can be of immediate use is in toxicity testing and drug discovery. From lead drug candidates to numerous chemical compounds to which we are increasingly exposed during our daily life, the need for toxicity testing has never been greater. Traditional toxicity testing relies on animal models, transformed cell lines or primary human cells. Animal models are limited by ethical constraints, cost, time and failure to predict species-specific toxicity. Transformed cell lines are generally karyotypically abnormal, and often do not accurately recapitulate the functions of their primary counterparts. Primary human cells are extremely limited in their tissue source, exhibit high variations in the quality between different donors, and often undergo dedifferentiation during *in vitro* culture such as primary

TABLE 30.1 Application of human iPSCs in disease modeling

Disease	Cell phenotype
Neuronal	
Spinal Muscular Atrophy[a]	Motor neuron with selective deficits.
Friedreich's ataxia (FRDA)[b]	GAATTC triplet hyperexpansion in FXN gene Repeat instability in FRDA iPS cells
Schizophrenia (SCZD)[c]	Diminished neuronal connectivity in conjunction with decreased neurite number, PSD95-protein levels and glutamate receptor expression
Parkinson's disease[d]	Elevated amount of alpha-synuclein in midbrain dopaminergic neurons
Familial Alzheimer's disease (FAD)[e]	Increased amyloid beta42 secretion, which responds sharply to gamma-secretase inhibitors and modulators
Cardiac	
LEOPARD Syndrome[f]	PTPN11 mutation; enlarged cardiomyocyte cell size with higher degree of sarcomeric organization and preferential localization of NFATC 4 in the nucleus
Long QT Syndrome[g,h]	Prolonged action potential in ventricular and atrial cells, reduced I(Ks) current
Hepatic	
α1-Antitrypsin Deficiency[i]	Aggregation of misfolded α1-antitrypsin in endoplasmic reticulum
Familial Hypercholesterolemia[i]	Deficient LDL receptor-mediated cholesterol uptake
Glycogen Storage Disease Type 1a[i]	Elevated lipid and glycogen accumulation
Aging	
Hutchinson-Gilford progeria syndrome (HGPS)[j]	LMNA mutation, premature senescence phenotype, absence of nuclear DNAPK holoenzyme

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[c]*Brennand KJ, Simone A, Jou J, Gelboin-Burkhart C, Tran N, Sangar S, Li Y, Mu Y, Chen G, Yu D, McCarthy S, Sebat J, Gage FH. Modelling schizophrenia using human induced pluripotent stem cells. Nature 2011;473:221–5.*
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hepatocytes and cardiomyocytes. Compared to these test materials, human iPSCs can provide a better alternative with unlimited availability and consistent quality. For example, about one third of withdrawals of pharmaceuticals for safety reasons were because of their cardiotoxicity. Thus an effective early screen for cardiotoxicity can significantly decrease late-stage drug attrition. As a proof of concept, in a study by Guo et al. [72], human iPSC-derived cardiomyocytes were shown to predict the performance of 28 different compounds with known cardiac effects with much higher accuracy than the traditional cell line and animal models. These cells were able to identify compounds with severe cardiac toxicity where the traditional models failed, as well as those compounds without clinical manifestation that tested positive with traditional models. In addition to toxicity testing, human disease-specific iPSC-derived differentiated derivatives can provide an effective substrate for drug screening. For example,

human iPSCs derived from patients with Rett syndrome (RTT) were able to generate functional neurons with fewer synapses when compared to controls. These RTT neurons were used to test the effects of drugs in rescuing synaptic defects, thus providing a previously unexplored developmental time window before the disease onset for potential therapeutic intervention [73]. Also human Alzheimer's disease iPSC-derived neuronal cells were capable of amyloid beta peptide (Abeta) production, which was sensitive to the inhibition of beta- and gamma-secretase inhibitors. Thus these cells potentially could be used for anti-Abeta drug screening upon sufficient neuronal differentiation [74].

Perhaps the most captivating application of human iPSCs is their therapeutic potential. With their unlimited proliferation potential and the ability to differentiate into clinically relevant cell types, human iPSCs can provide an essentially unlimited cell source for autologous cell replacement therapies. Patient-specific human iPSCs can be derived from easily accessible donor cells such as those from skin, blood or adipose tissue. These cells can be either used directly for differentiation, or be genetically modified to repair specific genetic defects or to deliver therapeutically important proteins prior to differentiation. Methods for efficient gene targeting of human iPSCs and ESCs include helper-dependent adenoviral vectors [75] and engineered nucleases such as zinc-finger nucleases [76] and TALENs [77]. Differentiated cells such as cardiomyocyte, hepatocytes, neural cells or blood cells can be enriched to high purity to avoid contamination with iPSCs and transplanted back into patients for therapeutic intervention. Many proof of principle experiments have been successfully carried out in animal models. For example, mice were rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPSCs in a mouse model of sickle cell anemia [78]. Mouse iPSC-derived dopamine neurons of midbrain character were able to improve behavior in a rat model of Parkinson's disease upon transplantation into the adult brain [79]. Mouse iPSC-derived pancreatic beta-like cells corrected a hyperglycemic phenotype in two mouse models of type 1 and 2 diabetes [80]. Human iPSC-derived retinal pigment epithelium cell transplantation was shown to facilitate the short-term maintenance of photoreceptors and the long-term maintenance of visual function in the retinal dystrophic rat [81]. Clinical trials of several human pluripotent stem cell-derived cell types (e.g., retinal pigment epithelium cells) are currently underway. Although promising, no final conclusion can yet be drawn about the therapeutic benefits of these trials.

CHALLENGES

Since the initial report of iPSC derivation in 2006, much progress has been made in both their derivation and applications. Human footprint-free iPSCs can now be routinely obtained from different donor cell types, and can be efficiently directed to differentiate into many clinically relevant cell types, under chemically defined conditions. However, as we gain more insight into these cells, more questions arise about their applications both *in vitro* and *in vivo*.

One question currently under debate is whether iPSCs are molecularly and functionally equivalent to ESCs. Since viable mice can be produced from iPSCs by tetraploid complementation, it is safe to conclude that the iPSC technology is able to produce pluripotent stem cells that are molecularly and functionally equivalent to ESCs. This, however, does not exclude the existence of significant clone-to-clone variations and the retention of epigenetic memory in many clones analyzed in published literature as a result of incomplete reprogramming. Due to the lack of better markers and efficient functional assays for authentic pluripotency, especially for human iPS cells, it will take some time to put this question to rest. However, it is technically possible to identify a reprogramming method that produces high quality iPSCs with minimal clone-to-clone variations by selecting appropriate donor cell types, the combination and stoichiometry of reprogramming factors, and reprogramming culture conditions including the use of small molecules. In addition, for practical purposes, it

might not be essential to produce human iPSCs molecularly and functionally equivalent to human ESCs.

A second question relates to the differentiation of iPSCs and ESCs. The application of human iPSCs relies on their ability to differentiate into clinically relevant cell types. Although efficient differentiation has been achieved from human iPSCs for many cells types such as cardiomyocytes, hepatocytes and neuronal cells, these differentiated derivatives appeared to be in fetal stages [82]. This presents problems for cells that show significant functional difference between adult and fetal stages. For example, adult hepatocytes have highly active drug metabolism activity that their fetal counterparts lack. Human iPSC and ESC-derived hepatocytes, although they have basic hepatic metabolism and the ability to support hepatic virus amplification [83], generally have significantly reduced functions in drug metabolism compared to the adult hepatocytes. This limits their usefulness in drug toxicity testing. Prolonged culture of human iPSC-derived cells appeared to improve their functions. However, normal tissue maturation takes place over an extended period of time, which is not entirely practical for industrial applications. It would be interesting to know whether such maturation can be greatly sped up by either small molecule treatment or genetic engineering via introduction of appropriate genes. Besides issues with cellular maturation, differentiation of human iPSCs and ESCs into certain clinically important cell types has yet to be achieved. For example, transplantation of hematopoietic stem cells (HSCs) from bone marrow, peripheral blood and umbilical cord blood is a well-established medical procedure to treat patients with certain life-threatening diseases. However, the application of this therapy is limited by the availability of donor cells and immune rejections. Patient-specific human iPSCs could potentially provide an unlimited number of donor cells without the complication of immune rejections. Additionally, genetically engineered HSCs can be used to treat certain illnesses that are currently without cure. For example, with the introduction of anti-HIV transgene(s), human iPSC-derived HSCs could provide a one-time-cure for HIV. Human iPSCs, however, have yet to produce HSCs that are capable of robust long-term engraftment *in vivo*.

The main concern for the *in vivo* application of human iPSCs stems from their genetic and epigenetic stability. Multiple mutations, copy number variations (CNVs) and aberrant epigenetic modifications have been found in iPSCs [84–86]. Although some of these changes can be attributed to pre-existing mutations in donor cells, mutations accumulated during iPSC expansion and incomplete reprogramming, there appears to be increased mutation rate during reprogramming. More recent data, however, suggest that the genetic and epigenetic instability of iPSCs observed could be partially technical. For example, in a study using iPSC lines derived with episomal vectors, no CNVs were found, and the single-nucleotide variants found were not selectively enriched for known genes associated with cancers [87]. Additionally, extended culture of iPSCs could be accomplished without a substantial increase in mutational burden [88]. Thus with the selection of donor cell types with low somatic mutation rate and appropriate methods for the reprogramming and culture of human iPSCs, it is possible to significantly reduce their genetic and epigenetic instability. Nevertheless, it will be essential to carefully examine the genetic and epigenetic status of human iPSCs before these cells can be used for transplantation therapies. Due to the genetic and epigenetic modifications, transplantation of human iPSC derivatives carries risks of tumor formation. Although human iPSCs can form benign teratomas *in vivo* when present in sufficient number, this risk can be greatly minimized by prior removal of human iPSCs from differentiated cells. Residual human iPSCs could efficiently activate and be lysed by natural killer cells [89]. Mutations and aberrant epigenetic modifications of human iPSCs, however, can result in malignant tumors. Moreover, they can also lead to the expression of developmentally inappropriate genes and other immunogenic antigens in human iPSC-derived cells that can elicit immunoreactions even in autologous transplantations [90].

CONCLUSION

iPSC technology has opened up many venues for research and applications previously unfeasible. In the past several years, we have seen increased applications both *in vitro*, such as disease modeling, toxicity testing and drug screening, and *in vivo* transplantations in various animal models, all with promising results. However, both technical and scientific hurdles exist, including finding an optimal reprogramming approach for producing high quality iPSCs with minimal clone-to-clone variations, minimal genetic and epigenetic mutations, and differentiation into functionally mature cell types. The genetic and epigenetic instability observed in human iPSCs raises cautions for their *in vivo* applications in clinical transplantation therapies. Nevertheless, the field is making rapid progress. Realizing the full potential of human iPSCs is not out of reach.

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CHAPTER 31

Stem Cells in Tissue Engineering

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POTENTIAL STEM CELL SOURCES FOR USE IN TISSUE ENGINEERING

Introduction

A stem cell is defined as a cell that has the capacity for self-renewal and the potential to differentiate into any cell type in the body. In contrast, a progenitor cell 'has the ability' to generate cells specific to the tissue or organ from which it was procured, but does not have the ability to cross germ lines.

Many types of adult stem cells have been reported over several decades. However, stem cells that would be useful in tissue engineering need to be:

1) Easily obtained in large numbers,
2) Safe to implant,
3) Able to differentiate into the cells needed.

Therefore, the types of stem cells which may be useful for tissue engineering are limited. Potential adult stem cells for use in tissue engineering, such as mesenchymal stem cells, hematopoietic stem cells, adipose stem cells, and skin stem cells are easy to access and relatively easy to obtain in large numbers. They are basically safe because they are harvested from adult tissues. Pluripotent stem cells, such as ES cells and iPS cells, are able to provide sufficient numbers of cells because they have an infinite proliferative potential, however, their tumorigenic potential often prevents their clinical application.

Hematopoietic stem cells

While diversification of cell types is largely completed at, or soon after birth, many tissues undergo self-renewal in response to acute injury or normal usage throughout life [1,2]. Therefore, some somatic stem cells are also maintained throughout the whole life span for the regeneration of specific tissues, such as the epidermis, hair, small intestine, and hematopoietic system.

Stem and progenitor cells, origins, phenotypes, lineage commitments, and transdifferentiation [3]

The existence of hematopoietic stem cells (HSCs) in bone marrow was first demonstrated by James Till and the late Ernest McCulloch in 1963 [4]. One of their important findings was the

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demonstration of the radiation sensitivity of mouse bone marrow (BM) cells by showing that limiting numbers of BM cells could give rise to clonal colonies of elytroid and myeloid cells in the spleens of the irradiated hosts. Although it was postulated that each spleen colony was genetically unique and had thus arisen from a single cell, the phenotype of those cells was still unknown. Through an investigation using cell sorters, Irving Weissman [5] showed that a population of mouse BM cells expressing the surface antigens Thy1 (low) and Sca1 from which lineage differentiation markers were absent (Lin negative) was able to rescue irradiated BM far more effectively than unsorted BM cells. The potency of the cells in that population was demonstrated by injecting as few as 30 cells into a lethally irradiated mouse, which rescued half of the recipients from BM failure, whereas 13,000–33,000 unsorted BM cells were needed to achieve the same effect. This result surely indicated that the proposed stem cells were greatly enriched by this protocol. This study led to many further studies, and human hematopoietic stem cells were isolated based on murine hematopoietic stem cell isolation protocols. Using long-term repopulation combined with a limiting dilution design, stem cell frequency in the bone marrow was determined to be 1 in 10,000 nucleated cells [6,7]. This type of assay is now a standard in hematopoiesis, for the evaluation of stem cell markers [3,5] and for evaluation of stem cell differentiation and regulation [8–10], and it has also been key in studying other populations of hematopoietic stem cells quantitatively [11–13]. According to the character of hematopoietic stem cells, the definition of stem cell is the potential of self-renewal and multi-lineage differentiation.

HSCs offer the advantage of being relatively easily to harvest from bone marrow, and to isolate and purify *ex vivo*. Recent studies indicate that the traditional wall separating the hemato-poietic and mesodermal tissue systems and lineages is being demolished. Interestingly, HSCs possess the unexpected potential for myogenesis and cardiomyogenesis [14–20]. Further-more, purified mouse HSCs can regenerate liver cells [31], and cells able to regenerate bone are also found in blood [21]. These studies suggest that HSC tendency to result in hematopoiesis is a result of environmental stimuli.

Skin stem cells

Since skin is a rapidly renewing tissue, similar to the gastrointestinal and hematopoietic systems, the epidermis is an attractive tissue in which to study stem cell biology. Although People empirically knew of the existence of epidermal stem cells, the culture of keratinocytes was quite difficult because of the contamination of fibroblasts which have high proliferative potential. Green and his colleagues discovered that co-culture with irradiated-3T3 cells results in differentiation to epidermal cells. This protocol resulted in many studies, and trans-plantation of epithelial cell sheets fabricated by this protocol is the first tissue engineering and regenerative medicine technique to be used in clinical application. The studies of epidermal stem cells *in vitro* were finally started in the late 1970s. In an effort to identify cells with a high proliferative capacity in the human epidermis, Barrandon and Green [22] studied the self-renewal ability of human epidermal cells in replating assays.

In their study, 50–100 keratinocyte cells (KCs) were individually plated. More than 50% of cultured human KCs formed colonies. Six days later, colonies were re-suspended and each plated into a 100 mm Petri dish. Different cell types resulted in different types of colonies in secondary cultures [22]. Cells that form mostly large smooth colonies with less than 5% of small abortive colonies are termed holoclones. Paraclones are terminally differentiating cells that form small and abortive colonies. Cells intermediate between stem and differentiated cells formed meroclones, intermediate in appearance and reproductive capacity. In the studies of Barrandon and Green [22], cultured KCs produced 28% holoclones, 49% meroclones, and 23% paraclones. It is believed that the holoclones are stem cells and the paraclones are transit-amplifying cells [22], thereby suggesting a hierarchy of epidermal stem cells beginning with an epidermal stem cell, which gives rise to a continuum of cell populations, with progressively

diminishing capacity to proliferate and self-renewal. These studies provided an important examination method for KCs capable of self-renewal *in vitro*.

In 1990s, the niche of epithelial stem cells was spotlighted. The bulge is a less morphologically distinct structure in the adult human compared with the adult mouse. However, studies of label-retaining cells indicated that human hair follicle stem cells were located in the bulge of the human hair follicle [23,24]. Human bulge cells were found to express Keratin 15, and Keratin15-expressing human KCs were slow cycling, proliferated at the onset of follicle growth, and were expressed a high level of β1 integrin [23,25]. Moreover, bulge cells isolated by laser capture micro dissection were highly clonogenic *in vitro* [24]. A recent study demonstrated that CD34 positive cells were predominantly in G0/G1 highly expressing α6-integrin. Therefore, CD34 was a specific marker of murine bulge KCs [26]. Although CD34 was not expressed in human bulge cells [24,27], the bulge has been accepted as a site of hair follicle stem cells in humans. A recent study utilized human bulge cell markers to determine that human hair follicle stem cells were maintained in men with androgenetic alopecia, but that CD200-rich and CD34-positive progenitors were decreased [28].

It is evident that methods to evaluate epidermal stem cells are inadequate, and long-term repopulation assays that measure sustained epithelial tissue regeneration, analogous to most acceptable assays in hematopoiesis, are essential for progress in epidermal stem cell biology.

Kaur and his colleagues developed a transplantation assay based on repopulation of the epidermis inside a rat trachea which had been implanted subcutaneously in a severely combined immuno-deficient mouse [29,30]. This assay allows long-term maintenance of transplanted epidermal cells *in vivo*. Similarly, Schneider and his colleagues developed an *in vivo* transplantation assay for epidermal stem cells [31] based on well-established functional assays for hematopoietic stem cells [32–35]. This assay was combined with limiting dilution analysis to enable the quantization of the frequency of long-term repopulating cells in a population of KCs.

The *in vivo* transplantation assay for epidermal stem cells allows quantization of epidermal stem cells. This assay has been used to demonstrate that the frequency of murine epidermal stem cells in this model is less *in vivo* than previously predicted, and more in line with hematopoietic stem cell frequency (i.e., of the order of 1 in 10,000[31]). It has also shown that rapidly adherent colony-forming cells are not enriched in long-term repopulating cells [36] and that although no significant difference in aged vs. young epidermal stem cell (long-term repopulating cell, nine weeks of repopulation) frequency could be detected, transient-amplifying cell (short-term repopulating cell, three weeks of repopulation) frequency was greater in the aged [37].

Engineering the correct shape and physical structure of a graft is relatively simple in epidermis and other surface epithelial tissues, and indeed can be achieved *ex vivo*. This reflects the essentially two dimensional organization of skin and other epidermal surfaces, and is in sharp contrast with other systems such as the skeleton, where effective engineering involves the reconstruction of complex shapes and architectures. These directly affect proper function, are normally generated over a long period of time, and cannot be achieved *ex vivo*.

As mentioned above, transplantation of cultured skin grafts was performed as the first example of tissue engineering and regenerative medicine. According to the study in 1987, only holoclones are the product of a true stem cells, as defined by their exceptional self-replication ability (>140 doublings), which is not ascribed to either meroclones (a population of transient-amplifying cells) or paraclones (a population of senescent or differentiating progenitors) [22]. In principle, a small but pure population of holoclone-generating cells would be all that is required for generating epidermal grafts. The recent identification of a keratinocyte stem cell marker (p63) may provide a new tool for this approach with respect to epidermis [38].

Skin autografts are produced by culturing keratinocytes to generate an epidermal sheet, and then transplanting this sheet along with a suitable dermis-like substrate [39]. But the success of these procedures has been variable, with graft failure resulting in many cases after a promising initial engraftment. The specific technological challenge in restoration of epithelial surfaces in turn consists of the definition of culture conditions, and of carriers that are specifically designed to maintain an adequate stem cell compartment in the engineered graft. Dependence of epithelial surfaces on relevant stem cell compartments is further highlighted by the successful reconstruction of damaged corneas using stem cells derived from the limbus in conjunction with an amniotic membrane [40,41]. One of the feature technologies is the discovery of temperature-responsive dishes. Using these dishes, fragile cultured epithelial cell sheets were easily harvested as an intact cell sheets without using any enzymes. Okano and his colleagues succeeded in applying these cell sheets for the surface deficiency of skin, cornea and trachea in a clinical study [42–44].

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are currently the most prolific cell source that could potentially be used therapeutically for the treatment of human disease, and numerous clinical trials are underway. MSCs may be isolated from human BM. The first experimental evidence for the existence of a stem cell population in this tissue compartment other than the HSCs was uncovered in the 1960s.

Transplantation of decellularized bone to ectopic sites demonstrated that cells present in other tissues could be induced to generate bone. Pursuing the source of this potential, Friedenstein identified adherent 'fibroblast-like' cells in those tissues arising from BM that were capable of osteogenesis *in vivo* [45].

Such cells were believed to be a component of the BM stroma. They existed to support and nurture the hematopoietic functions of the BM, but also possessed the ability to differentiate into other cells types, such as chondrocytes and adipocytes demonstrated additional lineage potentials. At this point, the multi-potency of such adherent fibroblastic cells was recognized and they became known as MSCs rather than simply marrow stromal cells [46]. The name change proved to be quite fortuitous because it soon became apparent that MSCs could be derived from sources other than BM. By the late 1990s, it seemed that umbilical cord blood, in addition to being a valuable source of HSCs, also contained multipotential cells similar to those found in the BM [47]. While it should have been no surprise that the umbilicus would require the presence of stromal cells to generate a niche capable of supporting its HSCs, some sources of MSC were less obvious. Recently, MSCs with similar characteristics to bone marrow-derived MSCs have been isolated from other tissue sources, including trabecular bone [48], periosteum [49], synovial membrane [50], skeletal muscle [51], skin [52], pericytes [53], peripheral blood [54], deciduous teeth [55] and periodontal ligament [56]. Although the stem cell populations derived from these sources are valuable, common problems include the small number of harvested cells and limited amount of harvested tissues. Therefore, almost all adult-derived stem/progenitor cells require at least some degree of *ex vivo* expansion or further manipulation before they can be used pre-clinically or clinically to satisfy efficacy and safety requirements.

The degree of similarity between MSCs from such seemingly diverse sources is still a matter of some debate, despite early suggestions that they are present in the connective tissues of many organs, surround blood vessels as pericytes, and may contribute to the maintenance of organ integrity.

We may find that other adult or tissue-specific stem cells are capable of similar feats, but to date the evidence for this is not strong, and the ubiquitous nature of MSCs suggests that they may have a degree of plasticity not enjoyed by other stem cell types. The ease of obtaining MSCs has

resulted in them being the subject of more intensive investigation which has brought them closer to the point of medical or commercial application than many other types of stem cell.

Adipose-derived stem cells

An entire field of medicine now centers on the use of adipose-derived stem cells (ASCs) [57] for tissue repair, since human adipose tissue is ubiquitous and easily obtained in large quantities with little donor site morbidity or patient discomfort. Therefore, the use of autologous ASCs in therapeutics seems to be feasible and has been shown to be both safe and efficacious in preclinical and clinical studies of injury and disease [58,59].

Although ASCs are an attractive cell source for tissue engineering, their characterization is not yet complete. To date, all attempts to discriminate clearly between ASCs and fibroblasts have been unsuccessful. Compared with ASCs from later passages, freshly isolated fat cells and early passage ASCs express higher levels of CD117 (c-kit), human leukocyte antigen-DR (HLA-DR), and stem cell-associated markers such as CD34, along with lower levels of stromal cell markers such as CD13, CD29 (b1 integrin), CD44, CD63, CD73, CD90,CD105, and CD166 [60–62]. Several studies have shown that CD34-positive ASCs have a greater proliferative capacity, while CD34-negative ASCs are more plastic [62,63]. ASCs share many cell surface markers with pericytes and bone marrow MSCs. Therefore, several studies suggest that ASCs may exist in the perivascular tissue *in vivo*, although their exact location remains undetermined.

ASCs exhibit telomerase activity to some extent, although it is lower than that in cancer cell lines, which indicates that ASCs have the capacity for self-renewal and proliferation [64]. In addition, Puissant and his colleagues [65] reported on the lack of HLA-DR expression and the immunosuppressive properties of human ASCs.

Routinely, 1×10^7 adipose stromal/stem cells have been isolated from 300 mL of lipoaspirate, with greater than 95% purity [66]. In other words, the average frequency of ASCs in processed lipoaspirate is 2% of nucleated cells, and the yield of ASCs is approximately 5,000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of approximately 100–1,000 CFU-F per milliliter of bone marrow [67].

To date, there have been numerous scientific publications demonstrating that ASCs possess the potential to differentiate toward a variety of cell lineages both *in vitro* and *in vivo* [68], and some of the research has been passed on to patients in the form of clinical trials. Although ASCs are of mesodermal origin, it is now clear that they can differentiate into ectoderm and endoderm lineage cells as well as mesoderm [69].

ASCs can differentiate into adipogenic [70–72], osteogenic [73], chondrogenic [73–75], myogenic [75], cardiomyogenic [76,77], angiogenic [78,79], tenogenic [80], and periodontogenic lineages [81], and tissue regeneration studies with suitable scaffolds and growth factors in appropriate external environment have been applied [82,83]. Some studies have reported the differentiation potential of ASCs toward cells of the ectodermal lineage. One study has also reported that ASCs cultured in monolayers with retinoic acid or on a fibrin matrix in the presence of EGF express the epithelial markers, cytokeratin 18 or E-cadherin and cytokeratin 8, respectively [84]. In another study, ASCs exposed to vasoactive intestinal peptide were induced to form retinal pigmented cells, which are of ectodermal origin, as indicated by the expression of retinal pigment epithelium (RPE) markers, namely best rophin, cytokeratins 8 and 18, and RPE 65 [85].

It has also been demonstrated that ASCs can differentiate into neuronal or neuronal precursor cells, both morphologically and functionally, under appropriate culture conditions [86]. In addition, recent studies have revealed that when ASCs are administered intravenously in a spinal cord injury model, they migrate to the injured cord, partially differentiate into neurons and oligodendrocytes and eventually may help to locomotor some functions [87].

Finally, it has been shown that ASCs can differentiate into endoderm lineage cells. Several reports have shown that ASCs have the potential to differentiate into hepatocytes as indicated by the presence of HGF, FGF-1 and FGF-4 [88,89].

DISCOVERY OF EMBRYONIC STEM CELLS

ES cells have attracted attention as a promising source of cells for tissue engineering, as they can self-renew almost indefinitely in culture and have the capacity to differentiate into all cell types of the body. ES cells are isolated from the inner cell mass of the developing blastocyst. *In vitro*, they can be maintained as undifferentiated cells via culture on murine embryonic fibroblasts (MEFs) or via culture in feeder-free conditions using a defined medium composition to supply essential cytokines and nutrients to promote the undifferentiated phenotype [90].

The concept of pluripotency, the ability of a single cell type to differentiate into multiple other types of cells arose from detailed histological studies of teratocarcinomas, which are neoplasms composed of seemingly undifferentiated cells randomly interspersed with somatic tissues. The fact that a single cell suspension produced from teratocarcinomas could give rise to completely new neoplasms in immuno-deficient mice suggested the presence of stem cells in their tissue mass. The nature of these somatic tissues in teratocarcinomas provided abundant information about various stages of differentiation. Lewis Klein Smith and Barry Pierce [91] were the first to develop conditions for isolating and culturing these cells, and these cells were published as 'embryonal carcinoma cells' (EC cells) in 1964. Not only the concept of pluripotency, but also the basal culture methods of pluripotent stem cells, which are come down to iPS cells, were established by the discovery of EC cells. Mouse embryonic stem cells (mESCs) were derived by the work of Martin Evans, Matthew Kaufmann, and Gail Martin [90]. Evans and Kaufmann demonstrated for the first time that they had succeeded in isolating the inner cell mass (ICM) of a mouse embryo at the blastocyst stage, and had allowed these cells to survive and develop [92]. Michael Sherman reported that an enriched culture medium containing 10% heat-inactivated fetal calf serum promoted 90% of mouse blastocysts to hatch from their zona pellucida and attach to the culture dish, although he was unable to prevent differentiation of the ICM into epithelial cells. Evans and Kaufmann solved this problem by isolating the ICMs from the blastocysts using the immune surgery method and disaggregating them into single cell suspensions. These cells were passaged onto irradiated MEFs, whereupon colonies of cells that were morphologically similar to EC cells finally appeared [93]. Unlike EC cells, the mESCs had normal karyotypes and were able to form embryoid bodies when cultured without their feeder fibroblasts. EC cells were widely used to study early embryonic cell differentiation, but within a few years after the discovery of mESCs, these were supplanted by mESCs principally because mESCs are thought to be more representative of those cells present in the ICM. A major contributing factor to the success of establishing the mES cell line was the introduction of leukemia inhibitory factor (LIF) into the mESC culture media to prevent differentiation [94]. LIF-supplemented culture medium allows mESCs to grow without feeder cells. This mechanism is based on the biology of the rodent, in that several rodent species possess the diapause phenomenon; which permits the generation of a new batch of fertilized embryos while the mother is still pregnant with a previous set. The new embryos arrest at the blastocyst stage of development until the uterus is vacated. This phenomenon is not observed in humans.

Seventeen years after the discovery of mESCs, the derivation of human ESCs was reported by James Thomson using cleavage stage human embryos produced by *in vitro* fertilization for clinical purposes. hESCs were more difficult to grow in culture than mESCs, but behaved in a broadly similar fashion in terms of their apparent immortality, expression of key surface antigens, and their ability to generate teratomas in immuno-deficient mice. It was not possible to examine germ line transmission following injection into blastocysts, since manipulation of

human embryos in this manner is illegal. The method used by Thomson and his colleague was similar to that of Evans in his mESC derivation work; however, the delay in obtaining hESCs may be attributed to ethical issues and the unavailability of human embryos for study. The original article describing the first five hESC lines was published in Science in November 1998 [95], and although it is a fairly brief report, the significance of this development cannot be underestimated since it is the forerunner of hundreds of new hESC lines derived in the following 13 years. Subsequent research has shown that hESCs, while similar to mESCs, have many unique characteristics for modeling human development.

Control over mES/hES cell differentiation has been extensively studied via manipulation of the parameters of the stem cell microenvironment; which is referred to as the stem cell niche. Thus, both soluble and insoluble factors of the niche which modulate differentiation have been thoroughly investigated, to determine the parameters amenable to inducing a particular cell fate.

ES cells require the EB formation to produce progenitor cell populations. One typical strategy for clinical applications using ES cells is that partially differentiated or tissue-restricted progenitor cells are isolated from the ES cells, and expanded *in vitro* to generate adequate progenitor cell populations before clinical application. ES cells differentiate into various mature somatic cell types, presumably via precursor cells, when the appropriate stimuli are applied. For instance, mesodermal progenitor cells were obtained after EB formation stimulated with BMP [96]. Moreover, multipotent hematopoietic progenitor cells [97,98], cardiac progenitor cells [99,100], endothelial progenitor cells [101] and neuronal progenitor cells [102–104]have been isolated from ES cells after appropriate stimuli. Recently, Lu and his colleague have reported an efficient and reproducible method for generating large numbers of such bipotential progenitors (hemangioblasts) from hESCs using an *in vitro* differentiation system [105]. In addition to obtaining a relatively high purity of progenitor cells by separation methods such as FACS and magnetic affinity cell sorting, stage-specific growth factor treatment or genetically altering the ES cells may greatly enhance their differentiation into desired progenitor lineages.

MSCs are generated from a number of different developmental origins [106]. Recent advances in stem cell biology have identified mesenchymal progenitor cell populations that can undergo multi-lineage differentiation into different mesenchymal tissues, such as cartilage, fat, and bone. These cells efficiently engrafted when injected into SCID mouse, and did not show any tumorigenic potential. A similar strategy has been applied to isolate clinically applicable mesenchymal precursor cells from hESCs without feeder support and EB formation [107].

There are potential limitations when using MSCs for tissue engineering and repair. These limitations include the relatively low frequency with which these cells occur in the marrow stroma, and also donor site morbidity. Additionally, the self-renewal and proliferative capacity of MSCs is limited and decreases with age [108]. The greater proliferative capacities of hESC-derived mesenchymal cells compared to human MSCs, and lack of teratoma formation *in vivo* highlight the former's significant potential for tissue engineering and regenerative medicine applications [109]. hESC-derived progenitor cells such as mesenchymal cells may have potential application in tissue regeneration and provide a tool for elucidating the mechanism of lineage commitment specification from ES cells, which may provide a platform for efficiently generating specialized transplantable cells for clinical applications.

INDUCED PLURIPOTENT STEM CELLS

In 2006, the generation of induced pluripotent stem cells (iPSCs) was reported by the laboratory of Shinya Yamanaka. The study demonstrated the reprogramming of mouse somatic cells to pluripotency. Retroviral transduction of just four genes (Oct4, Sox2, Klf4, and c-Myc) reprogrammed mouse embryonic fibroblasts and adult tail fibroblasts to show characteristics reminiscent of mESCs. The resulting cells were named iPSCs. They are capable of contributing

to chimeric animals with germ line transmission, and their contribution to all tissues of the resulting offspring indicative of their pluripotency [110–112]. Yamanaka's group extended their earlier work and showed that human adult dermal fibroblasts could be reprogrammed by retroviral transduction of Oct4, Sox2, Klf4, and c-MycOCT4, SOX2, KLF4, and C-MYC with good efficiency [113]. James Thomson's laboratory found that a slightly different set of factors, OCT4, SOX2, NANOG, and LIN28, were sufficient to allow iPSC generation from fetal and adult fibroblasts [114] via lentiviral rather than retroviral transduction. Lentiviral transduction allows the transduction of non-dividing cells, which was not previously possible using retroviruses. Initial iPSC studies used retroviruses for gene transfer into target cells, since it was believed these genes would be silenced [115,116], but alongside their inability to infect non-dividing cells [117], it was noted that gene silencing was not maintained in iPSC, giving the risk of tumorigenesis.

Constitutive lentiviral use, in which transgene silencing is poor [116,118] was superseded by inducible lentiviral methods which hoped to attain full silencing of transgene expression upon attainment of the pluripotent state. However, a common problem with these vector types is the possibility of mutations upon integration or reactivation of the transgenes, which has been shown to lead to tumorigenesis. New vectors have since been developed that can be removed from the programmed genome, and further developments of RNA-based reprogramming systems, such as those using micro-RNAs, isolated proteins, and small molecules, show some promise for deriving integration-free iPSC lines but there are still concerns that the somatic genome may not have been fully reprogrammed to pluripotency [119,120]. Some of the concerns are manifested as an epigenetic trace left over from the cell of origin. Even though this trace may be erased by overextended passage, the observation raises the concern over the impact and importance of cell of origin and its subsequent translational value.

Another issue in the iPSC field is the representative nature of a single iPSC line – this again points to the fact that 'not all iPSCs are equal', and again should act as a caution when considering patient-specific iPSCs (see below). These problems could restrict the clinical utility of iPSCs and are under intense investigation by many groups. However, generation of *in vitro* models of human disease using patient-specific iPSCs is allowing investigation and the generation of a wealth of data that promises to make a major impact on science and medicine. As an illustration of the use of iPSCs for modeling disease in a dish, we can turn to the work of Rusty Gage. At a time when Pharma is retreating from neurodegeneration/neuropsychiatric research because of cost and paucity and inadequacy of animal models, the use of iPSCs as

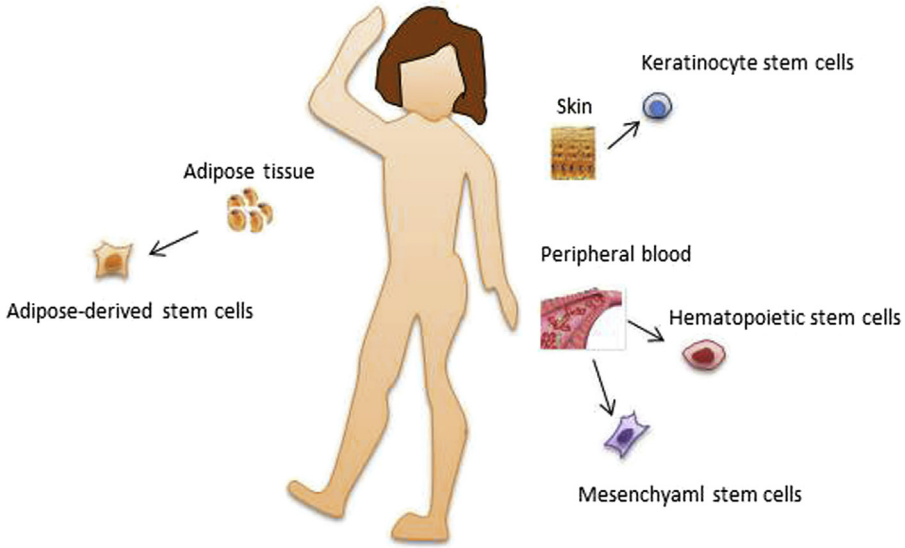

FIGURE 31.1
Adult stem cells for tissue-engineering applications. Blood, skin and adipose tissue are considered to be appropriate cell source for tissue engineering.

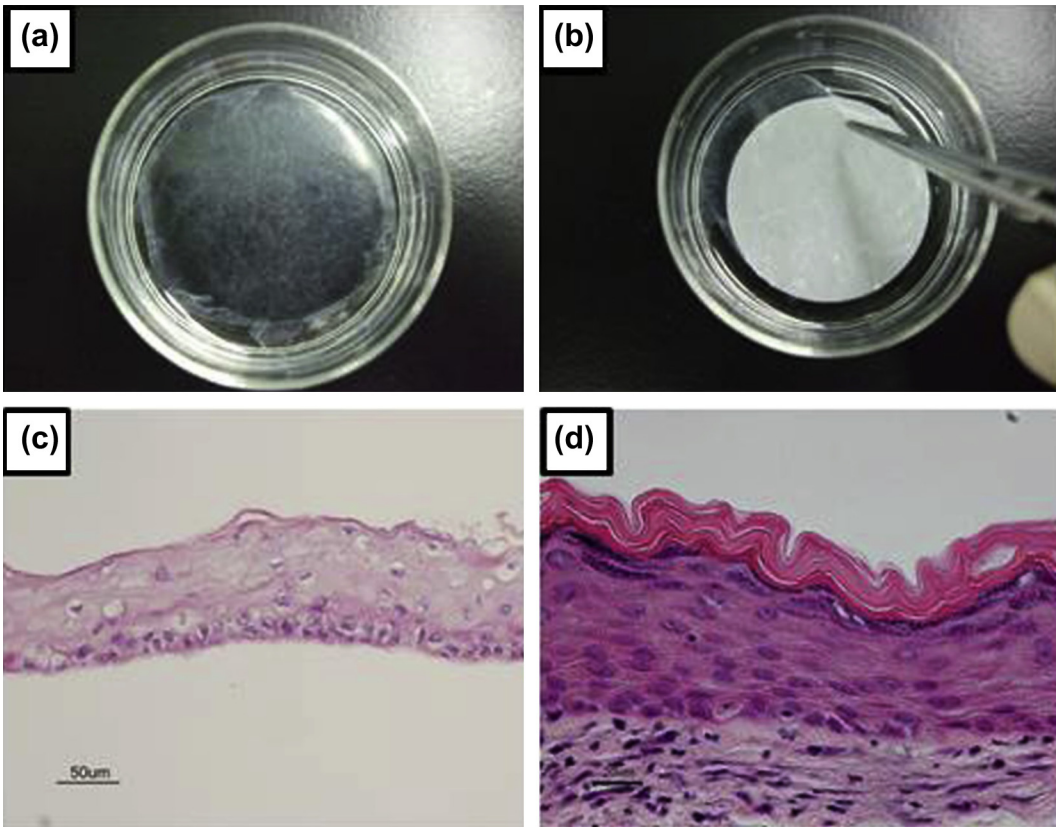

FIGURE 31.2
Temperature-responsive dishes and keratinocyte cell sheets. (a) Confluent human keratinocytes on a temperature-responsive dish. (b) Cell sheet manipulation using supporting membrane. (c) Harvested human keratinocyte cell sheet. (d) Transplanted human keratinocyte cell sheet on murine connective tissue.

a 'disease in a dish' assumes massive potential. Gage derived iPSCs from the fibroblasts of schizophrenic patients, and subsequently differentiated them into neurons. These cells showed reduced connectivity and synapse formation. Most dramatic was the rescue of this 'disease phenotype' by application of the antipsychotic, loxapine [121]. There is a long road between disease in a dish and cures for psychiatric disorders, but at least iPSCs offer a tractable system to interrogate cellular and molecular mechanisms.

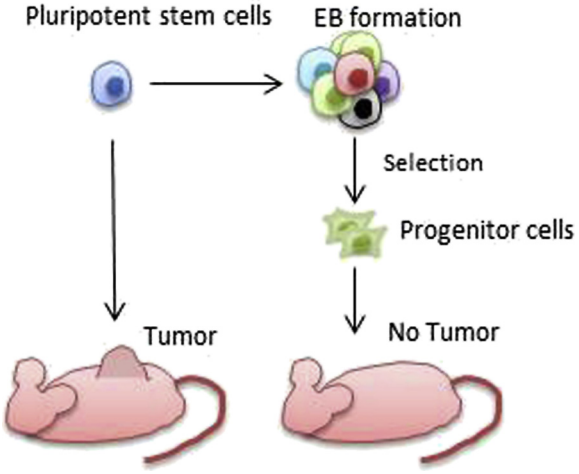

FIGURE 31.3
Strategy for harvesting progenitor cells from ES cells.

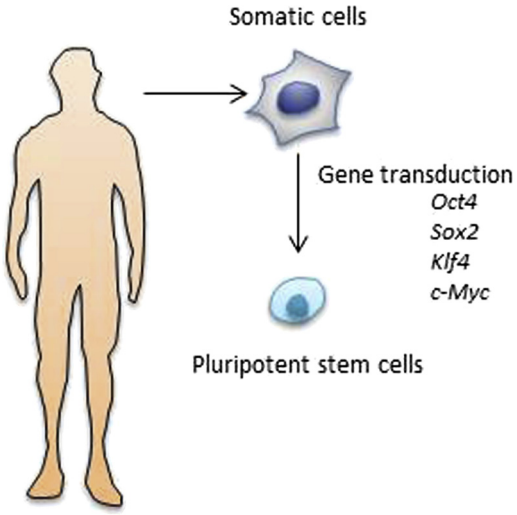

FIGURE 31.4
iPS cell generation.

CONCLUSION

As introduced in this chapter, each cell source for tissue engineering poses different challenges. Blood, skin and adipose tissue have been considered to be good stem cell sources because they are easy to access and allow relatively non-invasive harvesting. However, stem cell harvested from adult tissues always raises concerns about the limited number of cells procured. In recent years, it was hoped that ES cells and iPS cells would be appropriate cell sources for tissue engineering because of their pluripotency and proliferative potential. It has not been determined which cell source is most appropriate for which tissue-engineering application. To elucidate the true nature of stem cells, further data will be needed to open the door to potential applications.

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CHAPTER 32

Embryonic Stem Cells as a Cell Source for Tissue Engineering

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INTRODUCTION

It has been estimated that approximately 3,000 people die every day in the US from diseases that could have been treated with stem cell-derived tissues [1]. Given the therapeutic potential and growing public awareness of stem cells to treat disease, it is not surprising that embryonic stem cell (ESC) research has been rapidly expanding since mouse ESCs (mESCs) were first isolated in 1981 [2,3] followed by the isolation of human embryonic stem cells (hESCs) in 1998 [4,5] from the inner cell mass (ICM) of human blastocysts (Fig. 32.1).

Adult stem cells have been used clinically since the 1960s for therapies such as bone marrow transplantation, and these cells hold great therapeutic promise. ESCs also offer major benefits, including their ease of isolation, ability to propagate rapidly without differentiation, and — most significantly — their potential to form all cell types in the body. Additionally, ESCs are an attractive cell source for the study of developmental biology, drug/toxin screening studies, and the development of therapeutic agents to aid in tissue or organ replacement therapies. Regarding the latter application, which is the focus of this chapter, ESCs have the potential to exhibit a considerable impact on the field of tissue engineering, where current treatments for large tissue defects involve graft procedures which have severe limitations. Specifically, many patients with end-stage organ disease are unable to yield sufficient cells for expansion and transplantation. In addition, there exists an inadequate supply of harvestable tissues for grafting, and that which is available has associated risks, such as donor site morbidity, infection, disease transmission and immune rejection.

Tissue-engineering-based therapies may provide a possible solution to alleviate the current shortage of organs. Expectations for the potential of stem cells have increased even more after

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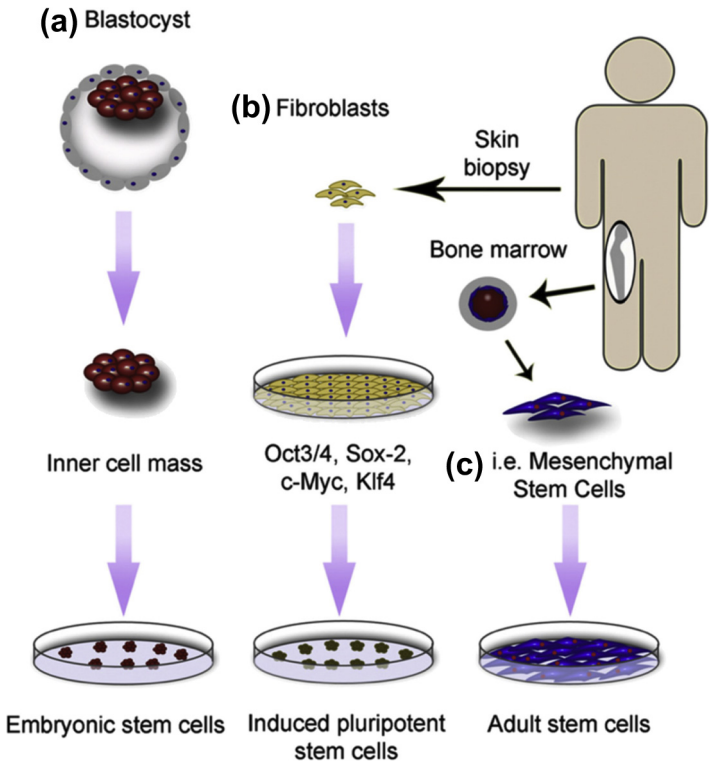

FIGURE 32.1
Schematic diagram of the derivation of stem cells. (a) Derivation of embryonic stem cells from the inner mass of the blastocysts and differentiation to different cell types; (b) Generation of induced pluripotent stem cells from somatic cells overexpressing Oct3/4, Sox2, c-Myc and Klf4; (c) Formation of adult stem cells (ASCs) during ontogeny (e.g., bone marrow mesenchymal stem cells). *(Adapted from [131]).*

the revolutionary finding of the generation of induced pluripotent stem cells (iPSCs) that profoundly modified the principles of cell fate and plasticity, and at the same time may represent a novel remarkably important cell therapy tool. iPSCs were originally generated by the introduction of four transcription factors (Oct3/4, Sox2, Klf4 and cMyc) in a somatic committed cell, the fibroblast, converting it to a pluripotent ESC-like state [6]. This work gave rise to a completely new field that is not covered in this chapter, and the reader is directed to a number of excellent papers and reviews on this topic.

Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering, materials science and life sciences toward the development of biologic substitutes that restore, maintain, or improve tissue function [7]. Thus, tissue engineering may provide therapeutic alternatives for organ or tissue defects that are acquired congenitally or produced by cancer, trauma, infection, or inflammation. Tissue-engineered products would provide a life-long therapy and may greatly reduce the hospitalization and health care costs associated with drug therapy, while simultaneously enhancing the patients' quality of life.

A central part of such strategy is the cell source to be used and the methods whereby sufficient numbers of viable differentiated cells can be obtained. ESCs represent a powerful source of cells capable of multi-lineage differentiation because they can potentially provide a renewable source of cells for transplantation. ESC-derived cells can be used directly as cellular replacement parts, or in combination with other materials (typically in the form of scaffolds, Fig. 32.2). Despite this promise, the application of ESC to tissue engineering faces numerous challenges, including appropriately differentiating the cells to the desired lineage in a controlled and homogenous fashion, and avoiding implantation of undifferentiated ESCs which can potentially form teratomas. Currently, ESC-based tissue-engineering research is focused on elucidating soluble and immobilized cues and respective signaling mechanisms that direct cell fate, on characterization and isolation of differentiated progeny, and on establishing protocols to improve the expansion and homogeneity of differentiated cells.

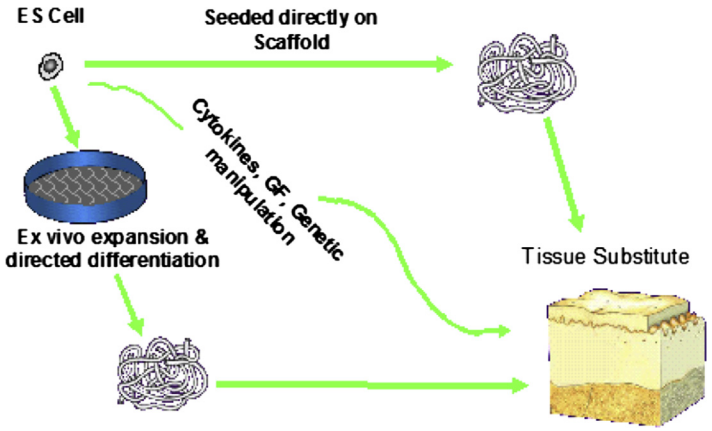

FIGURE 32.2
Approaches for using ES cells for scaffold-based tissue engineering. ES cells can be used in tissue-engineering constructs in a variety of methods. ES cells can be expanded in culture and then seeded directly onto scaffold where they are allowed to differentiate. Alternatively, stem cells can be directed to differentiate into various tissues and enriched for desired cells prior to seeding the cells onto scaffolds.

This chapter discusses key concepts and approaches for:

1) The propagation of undifferentiated ESC,
2) The directed differentiation into tissue specific cells,
3) The isolation of progenitor and differentiated phenotypes,
4) The transplantation of progenitor and differentiated cells, and
5) The remaining challenges for translating ESC-based tissue-engineering research into the clinical therapies.

Whenever possible, approaches using hESCs are reported.

MAINTENANCE OF ESCs

The self-renewal of ESCs is a prerequisite for generating a therapeutically viable amount of cells. Over the past few years much insight has been gained into the self-renewal of ESCs. Both murine and human ESCs (mESC and hESCs) were first derived and maintained in culture using mouse embryonic fibroblast feeder (MEF) layers and media containing serum. Considerable behavioral, morphological, and biochemical differences have been observed between mESCs and hESCs, and the research of animal ESCs is not easily translated to human ESCs [5,8,9]. For example, mESCs form tight, rounded clumps whereas hESCs form flatter, looser colonies, grow more slowly and demand strict culture conditions to maintain their normal morphology and genetic integrity. Unlike mESCs, which can be maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), hESC cultures require, in addition to LIF, supplementation of the basic fibroblast growth factor (bFGF) or the use of a feeder layer. Although both mESCs and hESCs express common transcription factors of 'stemness', such as Nanog, Oct4, and alkaline phosphatase, in the human system undifferentiated ESCs express Stage Specific Embryonic Antigen-3 (SSEA-3) and SSEA-4 and SSEA-1 is only expressed upon differentiation, whereas the opposite expression is observed in the mouse system. Due to these differences, efforts in hESC research focus on understanding the mechanisms of hESC self-renewal.

Mouse and human iPSCs have marker expression profile and biological properties very similar to the mouse and human ESCs, respectively, and this is a general proof of principle of their real pluripotent state. However, after initial studies, data are emerging that iPSCs are actually not identical to ESCs [10], and a growing body of evidence indicates that the epigenetic memory of the original cell type reprogrammed is at least partially maintained (reviewed in reference [11]). This may result in a more limited level of pluripotency in terms of spectrum of differentiation if compared to ESCs, but not necessarily a more limited spectrum of possibility of tissue differentiation for transplantation purposes if the original cell type is chosen accordingly [12].

Therapeutic applications of stem cells require moderate to large numbers of cells, hence requiring methods amenable to scale-up. Therefore, xenograft cell sources have also been considered. Using cultures of hESCs on human feeders it was found that human fetal muscle fibroblasts, human fetal skin fibroblasts and adult fallopian tubal epithelial cells supported the pluripotency of hESC culture *in vitro* [13]. The same group derived and established a hESC line on human fetal muscle fibroblasts in entirely animal-free conditions [13]. Since then, different fetal and adult cells have been examined and shown to support the continuous growth of hESCs [14–17]. However, the use of hESCs for therapeutic application requires defined culture medium and controlled cell derivation, maintenance and scale-up. To overcome these obstacles, combinations of self-renewal signals for hESCs have been investigated including soluble factor, extracellular matrix (ECM), cell-cell interactions and mechanical forces.

Significant attempts have been made to identify culture conditions and media components which can regulate hESCs self-renewal. Growth factors in culture media can bind the cell surface receptors to promote self-renewal. These soluble factors include bFGF [18,19], tumor growth factor β1 (TGFβ1)/ActivinA/Nodal ligands [20,21], insulin-like growth factor (IGFs) [18,22], Wnt ligands [23,24] and glycogen synthase kinase-3 GSK-3 inhibitors [25]. In one study, it was shown that hESCs can be expanded on human fibronectin using a medium supplemented with bFGF and TGFβ1 [26]. Noggin, an antagonist of bone morphogenetic protein (BMP), was found to be critical in preventing the differentiation of hESCs in culture. The combination of Noggin and bFGF was sufficient to maintain the proliferation of undifferentiated hESCs [27].

It has been also demonstrated that Wnt ligands affect ESCs self-renewal and differentiation. For example, spontaneous differentiation of mouse embryonic fibroblast inhibits by addition of Wnt1 to culture media [23]. In addition, hESC differentiation induces by using Wnt3 [28]; however, hESC self-renewal perturbs by the activation of canonical Wnt/β-catenin pathway through the expression of stabilized β-catenin [29]. Furthermore, hESCs maintained in media containing high concentrations of bFGF (24–36 ng/ml), alone or in combination with other factors, show characteristics similar to cultures maintained with feeder cell-conditioned medium [30,31].

The derivation of hESCs has also been achieved with minimal exposure to animal-derived material, using serum replacement (SR) and human foreskin fibroblasts as feeder cells [32], instead of the feeder layer [33], providing well-defined culture conditions [34]. Research is currently under way to determine how these conditions maintain cell integrity over long-term culture. For example, mTeSR, which contains TGFβ1, LiCl, bFGF, pipecolic acid, and GABA, supports long-term self-renewal of feeder-independent hESC cell culture [34]. In addition to growth factors, lipid molecules such as sphingosine-1-phosphate (S1P) [35–37], albumin [38], and synthetic lipid carriers [39] have been shown to regulate the self-renewal and differentiation of hESCs. Although growth factor and media compositions can control hESC self-renewal, challenges including maintenance of pluripotency, and production of biologically and functionally identical cells still remain.

In addition to soluble factors, a defined ECM or biomaterial may be required for maintaining the hESC self-renewal ability. Various biomaterials, such as Matrigel [40], human fibronectin [41], human vitronectin [42], collagen I [43], complex humanized matrices [44], hyaluronic acid hydrogels [45] or calcium alginate hydrogel [46], have been used as a structural support for hESC self-renewal. As an example, Xu et al. showed that hESCs can be maintained on Matrigel or laminin and MEF-conditioned media [47]. Cells grown in these conditions meet all the criteria for pluripotent cells: they maintain normal karyotypes, exhibit stable proliferation rate and high telomerase activity and they differentiate into derivatives of all three germ layers, both *in vitro* and *in vivo*. In an attempt to find ideal ECM components or biomaterials for *in vitro* feeder-cell-free culture of hESCs, Hakala et al. compared various biomaterials including ECM proteins (i.e., collagen IV, vitronectin, fibronectin, and laminin), human and

animal sera matrices, and Matrigel in combination with a variety of unmodified or modified culture media. Matrigel in combination with defined mTeSR1 culture medium was found to be superior matrix for hESC culture compared to other biomaterials used in this study [40]. Similarly, in a combinatorial study, Brafman et al. developed a high throughput technology, an arrayed cellular microenvironment, to assess the self-renewal of hESC cultured on different ECMs in media composed of different growth factors. Long-term self-renewal of hESCs was obtained on a biomaterial consisting of collagen IV, fibronectin, collagen I, and laminin in defined StemPro media and MEF-conditioned media [48].

Self-renewal and differentiation of ESCs can be also regulated through intercellular interactions [49–52] and mechanical forces [53–55]. Cell-cell interactions and formation of ESC colonies affect the self-renewal and spontaneous differentiation of ESCs. It has been shown that the size and shape of colonies play an important role in controlling ESC expansion [49,52]. Various microfabrication technologies have been employed to control ESC shape and size, such as micropatterning of substrate with ECMs [49] to confine colony formation to patterns, or formation of hESCs colonies in 3D microwells [50,51]. Another important factor in hESC self-renewal is the application of mechanical forces (e.g., cyclic biaxial strain [53,54] or shear stress [55]) to the cells. Although the physiological effects of mechanical forces on self-renewal and proliferation of ESCs remain unknown, it has been shown that these forces can regulate cellular differentiation. For example, fluid flow-induced shear stress has been demonstrated to enhance the elongation and spreading of undifferentiated hESCs and induce vascular differentiation of hESC at higher shear stress [55].

Large-scale production of hESCs is critical for tissue-engineering applications, which require large numbers of cells. It is generally accepted that 'classical' laboratory culturing methods are not suitable for the large-scale production of ESCs for therapeutic applications, and new culture systems are needed. Although two-dimensional (2D) methods such as the high density cultures of ESCs have been developed by combining automated feeding and culture methods [56], three-dimensional (3D) culture may be a more suitable technology for large-scale expansion of ESC production.

At the present time, the aggregation of multiple ESCs is necessary to initiate EB formation. The formation of large cellular aggregation may prevent nutrient and growth factor diffusion as well as metabolic waste removal from the aggregates in suspension cultures in large-scale systems. A small number of methods have been developed for the differentiation of mESCs in controlled cultures. Hanging drops and methylcellulose cultures have been shown to be somewhat efficient in preventing the agglomeration of EBs, but their complex nature makes their upscaling a rather difficult task.

A much simpler process in spinner flasks resulted in the formation of large cell clumps within a few days, indicative of significant cell aggregation in the cultures [57]. Compared to static culture system, spinner flasks enhance homogenous expansion of hEBs and can be easily scaled up to 10,000L bioreactor tanks [58]. In one study, it was demonstrated that the growth rate of hEB is higher when cultured in stirred vessels than in other culture systems (e.g., static culture and rotary cell culture system) [59]. However, an increase of the culture medium stirring rate to avoid agglomeration within the stirred vessels resulted in massive hydrodynamic damage to the cells due to the extensive mixing in the vessels. Therefore, in order to establish a scaleable process for the development of EBs, there is a need for dynamic cultivation under controlled mixing conditions. One approach used a static system for an initial aggregation period of four days, followed by a period in dynamic culture in spinner flasks, to successfully achieve the bulk production of cardiomyocytes from differentiating mES cells [60].

In addition to suspension cultures using hEBs, stirred vessels can be also used for the scale-up expansion of undifferentiated hESCs through the combination of a microcarrier with the stirred culture systems. Various microcarriers such as polystyrene [61], collagen-coated dextran

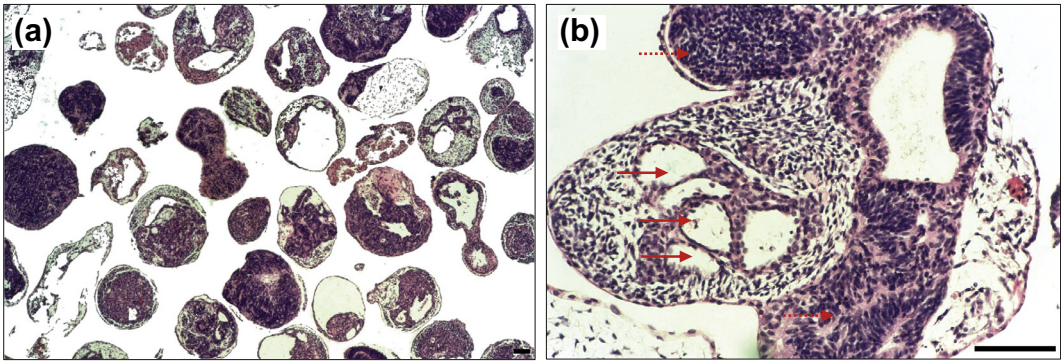

FIGURE 32.3

Formation of human EBs in a rotating cell culture system. (a—b) Haematoxilin- and eosin-stained sections of EBs after one month in culture, showing the formation of (a) a small and relatively homogenous population of human EBs, and (b) a variety of cell types such as: epithelial neuronal tubes (dashed arrows), and blood vessels (solid arrows) (Scale bars: 100 μm). *(Adapted from reference [66].)*

[62] and Matrigel-coated cellulose [63] have been used to promote hESC expansion in spinner flasks. In addition to hESC expansion, this combination of microcarriers and stirred culture systems has been used for directing hESC differentiation to definitive endoderm [64] and cryopreservation and recovery of undifferentiated hESCs adhered to microcarriers [65].

Another dynamic approach which was highly effective for hESCs is to generate and culture EBs within rotating cell culture systems [66]. These bioreactors provide exceptionally supportive flow environments for the cultivation of hESCs, with minimal hydrodynamic damage to incipient EBs, reduced EB fusion and agglomeration, and they allow the uniform growth and differentiation of EBs in three dimensions, as they oscillate and rotate evenly (Fig. 32.3a). hESCs cultured within these systems formed aggregates after 12 hrs that were smaller and more uniform in size and evenly rounded due to minimal agglomeration; the yield of EBs was three times higher than that measured for static cultures. Also, dynamically formed EBs exhibited steady and progressive differentiation, with cyst formation and elaboration of complex structures such as neuro-epithelial tubes, blood vessels and glands (Fig. 32.3b) [66].

Different rotary cell culture systems, including slow turning lateral vessel (STLV) and high aspect rotating vessel (HARV), have been used to promote the efficiency of EB formation and differentiation of stem cells [66—69]. Generally, STLV systems are preferable to HARV for the EB aggregate formation and differentiation. It has been shown that the HARV system can lead to significant aggregation with large necrotic areas at the center and differentiations at the peripheries of aggregates. The aggregation rate of hESCs can be controlled by using the STLV system, which results in the formation of small-size hEBs [58,66].

To further enhance the large-scale differentiation of hEBs, a perfused STLV system was combined with a dialysis chamber to allow the diffusion of media as well as removal of waste products from the bioreactor [70]. Compared to static cultures, uniform growth and differentiation of hEBs to neural lineage was promoted when the combined rotary cell culture system/dialysis chamber was used [70]. Although rotary cell culture systems provide low-shear environments for hESCs cultivation and differentiation, they can only be scaled-up to volumes of 5—500 mL, which is much lower than the scalability of stirred culture systems. Although still an area of active research, these technologies have demonstrated the potential of engineering for the development of scalable technologies to expand ESC provision for research and therapies.

DIRECTED DIFFERENTIATION

Perhaps the biggest challenge in the clinical use of ESCs is the lack of knowledge of how to predictably direct their differentiation. For example, although ESCs can generate cells of

hematopoietic, endothelial, cardiac, neural, osteogenic, hepatic and pancreatic tissues, it has been very difficult to achieve directed differentiation into these tissues. The lack of homogeneous differentiation may be attributed to the intrinsic property of ESCs of differentiating stochastically in the absence of proper temporal and spatial signals from the surrounding microenvironment. Various techniques have been employed to control the differentiation of hESCs and to isolate a specific germ layer for tissue regeneration applications. The limitation of current techniques used for controlled differentiation is the low transformation efficiency, which results in a cell population containing ectoderm and mesoderm germ layers. The segregation of these germ layers can be achieved by using appropriate differentiation protocols. In this section we describe some of the current approaches used to direct the differentiation of ESCs and give examples of their use.

Genetic reprogramming

This approach includes the introduction of specific gene(s) into hESCs, which enable the production (by enhancement or selection) and propagation of specific cell type populations. Different techniques for knocking-in and knocking-out genes into hESCs have already been established. Transfection of undifferentiated hESCs with specific plasmid was established using either chemical reagents or electroporation. The latter was further shown to be useful for the generation of homologous recombination events [71]. Another technique is the introduction of transgenes into hESCs by self-inactivated lentiviruses. This transduction technique was shown to be efficient, with sustained expression in undifferentiated hESCs as well as in hESCs, which undergo differentiation [72,73]. However, both undifferentiated and differentiated hESCs were successfully infected by using adenoviral and adeno-associated viral vectors [74]. Another approach, which uses genetic manipulation, is the introduction of suicidal genes, which permit the ablation of the cells if necessary [75]. Using this approach, hESCs were transfected to express the herpes simplex virus thymidine kinase gene [76].

Genetic techniques involve both positive and negative regulators. The positive regulators include the constitutive or controlled expression of transcription factors that have been shown to derive the differentiation into particular tissues. For example, the over-expression of the Nurr transcription factor has been shown to increase the frequency of ESCs that differentiate into functional neural cells [77]. Alternatively, the negative regulators can be incorporated to induce the apoptosis of cells that differentiate to varying pathways. For example, neomycin selection and suicide genes that are activated by certain transcription factors can be used [78]. In a recent study, Zoldan et al. developed a 3D siRNA delivery system using lipid-like materials, lipidoids, for the efficient transfection of hESCs. This system was used to direct differentiation of hESCs to a specific lineage by knocking down Kinase Insert Domain Receptor (KDR) to prevent the differentiation of endoderm layer, leading the separation of this germ layer from mesoderm and ectoderm [79]. The developed 3D RNA delivery technique have shown to be preferable over a 2D environment for directing hESC differentiation, in which the transfection reagents are added to the media used for *in vitro* culture of hESCs-seeded 2D substrates. Clearly, all these techniques would benefit from a deeper understanding of inner workings of transient cells and knowledge of the differentiation pathways and lineages. Further analysis of the stem cell and progenitor hierarchy through high-throughput analysis of gene and protein profiles should accelerate this process. Despite the power of these approaches, one potential concern is that the genetic modifications may make the cells unsuitable for transplantation.

Microenvironmental cues

Another approach to directing ESC differentiation is through the use of microenvironmental cues that are important in regulating adult and ESC fate decisions. During development, cells of the inner cell mass are exposed to a series of tightly regulated microenvironmental signals. However, in tissue culture the complex expression patterns and spatial orientation of these signals can be lost. Currently, ESCs are grown in their primitive state as aggregated colonies of

cells. To stimulate differentiation, two main methods have been examined. In one method, differentiated cells are derived from EBs. EBs can be formed from either single cell suspensions of ESC or from aggregates of cells. EBs mimic the structure of the developing embryo and recapitulate many of the stages involved during its differentiation, and clonally derived EBs can be used to locate and isolate tissue specific progenitors. EBs initiate many developmental processes and create suitable conditions for differentiation of cells into all three germ layers and are generally formed through suspension or hanging drop methods.

In general, the differentiation of ESCs in EBs produces a wider spectrum of cell types, due to the EBs' ability to better mimic the temporal pattern of cell differentiation seen in the embryo. In some applications, the combined use of EBs and adherent cultures has resulted in better cell yields. For example, to induce ESC differentiation to cardiomyocytes, EB formation in suspension cultures followed by differentiation in adhesion cultures has been shown to optimize the percentage of cells that give rise to cardiomyocytes [80,81]. Similarly the production of hepatocytes has been shown to be induced by first culturing the cell in EBs and then in 2D cultures [82].

Neural progenitor cells were isolated from hESCs that showed positive immunoreactivity to neuron-specific antigens, responded to neurotransmitter application, and presented voltage dependent channels in the cell membrane [83–86]. Various differentiation approaches, including adherent culture or EB suspension culture, have been used to direct the hESC differentiation to the neural lineage [87] (Fig. 32.4a).

To promote neural differentiation, different soluble factors such as BMP-inhibitors, retinoic acid (RA), and other supplements (e.g., N2, B27, ITS) are added to the media in adherent culture methods. In EB suspension culture systems, neural induction factors should be added during differentiation to induce neural differentiation of hESCs. The differentiated cells are then cultivated on adherent culture to allow for the neural cell growth. In both approaches, the morphological characteristics of the neural progenitors can be maintained and expression of NP-markers in the medium supplemented with FGF2 and B27 [87] (Fig. 32.4b). Highly enriched cultures of neural progenitor cells were isolated from hESCs and grafted into the stratum of rats with the Parkinson's disease [88]. The grafted cells differentiated *in vivo* into dopaminergic neurons and corrected partially behavioral deficits in the transplanted animals. A subsequent study showed that hESCs implanted in the brain ventricles of embryonic mice can differentiate into functional neural lineages and generate mature, active human neurons that successfully integrate into the adult mouse forebrain [89].

Oligodendrocytes and their progenitors were also isolated in high yield from hESCs [90]. Transplantation of these cells into animal models of dysmyelination resulted in integration, differentiation into oligodendrocytes and compact myelin formation, demonstrating that these cells displayed a functional phenotype. In addition to *in vivo* differentiation of hESCs to neural lineages, ESCs can be combined with a biomaterial to induce the *in vitro* differentiation of ESCs to specific neural lineages in the presence of differentiation-inducing agents. For examples, electrospun fibrous scaffolds not only enhanced the differentiation of mouse ESCs into specific neural lineages such as neurons, oligodendrocytes and astrocytes, but also supported the neurite outgrowth [91]. In recent years, the use of carbon nanotubes (CNTs) for the neuron differentiation from hESCs and neural growth has been also explored [92,93]. It has been shown that 2D scaffolds composed of poly(acrylic acid) grafted CNT thin films promoted hESCs' neuron differentiation efficiency as well as protein adsorption and cell attachment compared to poly(acrylic acid) scaffolds without CNTs [92].

The differentiation of hESCs to neural lineages is induced by supplementation of the culture medium with biochemical agents. Recently, it has been demonstrated that nanopatterning of the substrate can effectively control hESC differentiation to neural lineages in the absence of

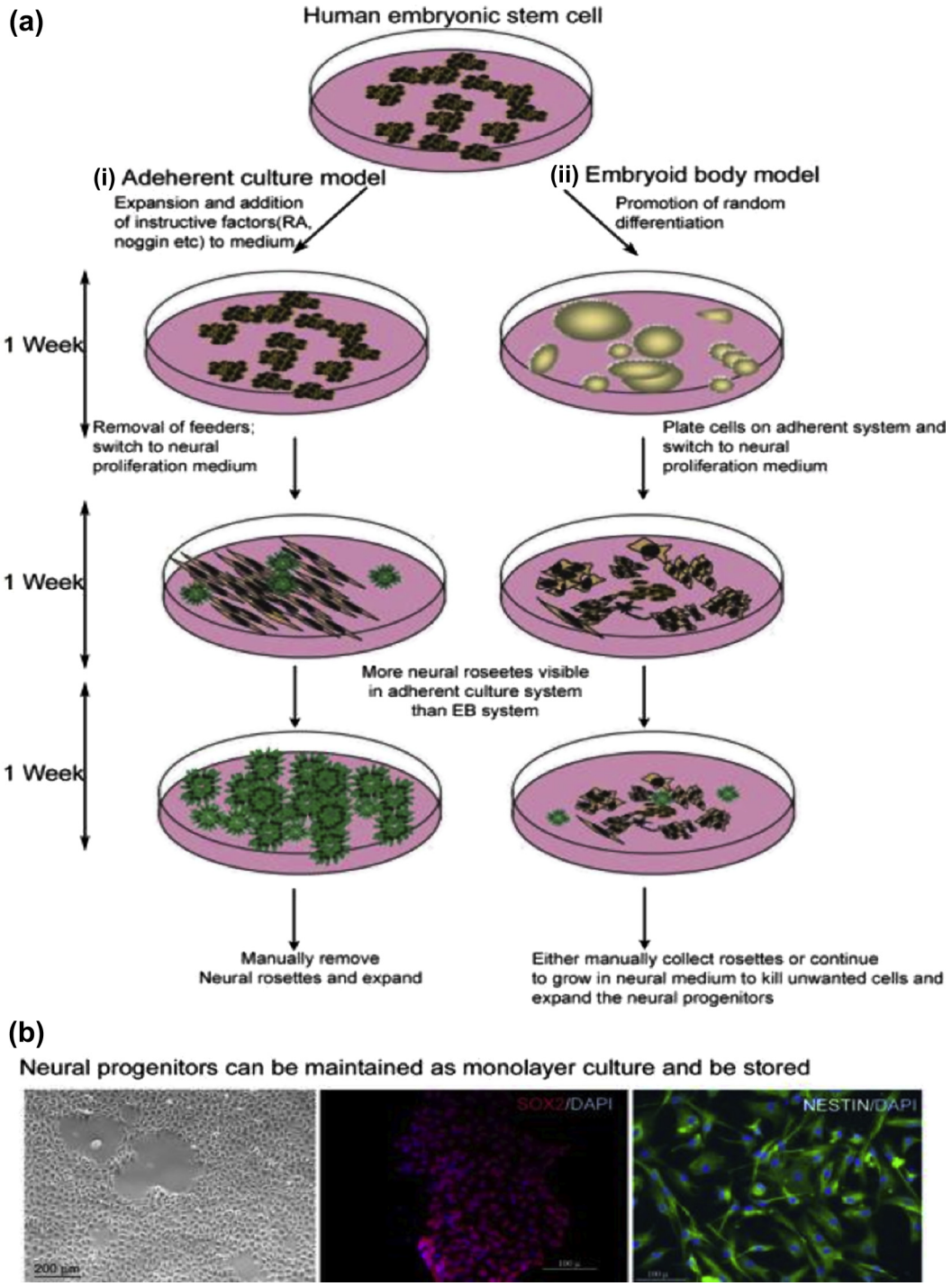

FIGURE 32.4

Differentiation of hESCs into neural progenitor cells. (a) Directed differentiation of hESC to neural lineages by adherent or EB suspension culture, (b) bright field image (left panel), and expression of neural progenitor markers including SOX2 (red, middle panel) and Nestin (green, right panel), indicating that the neural progenitor generated by these methods preserved their characteristic morphology and exhibited the expression of neural progenitor by culturing in medium containing FGF2 and B27. *(Adapted from reference [87]).*

any biological and biochemical agents. In one study, a UV-assisted capillary force lithography technique was developed to generate 350 nm pattern arrays using polyurethane acrylate [94]. The hESCs seeded on these patterns differentiated to neuronal lineage after five days of culture without the addition of differentiation-inducing agents [94].

ESCs have been shown to give rise to functional vascular tissue. Three different strategies have been employed to induce vascular differentiation of ESCs:

1) EB formation;
2) Uco-culture with fibroblast feeder layers or target cells; and
3) 2D monolayer culture of ESCs in defined chemical conditions combined with differentiation stimuli [95] (Fig. 32.5).

Spontaneous differentiation of ESCs to EB aggregates in a medium supplemented with cytokines has been shown to promote their differentiation to smooth muscle cell, pericytes, and endothelial cells [96,97]. One limitation of this strategy is that the ESCs differentiate to a heterogeneous cell population composed of vascular cells and other cell types from different germ layers. Various approaches have been employed to improve the efficiency of EB protocols to promote vascular cell differentiation, such as addition of VEGF-A [98] or BMP4 [99] to culture media and using magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) [100].

Another approach to directing the vascular differentiation of hESCs is the use of co-culture systems, where undifferentiated ESCs are seeded onto mouse fibroblast feeder layers such as stromal cells [101], MEF [102], or mouse ECs [103] to enhance vascular differentiation. Alternatively, 2D monolayer culture on Matrigel [104], collagen IV [105], and fibronectin [106] combined with differentiation stimuli (e.g., addition of GFs/Cytokines [104] or RA [107], mechanical stimulation [108], and hypoxia [109]) have been used for vascular hESC differentiation. Although these strategies increase the differentiation efficiency, isolation of progenitor cells expressing markers (e.g., CD34, Stem Cell Antigen [Sca]-] or Flk1) during ESC differentiation is a crucial requirement for deriving homogenous vascular cell populations [104].

Early vascular progenitor cells isolated from differentiating mESCs were shown to give rise to all three blood vessel cell types: hematopoietic, endothelial and smooth muscle cells [110]. Once injected into chick embryos, these vascular progenitors differentiated into endothelial

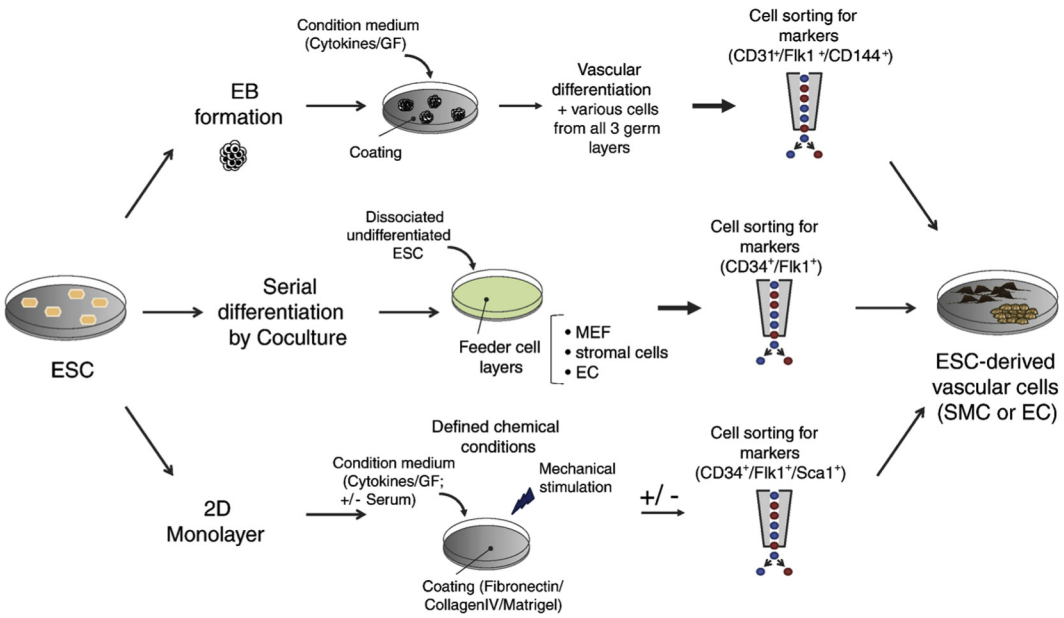

FIGURE 32.5

Approaches used for vascular differentiation of ESCs. Vascular cell differentiation is mainly induced by three culture methodologies: differentiation through EBs, co-culture with fibroblast feeder layers or target cells, and monolayer culture of ESCs in defined chemical conditions. *(Adapted from reference [95].)*

and mural cells and contributed to the vascular development. hESCs can also be differentiated into endothelial cells by using platelet endothelial cell adhesion molecule-1 antibodies [111]. *In vivo*, when transplanted into immunodeficient mice, these cells appeared to form microvessels.

Furthermore, it has been shown that monkey ESCs can give rise to endothelial cells when the embryonic cells were exposed to a medium containing combinations of growth factors. The isolated cells were able to form vascular-like networks when implanted *in vivo* [112]. Endothelial progenitor cells have been isolated from hES cells which presented hematopoietic [113] or smooth muscle cells competency. hESCs have been reported to differentiate into hematopoietic precursor cells when co-cultured with bone marrow and endothelial cell lines [114]. When these precursor cells are cultured on semisolid media with hematopoietic growth factors, they form characteristic myeloid, erythroid and megakaryocyte colonies.

Cardiomyocytes have been isolated from hES cells for the treatment of cardiac diseases. The most common approach to induce *in vitro* differentiation of hESC to cardiomyocytes is the formation of EB aggregates followed by few days post-plating on a 2D substrate to obtain cells with cardiomyocyte characteristics [115]. Cardiomyocyte differentiation of hESCs can be also induced by co-culturing undifferentiated hESCs with a mouse visceral endoderm-like cell line (END-2) [116]. Cardiomyocytes isolated from hES cells expressed sarcomeric marker proteins, chronotropic responses, and ion channel expression [116]. Upon differentiation, beating cells were observed after one week of culture under differentiation conditions. These increased in number over time, and could retain contractility for over 70 days [117]. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. Electrophysiology demonstrated that most cells resembled human fetal ventricular cells. Despite the progress made over the last decade, knowledge about the mechanism of the formation of functional cardiomyocytes from hESCs remains limited.

Insulin-producing β cells were also generated from hESCs [118], by spontaneous differentiation of hESCs in adherent or suspension culture conditions [119] and using media which contained growth factors [120,121]. Reverse transcription-polymerase chain reaction detected an enhanced expression of pancreatic genes in the different cells [120]. Immunofluorescence and *in situ* hybridization revealed high percentages of insulin-expressing cells [120].

Recently there has been great interest in examining the osteogenic potential of ESCs derived from both mice and humans. hESCs can differentiate into osteogenic cells with the same media supplements that are used to differentiate adult mesenchymal stem cells (MSCs). Due to their high self-renewal capability, ESCs are preferable to MSCs for bone regeneration, as the latter have limited abilities for self-renewal, proliferation, and differentiation into the osteogenic lineage [122]. Current issues associated with the osteogenic differentiation of hESCs include the formation of non-homogeneous cell populations and limited numbers of differentiated cells. To overcome these limitations, various growth factors and reagents such as β-glycerophosphate, ascorbic acid, dexamethasone, and osteogenic factors have been used to create osteoprogenitor cells from hESCs [123–126]. In addition, the differentiation efficiency of hESCs into a homogeneous osteogenic cell population was improved through co-culturing of hESCs with human primary bone-derived cells (hPBDs) in the absence of exogenous factors [127].

The osteogenic cells derived from hESCs (OC-hESCs) were seeded on a apatite-coated poly(D,L-lactic-co-glycolic acid)/nano-hydroxyapatite (PLGA/HA) composite scaffold and subcutaneously implanted in immunodeficient mice to examine *in vivo* bone formation [128]. The results of *in vivo* studies demonstrated that the implanted OC-hESCs and apatite-coated PLGA/HA scaffold induced the formation of large amounts of new bone tissue within the defect site, demonstrating the suitability of hESCs for bone regeneration [128]. Our group

showed that culturing hESCs without EBs leads to an over seven-fold increase in the number of osteogenic cells and to spontaneous bone nodule formation after 10–12 days [129]. In contrast, when hESCs were differentiated as EBs for five days followed by plating of single cells, bone nodules formed after four weeks only in the presence of dexamethasone.

We show that the cultivation of hESC-derived mesenchymal progenitors on three-dimensional osteoconductive scaffolds (derived from fully decellularized trabecular bone) in bioreactors with medium perfusion was shown to lead to the formation of large and compact bone constructs. Notably, the implantation of engineered bone in immunodeficient mice for eight weeks resulted in the maintenance and maturation of bone matrix, without a single incidence of the formation of the teratomas that were consistently observed when undifferentiated hESCs are implanted, alone or in bone scaffolds. This showed that tissue-engineering protocols can be successfully applied to hESC-progenitors to grow bone grafts for use in basic and translational studies [209].

In another study, native heart extracellular matrix (ECM) was successfully used to direct the cardiac differentiation of human embryonic stem cells (hESCs) *in vitro* [210]. A series of hydrogels was prepared from decellularized heart ECM blended with collagen type I at varying ratios. Maturation of cardiac function in EBs formed from hESCs was documented in terms of spontaneous contractile behavior and the mRNA and protein expression of cardiac markers. Hydrogel with high ECM content (75% ECM, 25% collagen, no supplemental soluble factors), increased the fraction of cells expressing cardiac marker troponin T (*c*TnT), when compared to either hydrogel with low ECM content (25% ECM, 75% collagen, no supplemental soluble factors), or collagen hydrogel (100% collagen, with supplemental soluble factors). The ability of native ECM to induce cardiac differentiation of hESCs without the addition of soluble factors makes it an attractive biomaterial system for basic studies of cardiac development and potentially for the delivery of therapeutic cells into the heart.

3D versus 2D cell culture systems

In an appropriate environment, ESCs can differentiate into complex 3D tissue structures. These environments are designed to resemble the key features of the hESC's niche and are favored over the 2D systems, which limit the cellular interactions and signaling, and hamper the subsequent differentiation of hESC into functional tissues [130,131]. The scaffold may act as a temporary ECM; providing physical cues for cell orientation, spreading, differentiation and the remodeling of tissue structures.

It has been demonstrated that the biochemistry, topography, and physical properties of the scaffold can regulate stem cell differentiation and function [131,132]. Culture of hESCs in PLGA scaffolds in specific media containing transforming growth factor β, activin A, or insulin-like growth factor induced the differentiation of the cells into 3D structures with characteristics of developing neural tissues, cartilage, or liver, respectively [133].

It was also demonstrated that the 3D environment created by cell encapsulation in Matrigel failed to support hESC growth and 3D organization, and this was likely due to the fact that this gel was unable to resist the force of cell contraction. Furthermore, when these cells were cultured in PLGA and poly(lactic acid) (PLA) scaffolds in the presence of media containing nerve growth factor and neurotrophin 3, enhanced numbers of neural structures were observed [134]. In one study, hESC-derived EBs cultured with a 3D collagen scaffold exhibited liver-specific genes expression and albumin production in the presence of exogenous growth factors and hormones [135]. The addition of signaling factors such as activin A and Wnt3a to this system may improve the efficiency of hESCs differentiation and production of functional hepatic endoderm [136].

Similarly, the culture of ESCs in a 3D collagen scaffold, stimulated with exogenous growth factors and hormones, led to the differentiation of the cells into hepatocyte-like cells. These

cells were characterized by the expression of liver-specific genes and synthesis of albumin, and the differentiation pattern observed compared favorably to cells differentiated in a 2D system. It was also reported that the differentiation of rhesus monkey ESCs in 3D collagen matrices was different from that which took place in monolayers [137]. Alginate scaffolds were also used for the differentiation of hESCs [138]. These scaffolds induced vasculogenesis in encapsulated cells to a larger extent than cells grown in bioreactors. Tantalum scaffolds also increased the differentiation of mESCs into hematopoietic cells as compared to traditional 2D cultures [139]. Therefore, the 3D culture systems can promote hESC differentiation and assembly into functional tissues, through better mimicking of the 3D structural organization of native tissue compared to 2D systems.

High-throughput assays for directing stem cell differentiation

Today, chemists and engineers are equipped with tools which give them the ability to synthesize molecules and test their effects on cells in a high-throughput manner. For example, libraries of small molecules, polymers and genes have been generated and used to screen candidate molecules to induce osteogenesis [140] and cardiomyogenesis [141] in ESCs as well as the dedifferentiation of committed cells [142]. The use of chemical compound libraries may provide a method of addressing the complexities associated with native microenvironments by directing cell behavior through interacting with transcription factors and cell fate regulators.

Microscale technologies can facilitate high-throughput experimentation and provide a powerful tool for screening whole libraries of molecules and biomaterials. Robotic spotters capable of dispensing and immobilizing nanoliters of material have been used to fabricate microarrays, where cell-matrix interactions can be tested and optimized in a high-throughput manner. For example, synthetic biomaterial arrays have been fabricated to test the interaction of stem cells with various extracellular signals [143]. Using this approach, thousands of polymeric materials were synthesized and their effects on differentiation of hESCs [144] and human mesenchymal stem cells (hMSC) [145] have been evaluated. These interactions have led to unexpected and novel cell-material interactions. Although the molecular mechanisms associated with the biological responses have yet to be clarified, such technology may be widely applicable in cell-microenvironment studies and in the identification of cues that induce desired cell responses.

Also, these materials could be used as templates for tissue-engineering scaffolds. Such an approach is a radical departure from traditional methods of developing new biomaterials, where polymers have been individually developed and tested for their effect on cells. In addition to analyzing synthetic material libraries, the effect of natural ECM molecules on cell fate can be evaluated in a high-throughput manner [143]. In one example, combinatorial matrices of various natural extracellular matrix proteins were tested for their ability to maintain the function of differentiated hepatocytes and to induce hepatic differentiation from murine ESCs [146]. Recently, Huang et al. used a micro-scale direct writing (MDW) technique to print ECM components (e.g., collagen IV, gelatin, and fibronectin) into diverse geometries and compositions on 2D surfaces for assessing the effect of ECM geometry and composition on ectodermal differentiation of murine ESCs [147]. It was shown that ECM compositions, soluble factors, and surface topography could regulate ESC attachment and differentiation [147].

Microfabrication techniques have been also used to control cell-cell interaction and to form hESC-derived EB aggregates with defined sizes and geometries. For example, in one study, soft lithography was used to fabricate cell-repellant poly(ethylene glycol) (PEG) microwells for the formation of EB aggregates with controlled sizes and shapes, determined by the geometry of the microwells (Fig. 32.6a). The EB cell aggregates formed within the microwells remained viable and maintained their geometries over at least 10 days of culture. Using this system, the EB aggregates could pattern into various shapes and sizes (Fig. 32.6b–d) [148]. To control the

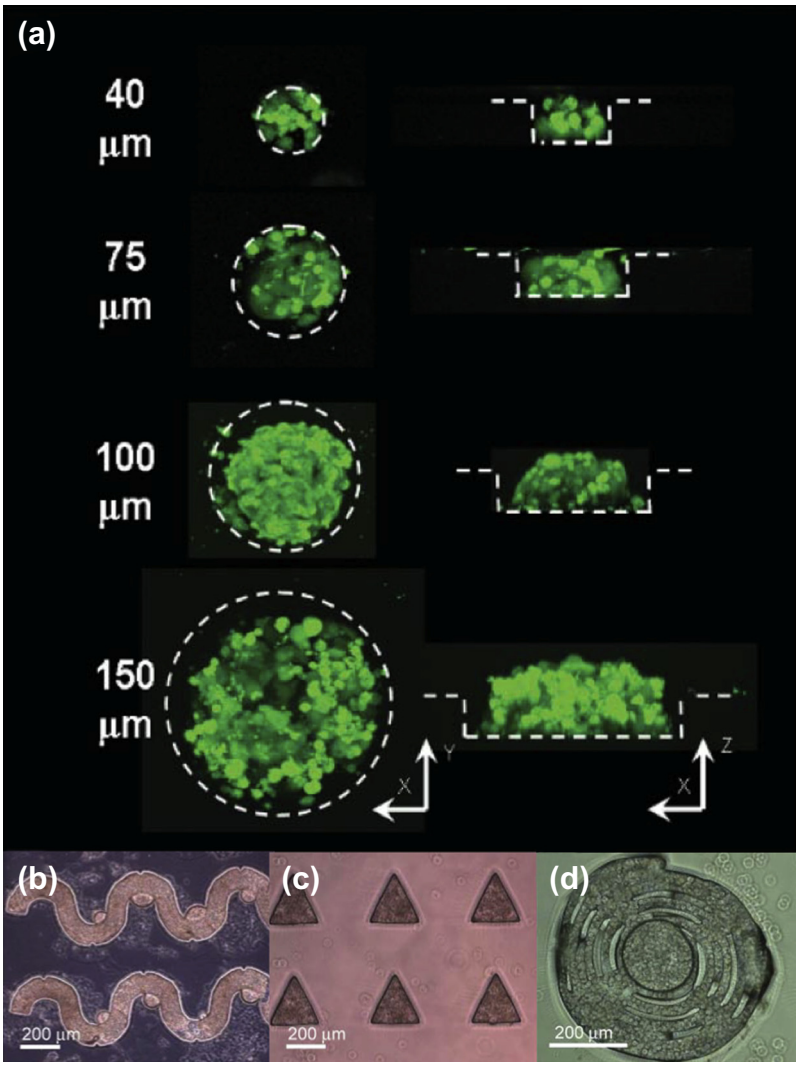

FIGURE 32.6
Microwells for the formation of EBs with controlled size and shape. (a) Confocal images of fluorescently labeled EB cell aggregates within microwells with different diameter raging from 40 μm to 150 μm on day 5 of culture; formation of EBs with different shapes including (b) curves, (c) triangles, and (d) swirls. *(Adapted from reference [148].)*

shape and direct the differentiation of EBs, RA-loaded PLGA microspheres were used to deliver morphogenic factors within EB microenvironments in a spatiotemporally controlled manner [149]. Homogenous differentiation of cystic spheroids with a bi-epithelial morphology was obtained when EBs were cultured on the fabricated microspheres [149].

In another study, the effect of EB aggregate size on its differentiation was investigated by seeding ESC on PEG microwells of various diameters [150]. It was found that larger microwells (450 μm diameter) induced differentiation of ESC to cardiogenesis through the expression of Wnt11. However, EBs formed in small (150 μm) microwells differentiated to endothelial cell by increased expression of Wnt5a [150].

Cell arrays have been also used to pattern stem cells on substrates. Arrays of cells can be used to localize and track individual cells, enabling the clonal analysis of stem cell fates. For example, clonal populations of neural stem cells were immobilized within microfabricated structures and their progeny were tracked using real-time microscopy, yielding information about cellular kinetics and cell fate decisions in a high-throughput manner [151]. Using this approach, it is possible to study the response of individual stem cells to various micro-environmental signals.

Cell patterning on geometrically defined shapes has been used to study the effects of cell shape on cell fate decisions. As cells adhere onto a micropatterned substrate, they align themselves to

the shape of the underlying adhesive region. A change in shape induces changes in the cell cytoskeletal features, which in turn influence cell apoptosis, proliferation [152] and differentiation [153]. Co-culturing ESCs with secondary cells can promote their differentiation into specific cell lines [154]. For this purpose, Fukuda et al. developed a technique to fabricate micropatterned co-cultures of ES with secondary cell lines on surfaces containing three different layers of hyaluronic acid, fibronectin, and collagen [155]. First, the hyaluronic acid was micropatterned on a glass substrate. Then, fibronectin was then deposited on the areas of exposed glass to create cell adhesive regions. After the cell attachment on fibronectin-coated areas, a layer of collagen was added to hyaluronic acid patterns to switch surface properties and facilitate the adhesion of the second cell type. Using this system, the patterned co-cultures of ECs with NIH-3T3 and AML12 cells could be obtained [155]. Further elucidation of the molecular mechanisms indicated that cell shape regulated the activation of the RhoA pathway demonstrating that mechanical stresses can be crucial for directing stem cells differentiation. Therefore, controlling cellular microenvironment using micropatterning may be used for directing cell fate for tissue-engineering applications.

Physical signals

Mechanical forces affect the differentiation and functional properties of many cell types, and are being increasingly used in tissue engineering. For example, functional autologous arteries have been cultured using pulsatile perfusion bioreactors [156]. Although it is known that mechanical stimuli (such as cyclic stretching and fluid shear stress) may be required to direct the differentiation of ESCs, understanding their effects is still in its infancy [157].

In one study, fluid shear stress was applied to induce Flk-1-positive ES differentiation into vascular endothelial cells through the activation of Flk-1. The expression of vascular endothelial cell-specific markers such as Flk-1, Flt-1, VE cadherin, and PECAM-1 enhanced in the presence of shear stress; however, shear stress had no effect on markers of epithelial or smooth muscle (keratin, or α-SMA) [157]. In another study, Shimizu et al. demonstrated that cyclic uniaxial stretching on Flk-1-positive ES cells for 24 hrs significantly increased the expression of VSMC markers α-SMA and smooth muscle-myosin heavy chain (SM-MHC), decreased the expression of EC marker Flk-1, and had no effect on the other EC markers (Flt-1, VE cadherin, and PECAM-1) [158]. Platelet-derived growth factor (PDGF) receptor beta kinase inhibitor blocked cell proliferation and VSMC marker expression that were induced by applying mechanical stimulation [158]. Mechanical stretching and fluid shear stress have been also used to direct ES cell differentiation into cardiovascular lineages [159,160]. Taken together, these studies demonstrate that mechanical stimulation can enhance the ability of ESCs to respond to exogenous signals, and promote their differentiation into a specific lineage. In one study, the hESC differentiation on deformable elastic substrates was inhibited by applying a 10% cyclic stretch [54]. The expression of Oct4 and SSEA-4 was promoted in the presence of mechanical stimulation, demonstrating an increase in hESC self-renewal. It was also found that mechanical stretch inhibited hESC differentiation when the cells were cultured in a mouse MEF-conditioned medium. However, differentiation of hESCs was not affected by mechanical stimulation when an unconditioned medium was used [54].

Other environmental factors that may be required include electrical signals. For example, it was found that electrical field stimulation could affect cardiac differentiation and reactive oxygen species (ROS) generation in hESC-derived EBs [161]. Hopefully, with time, such techniques will allow for the development of ESC-based tissue-engineering applications. The design of bioreactors that control the spatial and temporal signaling that induce ESC differentiation requires further collaborative efforts between engineers and biologists.

Microfluidic systems can be also used to investigate the effect of growth factor and chemical environments on stem cell differentiation in a high-throughput manner. For example, a microfluidic device was developed to generate a concentration gradient of growth factors for

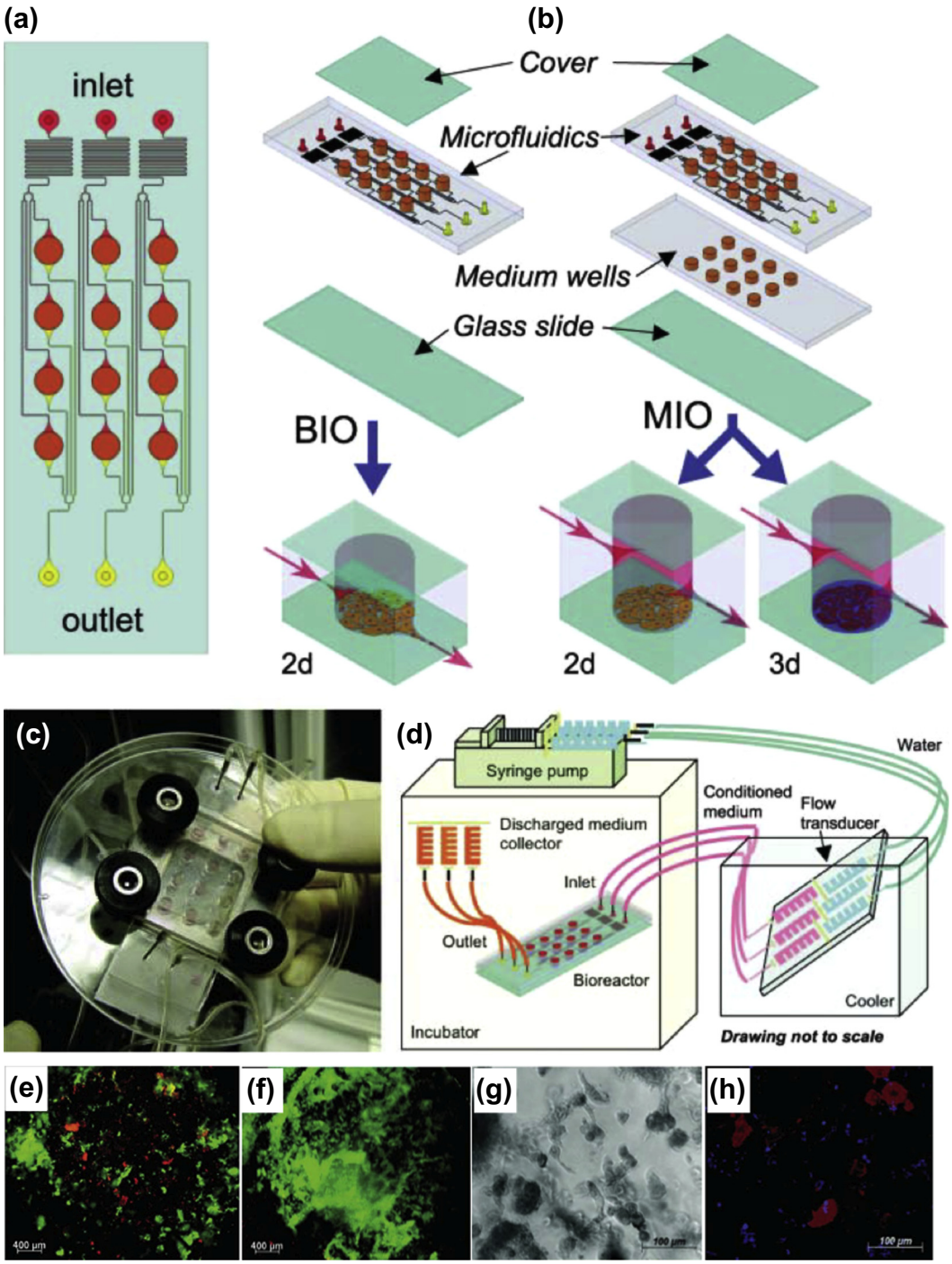

FIGURE 32.7

Microarray bioreactors. (a) The micro-bioreactor wells with 3.5 mm in diameter are arranged in an array. The medium (red) is delivered using three inlets through the flow transducers to four wells (orange) via microfluidic channels (100 mm wide) and waste medium removes from each bioreactor through a separate set of channels (yellow). (b) Two configurations were used: a bottom inlet/outlet (BIO) configuration, and a middle inlet/outlet (MIO) configuration (right) that allows for 3D cultivation. (c) Image of a single MBA with compression frame and fluidic connections. (d) Experimental setup. MBAs and medium collectors are placed in an incubator, and the medium reservoirs are maintained external to the incubator in an ice bath; (e—f) Representative images of hESCs on day 4 of culture (e) without and (f) with perfusion of culture medium (live cells are red and deal cells are green); (g) bright field image of differentiated hESCs, demonstrating that hVEGF addition to culture media resulted in hESC sprouting and elongation outside the colonies. (h) Confocal image of vascular differentiated hESC indicating the expression of a-SMA (shown in red). *(Adapted from reference [55].)*

optimizing the proliferation and differentiation of stem cells. The developed platform enabled rapid optimization of media compositions by exposing the cells to a continuous gradient of various growth factors within the microfluidic environment to induce proliferation and differentiation in a graded and proportional manner, depending on growth factor concentration [162]. In another study, micro-bioreactor arrays (MBAs) system composed of a microfluidic platform and an array of micro-bioreactors was designed to investigate the effect of culture microenvironments on hESCs differentiation both in 2D and 3D culture conditions [55,163] (Fig. 32.7a–d). Medium perfusion promoted the viability of encapsulated hESCs within hydrogels (67% viability in perfused culture compared to 55% in static culture) (Fig. 32.7e,f). In addition, using this system, it was possible to induce the vascular differentiation of hESCs through the addition of vascular growth factor (hVEGF) to the culture media [163] (Fig. 32.7g,h).

ISOLATION OF SPECIFIC PROGENITOR CELLS FROM ESCs

Although hESCs can generate specific functional cell types from all three germ layers, it is typically not possible to directly differentiate the cells in culture and obtain pure cell populations. Isolation of a specific differentiated population of cells for transplantation will eliminate the presence of undifferentiated hESCs which have tumorigenic potential, and allow for efficient use of the various cell populations for therapeutic purposes. With the exception of few cases where the enrichment of cells of interest was almost fully achieved [83,88,90], the protocols adopted for the differentiation of hESCs do not yield pure cell populations. Therefore, there is a need for suitable techniques to isolate desired cells from heterogeneous cell populations (Table 32.1). One approach for achieving this is to isolate specific cells by using cell surface markers and FACS. In this case, the initial population of cells is immunostained by a single or a combination of different markers, and the desired cell type is isolated by FACS. Part of the initial population of cells is also labeled with isotype controls to gate the populations. The use of FACS yields a pure population of cells, and allows one to select cells using different markers [113], but the limitations of this technique may hamper the final cell survival.

Magnetic immunoselection has been used very often to isolate specific differentiated cells [84,114,164]. Initially, the cells are labeled with relevant cell surface antibodies conjugated with magnetic beads. The magnetically labeled cells are then separated from the other ones by a magnetic column, to purities that are generally higher than 80% [164]. Although these purities are slightly lower than the ones obtained by FACS, the magnetic selection is less harmful to the cells than FACS. Recently, different surface markers specific for cardiomyocytes have been identified: Emilin2 [165] and later SIRPA [166,167] and VCAM [167,168]. These findings allow the prospective isolation of live cardiomyocytes, from ESCs or iPSCs-differentiated mixed cardiac population, with purities above 95%.

Another potential method for cell isolation is through reporter gene knock-in modifications [169]. For example, to trace hepatic-like cells during differentiation of hESCs in culture, a reporter gene expressed under the control of a liver-specific promoter was used [169]. For that purpose, hESCs underwent stable transfection with eGFP fused to the albumin minimal promoter sequence. This methodology allowed one to follow the differentiation pattern of hESCs into hepatic-like cells and to isolate those cells by FACS using the fluorescence of eGFP. Similarly, hESCs genetically manipulated to carry the Nkx2.5-eGFP reporter construct allow the isolation of cardiac cells [167]. Since Nkx2.5 is an early cardiac transcription factor, it allows the identification and isolation of early cardiac progenitors.

Isolation of a specific differentiated population of cells may also be accomplished by mechanical/enzymatic separation of cells exhibiting specific morphology, functional activity or adhesion to a substrate. For example, cardiomyocytes have been isolated by dissecting contracting areas in embryoid bodies and dissociating those areas using collagenase [170].

TABLE 32.1 Summary of methodologies to enrich specific lineages from hES cells

Cell type	Methodology followed to enrich specific lineages	Cell lines	Reference
Cardiomyocytes	Flow activated cell sorting	hES2	[165]
Cardiomyocytes	Flow activated cell sorting, Magnetic immunoselection	hES2, 3	[166]
Cardiomyocytes	Introduction of a reporter gene and cell selection by flow activated cell sorting	hES3Nkx2.5^{eGFP}	[167]
Cardiomyocytes	Flow activated cell sorting	KhES1	[168]
Cardiomyocytes	Flow activated cell sorting	cmESC, KhESC1, 2, 3	[171]
Cardiomyocytes	Flow activated cell sorting	hES2	[165]
Cardiomyocytes	Discontinuous percoll gradient	H1,H7,H9	[117]
Cardiomyocytes	Enzymatic and mechanical dissociation	N/A	[170]
Cardiomyocytes	Enzymatic dissociation	HES2	[116]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H1.1, H9.2	[114]
Hematopoietic progenitor cells	Flow activated cell sorting	H1, H9	[206]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H9	[164]
Leucocytes	Selective adhesion of cells	H1	[207]
Endothelial cells	Flow activated cell sorting	H9	[111]
Endothelial-like cells	Flow activated cell sorting	H1, H9	[113]
9 Ectoderm			
Neurons and glia	Magnetic immunoselection	H1, H7, H9	[84]
Neurons and glia	Enzymatic dissociation and selective adhesion of cells	H1, H9, H9.2	[85]
Oligodendrocytes	Selective adhesion of cells	H7	[208]
10 Endoderm			
Hepatocyte-like cells	Introduction of a reporter gene and cell selection by flow activated cell sorting	N/A	[169]

Oligodendroglial cells were isolated from stem cell aggregates that adhered to a specific substrate [90]. In addition, neuroepithelial cells were isolated from embryoid bodies attached to a tissue culture-treated flask by using dispase [85], an enzyme that selectively detached neuroepithelial islands from the embryoid bodies, leaving the surrounding cells adhering. It is also possible to take advantage of the cell body content of specific cell types. For example, cardiomyocytes have a higher mitochondrial density than their progenitors and other cardiovascular cells, such as smooth muscle and endothelial cells. It is thus possible to isolate cardiomyocytes by flow cytometric sorting using the mitochondrial dye tetramethylrhodamine methyl ester percholate without genetic modification or surface antigen staining [171].

TRANSPLANTATION

The first application of stem cells as a cellular replacement therapy is associated with bone marrow transplantation and blood transfusion in which donor hematopoietic stem cells repopulate the host's blood cells [172]. Today, modalities are being developed for cell-based therapies of numerous diseases, including diabetes, Parkinson's disease, spinal cord injury, liver failure, muscular dystrophy, bone and cardiovascular disease, among others. Despite the advances in the development of disease models [173], only a few studies have reported the *in vivo* functionality of hESC-derived cells. In most cases, the cells are injected into a disease area and their functionality is evaluated by immunohistochemistry and functional tests. Using such methods, partial functional recovery of a mouse model of Parkinson's disease after hESC-derived neural progenitor cells has been reported [88]. Also, transplantation of hESC-derived oligodendroglial progenitor cells into the *shiverer* model of dysmyelination resulted in myelin formation [174].

Studies of neural regeneration in animal models have given very promising results [175–177]. In particular, hESC-derived oligodendrocytes have been shown to repair injured spinal cord in animal models with rebuilding myelin sheets [178]. Based on this system, in 2010 Geron started the first clinical trial for the treatment of patients with spinal cord injury. In the same year, a clinical trial also started for the treatment of the Stargardt's macular dystrophy [179], a pathology characterized by the death of photoreceptor cells in the central part of the retina (called the macula). This trial was based on the promising observation that hESCs are able to differentiate into RPEs (retinal pigmented cells) [180].

A new important step towards the clinical application of hESCs for infarct therapy is the very recent finding that hESC-derived cardiomyocytes electrically couple and are protective against arrhythmias in the recipient heart when transplanted into guinea-pigs [181], an animal model with a much closer heart physiology to humans than that of rodents.

Despite the ability of stem cells to differentiate into cells with desired phenotypic and morphological properties, there has been very few scaffold-based tissue-engineering studies that use ESCs, by differentiating these cells in culture, selecting desired cell types and seeding these into scaffolds. Ideally, scaffolds provide cells with a suitable growth environment, facilitated transport of oxygen and nutrients, mechanical integrity and suitable degradation. The scaffold brings the cells into close proximity and thereby enhances the formation of tissue structures.

Tissue-engineering scaffolds are comprised of either synthetic or natural materials, or a composite of the two. Scaffolds are commonly made of synthetic materials such as hydroxyapatite, calcium carbonate, PLA, poly(glycolic acid) (PGA), PLGA, poly(propylene fumarate), and natural materials such as collagen, Matrigel or alginate. Natural materials typically have better biocompatibility, while synthetic materials provide better control of various properties such as degradation rate, biomechanics, and structure [7]. hESC-derived endothelial progenitors were seeded onto highly porous PLGA biodegradable polymer scaffolds to form blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice. These endothelial progenitor cells were also able to support the formation of vascularized skeletal muscle [182]. Osteoblast-like cells derived from hESCs were also transplanted into an animal model by using a poly(D,L-lactide) scaffold. After 35 days, regions of mineralized tissue could be identified within the scaffold by Von Kossa staining and expression of human osteocalcin [123]. For cardiac tissue engineering, synthetic materials were used in the form of injectable hydrogels and surfaces that can be treated to get detached cardiomyocyte layers [183,184].

Transplantation and immune response

One of the major obstacles for successful transplantation of hESC-derived differentiated cells is their potential immunogeneity. As long-term immunosuppressive therapy would limit clinical applications, the creation of immunologic tolerance would enable stem cell-derived therapy. Methods currently under development include:

1) The establishment of hESC line banks large enough to represent the majority of tissue types;
2) Nuclear reprogramming of the cells to carry patient-specific nuclear genome (therapeutic cloning);
3) Creation of 'universal cells' by manipulating the major histocompatibility complex (MHC) [185];
4) Deletion of genes for immune response proteins using homologous recombination (as mentioned above); and
5) The generation of hematopoietic chimerism, to create the required tolerance for tissues or cells derived from it [186].

The latter method was demonstrated using rat embryonic-like stem cells that permanently engrafted when injected into full MHC mismatched rats [187].

Although the rejection of ESC-derived tissues is triggered by minor histocompatibility antigens, simple host conditioning with monoclonal antibodies against CD4 and CD8 could be sufficient to induce transplantation tolerance of ESC-derived donor tissue, but not of primary animal tissue [188]. It was recently observed that hESCs-derived mesenchymal progenitors have strong immunosuppressive properties resulting, similar to bone marrow mesenchymal stem cells, in inhibiting CD4+ or CD8+ lymphocyte proliferation and being more resistant to natural killer cells [189].

As an alternative to the novel approach of iPSC generation for autologous transplantation or disease-specific drug screening, therapeutic cloning or somatic cell nuclear transfer (SCNT), the process through which Dolly the sheep was cloned in 1997, might be an important tool to create hESCs from patient-specific genome, and thus preventing immunorejection [190] (Fig. 32.8).

This is important for the application of hESCs in tissue engineering, where transplantable populations of cells can be generated with genes that are derived only from the patient. Studies to date have demonstrated that cells derived by SCNT can be expanded in culture and will organize into tissue structures if transplantated with biodegradable scaffolds. However, before SCNT research can be translated into human therapies, the reliability of the overall process needs to be improved, including prevention of the alterations in gene expression.

Immunoisolation systems may help overcome the problems with the immunological incompatibility of the tissue. Thus, immunoisolation of cells may prove to be particularly useful in conjunction with ESCs to overcome the immunological barrier associated with the ESC-based therapies. Cells may be immobilized within semi-permeable polymeric matrices that provide a barrier to the immunological components of the host. Membranes can be designed to be permeable to nutrients and oxygen while providing a barrier to immune cells, antibodies and other components of the immune system, by adjusting the cutoff size of membrane pores [191,192]. Within these systems, the engineered tissues can either be implanted or used as extra-corporeal devices. Such closed tissue-engineering systems have been used for the treatment of diabetes [193–195], liver failure [196–198], and Parkinson's disease [199–202]. For example, ESC-derived β-cells that can respond to insulin or dopamine-producing neurons can be used in clinics without rejection. In addition, closed systems can protect the host against potentially tumorigenic cells.

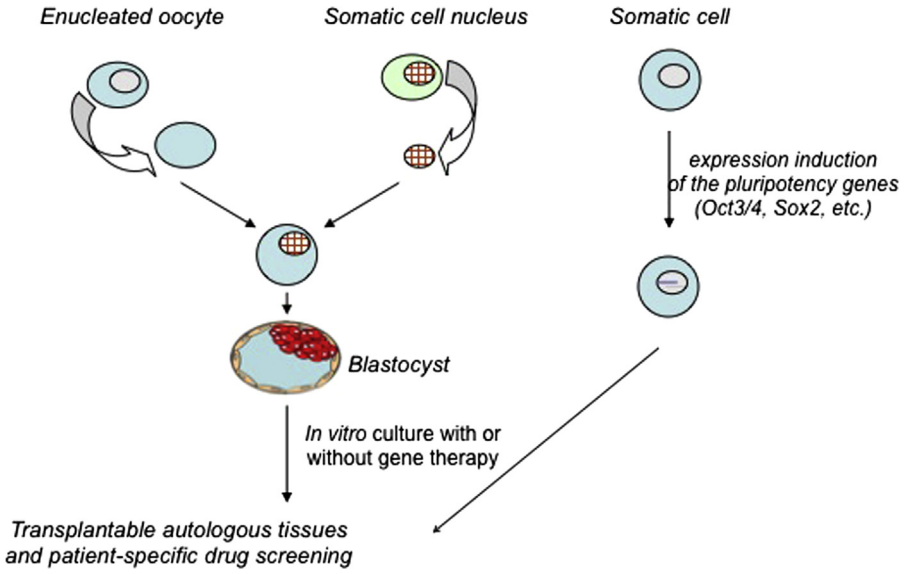

FIGURE 32.8
Schematic diagram of therapeutic cloning.
A somatic cell nucleus is transferred into enucleated oocyte to form a cell capable of giving rise to a blastocyst, which can be used to derive therapeutic cells. Alternatively, somatic cells are reprogrammed into iPS cells that can also be differentiated into therapeutic cells. In both cases, the resulting cells are used to engineer tissues for regenerative medicine and drug screening applications.

Currently, engineering and biological limitations such as material biocompatibility, molecular weight cutoff and immune system reaction to shed antigens by the transplanted cells are some of the challenges that prevent these systems from widespread clinical applications.

FUTURE PROSPECTS

Despite significant progress in the field of tissue engineering and ESC biology, there are a number of challenges that provide a barrier to the use of ESCs for tissue engineering. These challenges range from understanding cues that direct stem cell fate to engineering challenges on scale-up, to business questions of feasibility and pricing.

Although the derivation of hESCs from the ICM of preimplantation blastocysts has become a standard procedure and has been performed in a variety of laboratories, live human embryos must be destroyed in the process, which is ethically unacceptable. However, recent reports show that embryonic stem cells can be isolated without destroying blastocysts [203]. The generation and use of iPSCs require no embryo at all, overcoming the ethical issues associated with ESCs.

Since 2001, federal funding can be used for research using the existing 60 lines of embryonic stem cells, but not for the creation of new cell lines, even from surplus embryos normally discarded in fertility clinics. The existing federally approved lines are not adequate for human therapies, as they have been prepared using mouse cells and thus pose a risk of contamination. Major advancements since 2001 have established methods to culture hESCs without mouse feeder layers. It has recently been proposed that a common ground for pursuing hESC research may exist through assessing the death of a human embryo in the ethical context surrounding organ donation. Specifically, Landry and Zucker argue that a significant fraction of embryos generated for *in vitro* fertilization undergo irreversible arrest of cell division and thus can be considered as organismically dead, yet can still be used to harvest cells [204]. Donation of these embryos could ethically be considered analogous to the donation of essential organs from cadavers. Although criteria for determining the irreversible arrest of cell division have yet to be defined, it will certainly be interesting to see if these theories can be experimentally established and how these arguments will fare with those who currently oppose hESC research. In support of the therapeutic promises held by hESCs, in 2009 the limitations of the use of federal funding for research on hESCs have been reduced, encouraging research in this field.

Stem cells and their progeny reside in a dynamic environment during development, thus a scaffold should be designed to mimic the signaling and structural elements in the developing embryo. The use of 'smart' scaffolds that release particular factors and/or control the temporal expression of various molecules released from the polymer can help induce differentiation of ESC [205]. For example, by dual delivery of vascular endothelial growth factor (VEGF)-165 and PDGF, each with distinct kinetics, and from a single polymer scaffold, resulted in the formation of stable vascular networks [205]. An alternative approach to modifying the surface exposed to the cells is to immobilize desired ligands onto the scaffold. For example, RGD peptides, the adherent domain of fibronectin, can be incorporated into polymers to provide anchorage for adherent cells.

Another difficulty with the currently used materials is limited control over the spatial organization of the scaffold. Spatial patterning is necessary to create tissues that resemble the natural structure of biological tissues. In the direct cell patterning system, cells can be seeded into the scaffold at particular regions within the cells. For example, the direct attachment of two different cell types in different regions of the scaffold has been used to generate cells of the bladder. Cell patterning was critical for the effective co-culture of hepatocytes and fibroblasts.

CONCLUSIONS

A number of challenges are still ahead of us before ESC-based therapy can become clinically viable. These include directing the differentiation of ESCs (i.e., using controlled microenvironments or genetic engineering), ensuring their safety (i.e., by eliminating tumorogenicity), functionally integrating differentiated cells into the body, achieving long-term immune compatibility, and improving the cost and feasibility of cell-based therapies. Each of these challenges is currently being addressed. In particular, since ESCs can give rise to many different cell types, solving these challenges for the various possible tissue types will be a major undertaking. Further research is required to control and direct the differentiation of ESCs, in parallel with developing methods to generate tissues of various organs, to realize the ultimate goals of tissue engineering. We might be getting close to a day when ESCs can be manipulated in culture to produce fully differentiated cells that can be used to repair specific organs. Clearly, our ability to overcome these difficulties is not confined within any single scientific discipline but rather involves an interdisciplinary approach. Solving these challenges could lead improved quality of life for a variety of patients that could benefit from tissue-engineering approaches.

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CHAPTER 33

Postnatal Stem Cells in Tissue Engineering

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INTRODUCTION

In its first inception, tissue engineering was based on the use of natural or synthetic scaffolds seeded with organ-specific cells *ex vivo*. This approach was somewhat distinct from guided tissue regeneration, which utilized scaffolds and/or bioactive factors to encourage local cells to repair a defect *in situ*. These two approaches are now merged in the current field of tissue engineering, which encompasses multiple and diverse disciplines to use cells, biomaterials and bioactive factors in various combinations to restore and even improve tissue structure and function. Stem cell-based tissue engineering represents a major turn in the conceptual approach to reconstructing tissues. By expanding the repertoire of available cells, identifying potential targets, developing the technological means of generating functional tissues *ex vivo*, and above all, by making it possible (or promising) to engineer tissues that otherwise would not be amenable to recreation, advances in stem cell biology have a profound impact on tissue engineering at large.

In many cases, tissue engineering seems to have more use for postnatal stem cells than nature itself. This is especially apparent in the case of postnatal stem cells derived from tissues with low turnover, or no apparent turnover at all. If neural stem cells were able to repair the loss of dopaminergic neurons in the intact brain *in vivo*, there would be no Parkinson's disease for which to envision stem cell-based therapies. Dental pulp stem cells do not regenerate primary dentin *in vivo*, but vast numbers of cells can be made from the pulp of a single extracted tooth to generate copious amounts of new dentin. Current definitions of stem cells, in fact, include a technological dimension in many cases (a cell that can be expanded *ex vivo* and bent to the generation of differentiating cells). Importantly, the two neighboring fields of cell therapy (reconstruction of functional tissues *in vivo* using cells) and tissue engineering proper (reconstruction of functional tissues using cells and something else) merge significantly once a stem cell angle is adopted for either, not only with respect to the ultimate goals, but also to several biotechnological aspects.

The need for postnatal stem cells in tissue engineering

Given the fact that differentiated cells and their immediate progenitors can be used to successfully recreate a tissue in many instances, one may wonder why postnatal stem cells are such

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an important ingredient in tissue engineering. The answer lies in the need for tissue turnover. Although not a tissue-engineering approach *per se*, bone marrow transplantation provides the best example for the requirement of postnatal stem cells for the restoration of tissue homeostasis. While lethally irradiated recipients may survive for short periods of time when transplanted with different populations of hematopoietic cells, even those containing hematopoietic progenitor cells, the recipient will succumb if the true hematopoietic stem cell (HSC) is not provided, due to the rapid turnover of blood [1]. This is also the case, not only for tissues with rapid rates of turnover such as in the skin and the gastrointestinal tract, but also in tissues with slower rates such as bone and muscle, and for tissues with very low rates, such as in the pancreas and kidney. In reconstructing organs and tissues, if the postnatal stem cell is consumed, the construct will ultimately fail. That is not to say, however, that tissue-engineering approaches using more mature cells populations for tissues with very low rates of turnover are not useful, but rather to highlight the need for recognition of tissue dynamics when devising cell-based tissue-engineering approaches.

THE RESERVOIRS OF POSTNATAL STEM CELLS

During development, the inner cell mass of the blastocyst undergoes gastrulation, and becomes specified into the three distinct germ layers; ectoderm, mesoderm and endoderm (Fig. 33.1). Each of these embryonic germ layers contain fetal stem cells that become more and

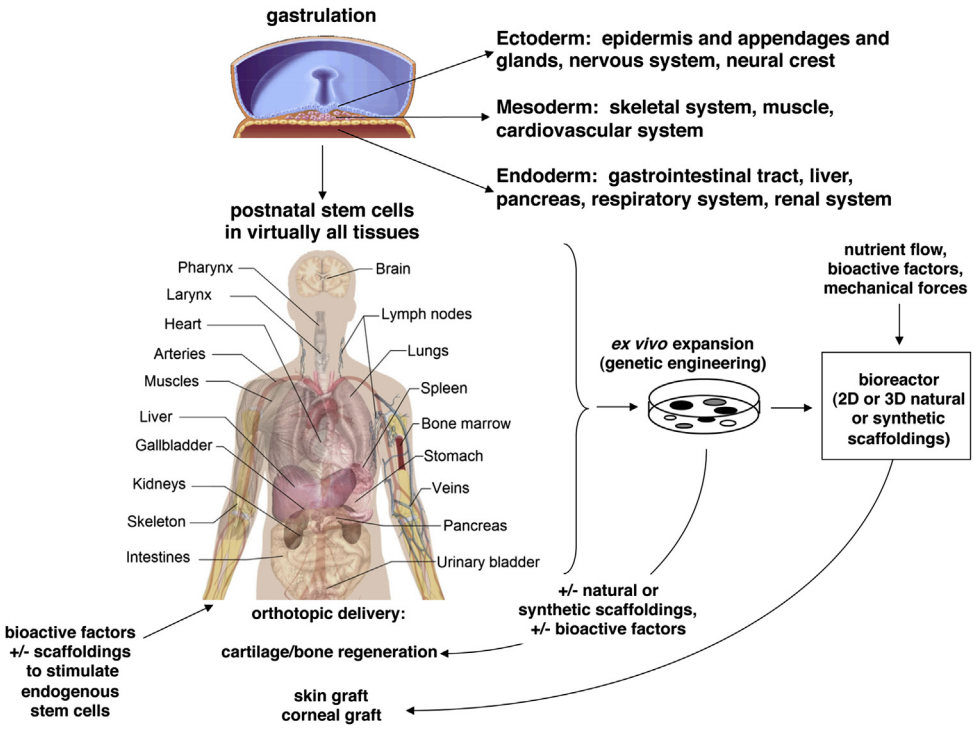

FIGURE 33.1

Current applications of postnatal stem cells in tissue engineering. During gastrulation, the three embryonic germ layers are formed that give rise to organ and tissue-specific stem cells. Virtually all tissues in the postnatal body have cells with some regenerative capabilities that can be potentially utilized in tissue engineering. For approaches other than hematopoietic cell transplantation, cell populations need to be expanded *in vitro* to obtain sufficient numbers of cells, and are delivered orthotopically in various combinations with bioactive factors and scaffolds. Generation of 3D structures *ex vivo* prior to transplantation requires the use of bioreactors in which cells are seeded onto scaffoldings and subjected to nutrient flow, bioactive factors and mechanical forces to induce formation of functional tissue for transplantation. In the future, it may be possible to stimulate endogenous local stem cells to repair damaged tissue, perhaps in combination of growth factors and scaffolds to guide regeneration.

more specified as embryonic development progresses. Even beyond embryonic development, it is now evident that virtually every tissue contains some kind of a postnatal stem/progenitor cell. How these tissue-specific stem/progenitors are maintained, or whether they are established *de novo*, in the postnatal organism, is not well understood at this time [2]. Clearly, however, an understanding of the developmental process of tissue specification is an essential point in devising tissue-engineering approaches.

Current approaches to stem cell-based tissue engineering rely primarily on the use of postnatal stem cells (Fig. 33.1), although new approaches utilizing pluripotent stem cells [human induced pluripotent stem cells (iPSCs)] may offer an alternative if hurdles such as genetic stability and strict differentiation can be overcome. First identified in constantly (and rapidly) self-renewing tissues such as blood (mesoderm origin), skin (ectoderm origin) and the gastrointestinal tract (endoderm origin), the repertoire of postnatal stem cells has expanded to include perhaps every single tissue in the body (Fig. 33.1), regardless of its rate of tissue turnover or ability to regenerate (reviewed in reference [3]). Not all of these tissue-specific postnatal stem cells, however, are equally accessible for safe harvest, or available in sufficient quantity (or amenable for *ex vivo* expansion) to generate the number of cells needed for tissue regeneration. However, lessons on the dynamics of tissue homeostasis (growth and turnover) that can be learned from these cells have an obvious impact on the design of future tissue-engineering strategies nonetheless. In addition, mechanisms whereby postnatal cells maintain differentiated functions in tissues and organs are relevant to future pluripotent stem cell-based approaches, and can only be learned from postnatal cells [4].

Mesodermally-derived postnatal stem cells

BONE MARROW — A SOURCE OF MULTIPLE POSTNATAL STEM CELLS

It has long been known that bone marrow is the home of at least two different types of stem cells, the HSC (reviewed in reference [5]), and the skeletal stem cell (SSC, also known as the bone marrow stromal stem cell, or 'mesenchymal' stem cell, ([reviewed in references [6] and [7])); each able to reconstitute the hematopoietic and skeletal system, respectively. AC133 (CD133) positive endothelial progenitors are also a marrow component [8], although they are somewhat different from endothelial precursors of non-marrow origins [9] (Fig. 33.2).

TRANSDIFFERENTIATION — A THING OF THE PAST

It was initially thought that bone marrow cells (either hematopoietic or non-hematopoietic) could differentiate into other cells types, based on studies that infused these cells systemically.

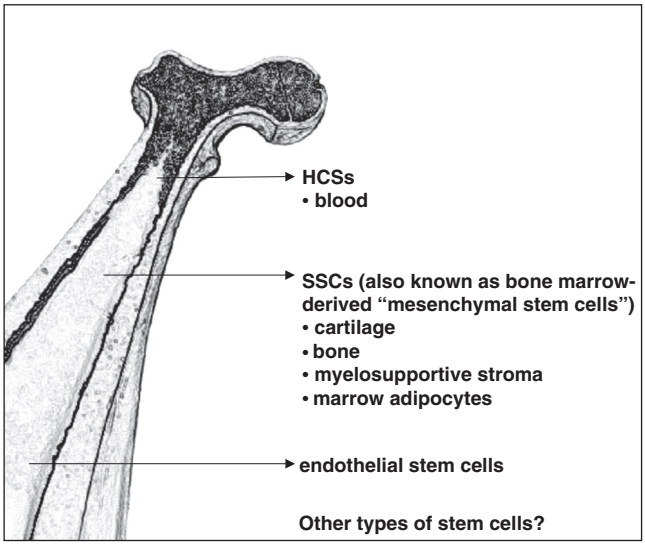

FIGURE 33.2
Bone marrow as a central source of postnatal stem cells. Bone marrow consists of at least two well defined populations of postnatal stem cells, the hematopoietic stem cell (HSC) and the skeletal stem cell (SSC), both of which form numerous phenotypes within their cellular system, but may also form cells outside of them. Endothelial precursors ($AC133^{+}$) have been identified, although they may differ from endothelial precursors from non-marrow sources. The presence of three different types of stem cells place bone marrow high on the list of tissues that are easily accessible and able to be harvested in sufficient quantity for use in tissue engineering.

Circulating, marrow-derived cells contributed to the regeneration of skeletal muscle in response to injury [10], and in mouse models of muscular dystrophy [11,12]. Donor-derived cells have also been detected in neuronal tissue, newly formed vasculature, in the kidney, and even in the oral cavity following bone marrow or mobilized peripheral blood transplantation (reviewed in reference [13]). The vast majority of these studies relied on the expression of a few markers, and did not convincing demonstrate functionality of the 'transdifferentiated' cells [4]. In some cases, formation of a differentiated cell of donor origin could be attributed to cell fusion with endogenous cells [14], which is a natural process that occurs in some tissues such as in the liver and in muscle. Nonetheless, bone marrow is a unique source for two of the most commonly used postnatal stem cells: the hematopoietic stem cell (discussed elsewhere in this volume) and the bone marrow stromal (skeletal) stem cell (described in more detail below).

STROMAL STEM/PROGENITOR CELLS (ALSO KNOWN AS 'MESENCHYMAL STEM CELLS')

The first concept of a stromal stem/progenitor cell emerged from the work of Friedenstein and Owen, based on the inherent skeletal properties of bone marrow stromal cells (BMSCs) and their subpopulation of stem cells, and was limited to the skeletal system. Upon *in vivo* transplantation with an appropriate scaffold in an open system, the progeny of a single cell, the Colony Forming Unit-Fibroblast (CFU-F), formed bone, hematopoiesis-supportive stroma and marrow adipocytes, and formed cartilage when transplanted in closed systems, or in cell pellet cultures *in vitro* (reviewed in reference [15]). Based on their mesodermal origin (except for in the facial bones, which derive from neural crest), it was later envisioned that BMSCs would not only regenerate skeletal cell types, but also other mesodermal derivatives such as muscle, tendon, ligament, etc., through the so-called 'mesengenic process'. This was originally modeled on BMSCs, which were then renamed 'mesenchymal stem cells' (MSCs) [16]. However, during development, there is no common mesodermal (or mesenchymal) cell even for bone formation [17], let alone for tendon, muscle, or other connective tissues. Nonetheless, reports emerged suggesting that BMSCs have the ability to differentiate into skeletal muscle, cardiomyoctes, tendons, etc. (reviewed in reference [18]), but usually needing extensive chemical modification of culture conditions, expression of a limited number of markers of the differentiated phenotype, and less than rigorous assays demonstrating their functionality [19].

In attempts to identify markers that could effectively isolate these multipotent bone marrow-derived 'MSCs', their cell surface was intensively investigated. Subsequently, BMSCs were described as being CD29, CD44, CD71, CD90, CD106, CD120a, CD124 positive (and Stro-1 positive in freshly isolated cells) (reviewed in reference [19]). However, none of these markers are specific for BMSCs, or for the SSC subset within the BMSC population. The majority of these markers are expressed by many connective tissue cell populations, whether or not they have been determined to contain a subset of stem cells by rigorous criteria (the ability of the progeny of a single cell to reform a tissue and to self-renew). Furthermore, it is well known that the pattern of expression of many of these markers change, depending on the culture conditions and length of time in culture [19].

Because of the lack of specificity of these markers, studies rapidly appeared describing the isolation of 'MSCs' from a long list of non-skeletal tissues. In addition, it became a mainstay to compare the differentiation potential of these non-skeletal 'MSCs' to that of BMSCs by using *in vitro* differentiation osteogenic, adipogenic and chondrogenic assays and expression of mRNAs representative of differentiated cell types [20], instead of by appropriate *in vivo* transplantation assays (e.g., as in reference [21]). However, current *in vitro* assays cannot be used to reliably predict the inherent osteogenic and adipogenic potential of a cell population, and chondrogenic differentiation is often not convincing [19]. In an attempt to clarify the nature of 'MSCs', a 'recommendation' was made that the name 'mesenchymal stromal cell' be used (due to the lack of convincing data of stemness in many cases) for cells that are: 1) adherent in

culture, 2) express CD105, CD73 and CD90, but lack expression of CD45, CD34, CD14, CD11b, CD79a and HLA-DR markers, and 3) able to differentiate into osteoblastic, adipogenic and chondrogenic cells *in vitro* [22]. Unfortunately, due to the widespread use (and abuse) of this definition, this recommendation has done little to clarify the nature of connective tissue stem/progenitor cells, to the extent that it is now commonly thought that 'MSCs' are ubiquitous [23], and are equivalent in their differentiation properties [24], in spite of the lack of rigorous analyses. In essence, the over-emphasis on these artificial assays has overshadowed efforts to reveal the inherent differentiation potential of non-skeletal connective tissue stem/progenitor cells.

In addition, based on the intense search for stem cell sources that would avoid the ethical and biological issues (use of allogeneic cells) associated with hESCs, the idea became prevalent that BMSCs (and other 'MSCs') are pluripotent [25,26]. This new twist emerged from early studies that aimed to treat generalized disorders of the skeleton and other mesodermal tissues by systemic infusion or direct orthotopic injection of BMSCs. What followed were suggestions that 'MSCs' were able to 'transdifferentiate' into cell types outside of the mesodermal lineage, such as neuronal cell types, hepatocytes, insulin-producing cells, etc. (reviewed in references [18,27]). However, it is now recognized that 'transdifferentiation' of BMSCs and other postnatal stromal cells is a rare event, if it occurs at all [19]. Furthermore, it is becoming increasing clear that 'MSCs' are tissue specific. Upon *in vivo* transplantation, BMSCs from bone support the formation of a hematopoietic marrow [21], whereas dental pulp 'MSCs' form dentin and a pulp-like complex, and do not support the formation of a hematopoietic marrow [28]. Yet while the notion of transdifferentiation outside of a specific lineage is no longer considered to be scientifically sound, numerous papers continue to be published suggesting the use of cells from one type of tissue to form another (e.g., adipose-derived stromal cells to form bone [29]). This is not to be confused with current efforts in directed reprogramming (e.g., skin fibroblasts into chondrocytes [30]), whereby cells are partially reprogrammed through the introduction of certain transcription factors, but without going back to a completely pluripotent state. Clearly, there is an urgent need for rigorous assessment of the differentiation capacities of different populations of postnatal connective tissue stem/progenitor cells, in order for tissue engineering efforts to progress into a clinical reality.

PERICYTES

The identity of local connective tissue stem/progenitors has long been sought, but the lack of specific markers of a stem/progenitor cell in any connective tissue has made this a challenging task. However, recent studies suggest that pericytes, cells located on the extravascular surface of blood vessels, may be the source of tissue-specific connective tissue stem/progenitors that emerge through what might be a common developmental process [19]. As an example, during development of bone marrow, blood vessels gain access to what will become the medullary cavity by invading through a layer of committed osteogenic cells [31]. Due to the association of the osteogenic cells with endothelial cells, they change not only their morphology, but also cease to synthesize and deposit bone matrix, and proliferate along with the developing blood vessel. These formerly osteogenic cells, now pericytic cells, further proliferate to establish the bone marrow stroma upon which hematopoiesis occurs [32]. It has been demonstrated that CD146 (Muc18/MCAM) is a useful marker for isolating pericytes from bone marrow [33]. Prospective sorting for $CD45^{-}/CD31^{-}/CD146^{+}$ cells isolated all of the CFU-Fs in bone marrow, a subset (1:5) of which were found to be multipotent by *in vivo* transplantation. Of note, these cells were positive for all of the other markers that have been associated with 'MSCs', making them connective tissue cells, but of a different flavor: $CD146^{+}$ pericytes [33]. Studies in other connective tissues have also alluded to the pericytic nature of the local/stem progenitor cell, such as in dental pulp and in adipose tissue based on Stro1 and CD146 expression [34,35], and in muscle based on alkaline phosphatase and CD146 expression [36]. Subsequently, it was speculated that 'MSCs' from all tissues are pericytes [24,37]. However, it is

yet to be determined in a systematic fashion if, indeed, all pericytes are the source of local stem/progenitor cells in all connective tissues. The developmental origin of pericytes varies from tissue to tissue, and their biology is complex and not well understood to date [38,39]. In support of the idea that pericytes are committed to a particular lineage, it can be noted that transcriptome analysis of 'MSCs' isolated from different tissues do in fact display their inherent differentiation preference; e.g., undifferentiated BMSCs express low levels of osteogenic transcription factors and bone matrix proteins [40], and undifferentiated adipose-derived stromal cells express low levels of adipogenic transcription factors [41].

Ectodermally-derived postnatal stem cells

Embryonic ectoderm gives rise to the dermis and its appendages (hair follicles, mammary glands and other types of subcutaneous glands), to the nervous system, and to neural crest cells that contribute to the development of multiple tissues (Fig. 34.2). There are numerous examples of the isolation and characterization of postnatal stem cells derived from all of these. In particular, the epidermis has long been known to contain a stem cell within the basal layer of the epidermis, and also within the bulge region of hair follicles (reviewed in reference [42]). As discussed below, these and other ectodermally-derived postnatal stem cells, in particular, limbal stem cells, are under intensive investigation for their potential use in tissue engineering. Other ectodermally-derived tissues, such as mammary gland and the nervous system, are well known to contain stem cells (reviewed in references [43,44], respectively), as discussed elsewhere in this volume.

Endodermally-derived postnatal stem cells

The gastrointestinal tract, and all derivatives thereof, including the liver and prancreas, the respiratory system, and the renal system are derived from embryonic endoderm (Fig. 33.1). More specific discussions of these types of stem cells and their use in tissue engineering are included elsewhere in this volume, but the reader is referred to recent review articles for more information on intestinal stem cells [45], liver stem cells [46,47], potential pancreatic stem cells an area that is hotly debated [48], lung stem cell [49] and renal stem cells [50].

CURRENT APPROACHES TO TISSUE ENGINEERING USING POSTNATAL STEM CELLS

Approaches for the regeneration of functional tissue using non-hematopoietic postnatal stem cells can be envisioned by three different scenarios:

A) Expansion of a population *ex vivo* prior to transplantation into the host (with or without an appropriate carrier);
B) Recreation of a tissue or organ *ex vivo* for transplantation;
C) Design of substances and/or devices for *in vivo* activation of stem cells, either local or distant, to induce appropriate tissue repair (Fig. 33.1).

In all of these cases, considerable knowledge of the stem cell population's dynamics is required in order to predict and control their activity under a variety of different circumstances.

Ex vivo culture of postnatal stem cells

Ex vivo expansion of tissue or organ-specific postnatal cells, used either alone or added to carriers and scaffoldings or with growth factors, at the time of transplantation has been the primary approach in tissue engineering to date. However, *ex vivo* expansion in a fashion that maintains an appropriate proportion of stem cells within the population is a significant hurdle that must be overcome. For example, in spite of enormous efforts, the culture conditions for maintaining HSCs (let alone expanding their number) are as yet undefined. It is perhaps for this very reason that currently there are only a handful of examples in which *ex vivo* expanded

postnatal stem cells are used successfully to restore structure and function (e.g., for skin and corneal regeneration). The key to successful expansion will lie in understanding cell proliferation kinetics (asymmetric versus symmetric division) [51,52]. The efficacy achieved by the use of *ex vivo* expanded populations, whether stem or more committed in character, may also depend, at least in part, on the nature of the tissue under reconstruction. As mentioned above, the rate of tissue turnover most likely defines the rate of success.

While optimizing culture conditions represents one hurdle, the issues of time and quantities represent others. In most cases, the amount of time required to generate the number of cells sufficient to repair defects induced by trauma or disease is in the order of weeks. In cases of trauma, this poses a large problem if the use of autologous stem cells is considered. There are numerous studies aimed at increasing the proliferation rate of various populations of postnatal stem cells; however, it is as yet unclear if this is in fact beneficial or desirable. The use of various cocktails and growth factors may indeed speed up the *ex vivo* expansion process, but may change the nature of the cell population by depleting the stem cells within it, or by altering the populations' biological activities. For example, basic FGF has been used to increase the proliferative capacity of BMSCs [53], but upon *in vivo* transplantation, cells grown with FGF-2 failed to support the formation of marrow (the home of the SSC [40]), which is where the SSC resides, indicating that the SSC had been lost. For these reasons, the use of allogeneic populations that could be used 'off the shelf' from unrelated donors would be preferable, but raises the same issue of rejection as in organ transplantation. Although it has been suggested that some postnatal stem cells appear to escape from immune surveillance in allogeneic settings and are immunomodulatory (BMSCs, as an example [54]), definitive proof is lacking. Furthermore, differentiation of stem cells imposes the expression of a mature tissue-specific phenotype, including a complete histocompatibility profile [55]. Use of allogeneic cells would most likely require concomitant immunosuppressive therapy, which has its own list of side effects. Co-transplantation of allogeneic bone marrow to reconstitute the hematopoietic system has been proposed as a way of inducing tolerance [56]. Studies in organ transplantation in conjunction with bone marrow or mobilized blood transplantation after immune ablation indicate a substantial improvement in long-term survival. In this type of approach, allogeneic cells could be better envisioned, provided that a single donor would be the source of cells for the tissue to be restored, along with the immune system.

Delivery of stem cells

As important as it is to control the *ex vivo* behavior of the cell population, consideration of how they will be delivered, and most importantly, how they will be integrated into the recipient in a functional fashion is of paramount importance. What the response of exogenous cells with or without scaffolds will be to the host environment, and vice versa, is not always predictable. In cases utilizing cells in association with scaffolds, integration relies on the rapid establishment of host-derived vascular and neuronal networks for viability, and cues must be provided. The host, in turn, must provide the signals that will dictate the differentiation if progenitor (not fully committed) populations are utilized. Furthermore, the recipient host tissue must also support the maintenance of the stem cell's niche, if not the creation of it *de novo*.

Systemic infusion and injection

Delivery of bone marrow or mobilized blood by systemic infusion for restoration of the hematopoietic system by HSCs is clearly an example of the efficacy of this method of delivery, but it is currently the only one. The success of systemic infusion is based on the fact that in terms of physical structure, blood is a simple, fluid entity. Furthermore, it is the physiological nature of blood cells to circulate through all parts of the body (and of HSCs as well under certain circumstances). Although systemic infusion of non-hematopoietic cells has been attempted for the delivery of cells to sites of injury, most adherent populations of cells lack the

cell surface characteristics needed for them to escape into extravascular spaces (reviewed in reference [57]), and the vast majority of the cells are trapped in the lungs and disappear very rapidly [58]. While it is thought that systemically infused non-hematopoietic cells 'home' or are 'recruited' to sites of injury, definitive proof of physiological signaling is lacking. The appearance of small numbers of cells at such sites is most likely due to vascular compromise, and it has been shown that these cells do not persist. Even upon direct injection into injured tissues, few cells of donor origin can be found after extended periods of time. Consequently, the reconstruction of two or three-dimensional structures requires different approaches, as discussed below.

CARRIERS AND SCAFFOLDS

Due to the solid state of tissues other than blood, transplantation of non-hematopoietic postnatal stem cells requires some form of support, whether it be for injection of cells (a semi-solid carrier to temporarily hold them in place and prevent their loss to the circulation), or for transplantation of constructs of cells with scaffolds (generally a solid structure of particular shape). Natural or synthetic, carriers and scaffolds must be biocompatable, bioresorbable and non-immunogenic. Furthermore, they must also be instructive, and dictate appropriate cell growth and differentiation, and support the recapitulation of a stem cell niche, which is essential for tissue renewal upon transplantation. The most commonly used natural scaffolds include either individual purified extracellular matrix (ECM) proteins such as collagen, fibronectin and laminin (or peptides derived from them), or devitalized ECM from skin, submucosa of the small intestine, urinary bladder and others, and these can be autologous, allogeneic or even xenogeneic. Such devitalized ECMs contain not only the structural proteins (which also have other important biological activities) that define the three-dimensional organization of the tissue, but also the local repertoire of growth factors that are stored within them (reviewed in reference [59]). Various chemical and non-chemical treatments have been applied to ECMs and their components in attempts to modify their biomechanical and immunological properties upon transplantation, but these have resulted in less than desirable effects due to inactivation of their conducive and inductive properties, and inhibition of their resorption and replacement. If left unmodified, ECMs generally promote infiltration and proliferation of cells and differentiation *in vitro* and *in vivo*, and are ultimately turned over upon transplantation (reviewed in reference [59]). New approaches include the use of recombinant human proteins that can be fabricated into scaffolds without the potential introduction of infectious agents, and with various combinations of proteins suited for a particular application [60].

Synthetic scaffolds are designed not only to mimic the biological properties of ECMs, but also to have enhanced material properties appropriate for a particular tissue. Polyglycolic acids, polyhyroxyalkonates and hydrogels are the most common examples, and all can be manipulated to form a broad range of structures with varying degrees of porosity and rigidity [61,62]. Synthetic scaffolds can also be functionalized [63], to include morphogenetic and growth factors, or even naked plasmid DNA to transduce in-growing cells to produce these important factors, such that they can become as instructive as ECMs [64,65].

RECONSTRUCTION OF SURFACE (2D) TISSUES

While more complicated than bone marrow transplantation, recreation of two-dimensional tissues is a relatively straightforward task in tissue engineering, although not routine to date. This is based not only on the relatively simple structures of surface tissues, but also on the fact that, generally speaking, surface tissues (e.g., skin) exhibit a high rate of turnover, thus making it easier to replace damaged tissue with applied exogenous cells. Reconstruction of surface tissues relies not only on appropriate *ex vivo* expansion conditions, but also on selection of appropriate scaffolds. As such, epithelial grafts (e.g., skin and cornea) are currently the only successful stem cell-based applications, other than bone marrow transplantation, and also provide an example of the impact of postnatal stem cell biology on the success of cell-based

tissue reconstruction. The use of *ex vivo* expanded keratinocytes for the generation of skin grafts was first brought to a clinical setting by the pioneering work of Green and coworkers [66], but persistent engraftment was not routine. It is now recognized that both the expansion culture conditions in which the cells are grown and also the scaffolding upon which they are placed, must support self-renewal of epidermal stem cells (cells that give rise to holoclones as opposed to transiently amplifying meroclones or more differentiated paraclones) [67]. Expansion of keratinocyte populations on fibrin substrates was found to maintain the stem cell population and to improve long-term maintenance of skin grafts [68]. Currently, there are a number of commercially available products, some of which incorporate dermal fibroblasts in a collagen gel, which is then layered with *ex vivo* expanded epidermal cells [69]. They provide coverage of severe wounds and are maintained while the recipient generates new skin. The standard starting material for establishing epidermal cultures is skin, however, there is evidence that in certain circumstances, a stem cell located in the bulge region of the root sheet of hair follicles has the ability to form not only new follicles and sebaceous glands, but also interfollicular epidermis (reviewed in reference [70]). These cells, which can be obtained non-invasively, are highly proliferative, and have been used to treat leg ulcers [71], and not surprisingly, to regenerate hair [72], albeit only in mice to date.

There have been a number of studies and a small clinical trials to molecularly engineer autologous epidermal stem cells to correct gene defects causing a form of severe blistering (junctional epidermolysis bullosa) prior to generation of skin grafts (reviewed in references [73,74]), and there is a current trial using a similar approach to treat dystrophic epidermolysis bullosa underway (clinicaltrials.gov # NCT01263379). But beyond generation of skin grafts and the treatment of genetic skin disease, perhaps the most significant success story in cell-based tissue engineering is seen in the use of stem cells from the limbus of the sclera for corneal epithelium regeneration. Limbal stem cells have been expanded in culture on fibrin substrates to generate sheets of epithelial cells that are able to reconstitute a damaged corneal surface with dramatic beneficial clinical results [75].

RECONSTRUCTION OF THREE-DIMENSIONAL TISSUES

Reconstruction of tissues with more complicated (and large) 3D structures than surface tissues presents a far more difficult challenge. In cases of large segmental defects treated with cell/scaffold constructs, there is a need for the rapid establishment of vasculature to meet nutrient demands, and also for neuronal networks in many cases. Furthermore, due to the generally slow turnover of complicated 3D tissues, replacement of existing damaged or diseased tissue by an exogenous cell/carrier application imposes an additional challenge. But again, selection of appropriate carriers and scaffolds for complex 3D structures is of utmost importance for ultimate success.

Using the skeletal system as an example, numerous pre-clinical studies in a variety of animal models have demonstrated that BMSCs, when used in conjunction with appropriate carriers, regenerate new bone in critical sized defects that would never heal without intervention (reviewed in references [76,77]). A wide variety of carriers were tested, ranging from collagen sponges, synthetic biodegradable polymers to synthetic hydroxyapatite derivatives (reviewed in reference [78]). To date, ceramic-based scaffolds appear to be superior to others, although there is no optimal scaffold to date [79]. A carrier that provides immediate stability, especially in the case of weight bearing bones, and yet be completely resorbed as new bone is formed and remodeled, has yet to be fabricated. Nonetheless, a small number of patients were treated with *ex vivo* expanded BMSCs in conjunction with hydroxyapatite/tricalcium phosphate ceramic particles with good outcome [80], and numerous clinical studies are in progress (see clinicaltrials.gov). In addition to healing segmental defects, BMSCs were also used to construct vascularized bone flaps. In cases where morbidity of the recipient site is an issue, it can be envisioned that a new bone rudiment could be grown elsewhere in the body and then transferred, with vasculature intact [81], as was attempted in the reconstruction of the mandible [82].

With respect to treatment of cartilage defects, appropriate scaffold selection presents different challenges. Suitable carriers must allow for chondrogenic differentiation of postnatal stem/progenitor cells capable of forming cartilage (BMSCs and periosteal-derived cells), and prevent vascular invasion while providing a conduit for nutrient flow. Furthermore, they must provide the appropriate microenvironment to maintain the cartilage phenotype and prevent hypertrophy. Natural polymeric gels such as hyaluronic acid, collagen, alginate and chitosan provide an adequate three-dimensional structure to maintain the chondrocyte phenotype, but are not well modeled into specific shapes and have very poor biomechanical properties. For these reasons, synthetic biodegradable polymers such as polylactic acid and polyglycolic acid, and mixtures of both, that can be crosslinked and molded to form porous three-dimensional structures are thought to provide more adequate scaffolding, and have been used to construct cartilage in with predefined shapes (reviewed in references [83–85]). In designing constructs of stem cells and scaffolds, the differences between the elastic cartilages of the ear and nose and articular cartilage must also be considered to ensure that tissues form with the appropriate biomechanical characteristics.

EX VIVO TISSUE/ORGAN RECONSTRUCTION

The creation of tissues and even organs prior to transplantation is a rapidly expanding area of research (as discussed elsewhere in this volume). This approach relies on expanding cell populations on, or in, either natural or synthetic scaffolds in some form of a bioreactor. The initial designs primarily provided nutrient flow through the developing tissue. However, many tissues grown under these circumstances fail to achieve the biomechanical properties required for tissue function *in vivo*. For example, the construction of blood vessels using a variety of biomaterials and cell populations using culture perfusion yields structures that histologically resemble native blood vessels. Yet when transplanted, these constructs failed due to their inability to withstand changes in pressure. A substantial improvement in such constructs was achieved by subjecting the developing tissues to pulsatile flow conditions (reviewed in reference [86]). Perfusion type bioreactors support the growth of tissue with only a nominal number of cell layers and are amenable for construction of relatively simple structures. Construction of larger, more complex, three-dimensional structures requires the development of even better bioreactors that will support cell survival to achieve a significant cell mass (reviewed in references [87,88]). Another critical aspect of using large constructs is the development of supporting vasculature. While constructs can be placed in vascular beds in some cases, it will be essential to design constructs that rapidly induce vascularization, perhaps by including the angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), or cells that produce it, or even by including endothelial cells and their precursors, which tend to assemble themselves into primitive tubular structures that may allow for more rapid establishment of organ perfusion (reviewed in references [89,90]).

Activation of local and distant endogenous stem cells

While the *ex vivo* reconstruction of entire organs that are functional is the goal the bioengineering world, what perhaps is even more challenging is the goal of inducing endogenous stem cells to become activated to reconstruct a tissue. Most tissues in the body display at least some sort of regenerative capability [91,92], but in many cases this remains insufficient to mount a spontaneous and successful repair response *in vivo*. The application of exogenous growth factors, cytokines and morphogens or their induction by genetic means, perhaps in combination with appropriate scaffolds, might be envisioned to 'encourage' local stem/progenitors to regenerate a functional tissue. In all cases, a regulated morphogenetic process is needed to establish normal structure and function. Definition of the intrinsic properties of regenerative cells and extrinsic signals that may trigger a recapitulation of developmental processes is critical.

LOCAL CELLS

Based on the reasoning that following an injury, signals that normally activate a local cell to initiate repair would be either obliterated or not of a high enough magnitude, numerous studies have attempted to provide appropriate morphogenetic and growth factors. However, this approach has been uniformly disappointing, most likely due to the short half-life of such soluble factors, and lack of an appropriate scaffolding. Scaffolding provides not only a template for the organized outgrowth of local cells, but also provides a substrate upon which to stabilize and/or orient factors (or parts thereof) for appropriate presentation to a cell (reviewed in references [93,94]). However, in using 'smart' constructs, tissue regeneration of large defects may not be complete due to exhaustion of the local stem cell population, hence the need to maintain a stem cell niche during the activation of local stem/progenitor cell populations. Furthermore, some clues about the negative impact of local constraints upon the native, albeit incomplete, repair capacity of cells and tissues may be found in studies of spinal cord regeneration. Following injury, there is phase characterized by neurite outgrowth from the severed end, however, this is short-lived due to the production of inhibitory factors by cells within the myelin sheath, and gliosis impedes neurite outgrowth (reviewed in references [95,96]). Thus, regeneration by endogenous cells must not only take into consideration bioactive factors and scaffolding that are necessary to maintain the in-growing population with the appropriate balance of stem cells, transiently amplifying cells and differentiating cells, but also the inactivation of local inhibitory factors that work against the process.

MOBILIZATION AND RECRUITMENT OF DISTANT STEM CELLS

Cytokine administration has become a well-established procedure for mobilization of HSCs into peripheral blood for the purposes of hematopoietic cell transplantation. Such mobilization is based on not only a complex series of events that liberates the HSC from its niche in bone marrow, but also on the inherent ability of HSCs to escape into and to survive in the circulation (reviewed in references [97,98]). However, it is not clear that other postnatal stem cell populations present in marrow or any other tissue are equally amenable to liberation by current procedures. It has been reported that bone marrow-derived endothelial precursors are liberated into the circulation following a number of different stimuli induced by trauma [99], however, it is unclear to date if they actually participate in neovascularization [9], and in fact, prevailing evidence suggests that they do not [100]. Likewise, there are numerous reports of stromal-like cells being present in the circulation, although their numbers are vanishingly small in humans [101,102]. Many reports also suggest that their numbers are increased in different pathologies, injury or after stimulation with various factors, and that these cells originate from bone marrow (i.e., that they are BMSCs) (reviewed in reference [103]). However, based on our current inability to do lineage tracing studies with a BMSC-specific promoter, the origin of these cells is not known. Furthermore, the question of whether these stromal-like cells play a significant role in repair of distant tissues is highly controversial. Lack of convincing evidence of mobilization of non-hematopoietic cells most likely rests in the facts that:

1) Without *ex vivo* manipulation, they lack the features that enable their escape into the circulation,
2) They appear to have limited ability to persist in the circulation due to their unanchored state and/or their entrapment in the lungs (and also the spleen and kidneys), and
3) They are unable to escape from the circulation except in the case of vascular defects, and even then, their numbers are limited.

Whether or not procedures can be developed that will overcome these hurdles is a major question.

CONCLUSIONS

The recent advances in pluripotent and postnatal stem cell biology have most certainly captured the public's attention, and have raised expectations for miraculous cures in the near future. While certain applications that utilize postnatal stem cells are in practice or will be shortly, the majority are in their infancy and will take much more effort. However, the current sense of urgency in translating recent findings into clinical applications should not lead this field to repeat the mistakes experienced in the field of gene therapy. The development of tissue engineering as a viable medical practice must proceed in an evidence-based fashion in all of the associated disciplines that are involved, and several hurdles are yet to be overcome. First, our current understanding of postnatal stem cell biology is rudimentary, at best. The manner in which we isolate stem cells and manipulate them and their progeny appropriately relies heavily on a clear understanding of cell population kinetics. This also requires a complete understanding of their response not only to bioactive factors, scaffoldings and delivery systems, but also to the host microenvironment in which they must survive and function. Secondly, more efficient bioreactors that adequately model the microenvironment and also allow for scale-up of the tissue-engineering process must be developed. Thirdly, in order to translate what we learn into clinical application, development of appropriate pre-clinical models to prove the principle that postnatal stem cells do indeed have a positive biological impact is absolutely essential. In analyzing these models, stringent criteria must be defined to determine efficacy, and we must remain principled in assessing them. Exciting times, yes, but also a time for due diligence to bring what started off as a scientific curiosity into medical reality.

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PART 7

Gene Therapy

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CHAPTER 34

Gene Therapy

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Gene therapy uses the transfer of genetic information to modify a phenotype for therapeutic purposes [1]. The application of gene transfer to tissue engineering has a myriad of possibilities, including the transient or permanent genetic modification of the engineered tissue to produce proteins for internal, local or systemic use, helping to protect the engineered tissue and providing stimuli for the engineered tissue to grow and/or differentiate. To provide a background for the application of gene transfer to tissue engineering, this chapter will review the general strategies of gene therapy, detail the gene transfer vectors used to achieve these goals, and discuss the strategies being used to improve gene transfer by modifying the vectors to provide cell-specific targeting and by regulating the expression of the targeted gene. Applications which combine gene therapy and stem cell therapy will be reviewed. Our overall goal is to provide a state-of-the-art review of the technology of gene therapy, including the challenges to making gene therapy for tissue engineering a reality. For details regarding the applications of gene therapy to specific organs and clinical disorders, several reviews are available [1–10].

STRATEGIES OF GENE THERAPY

The basic concept of gene therapy is to transfer nucleic acid, usually in the form of DNA (or RNA in retrovirus and lentivirus vectors) to target cells. The vector with its gene cargo can be administered *ex vivo*, where the gene is transferred to the cells of interest in the laboratory and the genetically modified cells restored to the patient, or *in vivo*, when the nucleic acid is administered directly to the individual (Fig. 34.1). Independent of the overall strategy, an

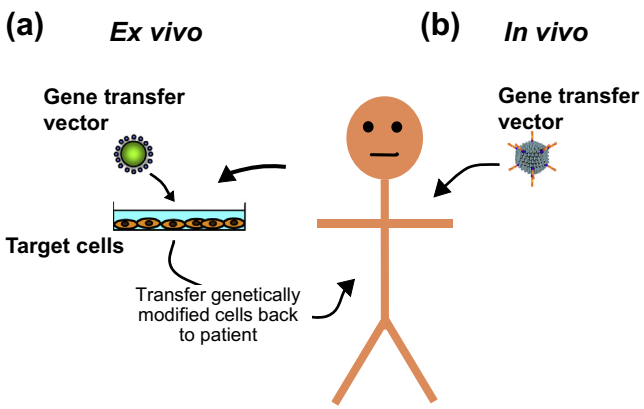

FIGURE 34.1
General strategies for gene therapy for tissue engineering. (a) *Ex vivo* strategies use a gene transfer vector to genetically modify autologous target cells (e.g., skin fibroblasts) *in vitro*, followed by transfer of the genetically modified cells back to the patient. (b) *In vivo* strategies transfer the therapeutic gene by direct administration of a gene transfer vector to the patient.

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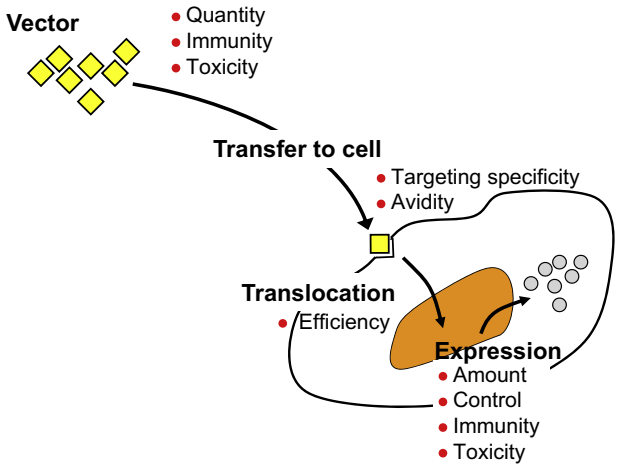

FIGURE 34.2
Issues relating to successful gene transfer. In addition to the choice of vector, successful gene transfer requires decisions regarding the quantity of vector to be used, and that the vector does not induce immunity and/or toxicity that will limit its use. The vector has to be transferred to the cell, a relatively easy task for *ex vivo* strategies, but *in vivo* may require enhancing targeting specificity and vector avidity to its receptor. Once reaching the target cells, the vector must insure its gene cargo is efficiently translocated to the nucleus. In the nucleus, the gene must be expressed in appropriate amounts, with control if necessary, and without appreciable immunity and/or toxicity induced by the gene product.

expression cassette containing the genetic sequences to be delivered, typically a cDNA along with regulatory sequences to control expression, is inserted into a 'vector', a non-viral or viral package used to improve efficiency and specificity of the gene transfer. Together, the choice of vector, the design of the expression cassette and the coding sequences of the gene determine the pharmacokinetics of the resulting gene expression.

Simple in concept, gene transfer is complex in execution. In addition to choosing an *ex vivo* or *in vivo* strategy, the major issues relating to successful gene therapy are the design of vector, how the vector is delivered to the cell population of interest, translocating the vector/expression cassette from outside the cell to the nucleus, and the expression of the gene to obtain the desired therapeutic effect (Fig. 34.2). Independent of the choice and design of the gene transfer vector, successful gene therapy requires decisions regarding the quantity of vector required to modify the numbers of target cells necessary to obtain the desired therapeutic effect, and consideration of whether or not the vector will evoke a host immune response and/or cause unacceptable toxicity. The gene therapist must also decide how to get the vector to the target cells, including targeting specificity and avidity of the vector for its relevant receptor. Once the vector reaches the cells of interest, the gene cargo within the vector must be translocated from outside of the cell to within the nucleus. In designing the gene transfer strategy, it is critical to decide whether or not the gene is to be inserted into the chromosomal DNA of the target cells. Finally, the transferred gene must be expressed, with the concomitant issues of amount and control of expression, and host immunity and toxicity that may be evoked by the expression of the gene. In the sections that follow, we will discuss all of these issues.

EX VIVO VS. *IN VIVO* GENE THERAPY

There are generally two strategies by which gene therapy technology can be used for genetic engineering; *ex vivo* transfer of genetic material with subsequent transfer of the modified cells or tissue to the host, and *in vivo* transfer, with direct administration of the gene therapy vectors to the patient (Fig. 34.1).

Ex vivo

The *ex vivo* strategy has the advantage that the cell population can be purified and carefully defined, and the transfer of the gene is limited to that cell population and not to other cells or tissues. The challenge for this approach is that, for most applications, once returned to the patient, the genetically modified cells need to have a selective advantage to modulate the therapeutic goal. For applications where long-term expression of the gene is required and

the transferred cells subsequently replicate within the patient, the vectors used to transfer the gene must mediate the integration of the gene, so that it persists when the cell divides [1,9].

The *ex vivo* strategy typically is used to genetically modify hematopoietic cells, such as CD34+ cells derived from bone marrow, or skin fibroblasts [4,9]. Examples of *ex vivo* gene transfer strategies include the correction of hereditary immunodeficiencies with retroviral vectors, transfer of the factor VIII gene to autologous fibroblasts to treat hemophilia A, and transfer of suicide genes to T cells to control graft vs. host disease and some leukodystrophies [11,12,14–16,27]. The *ex vivo* strategy is also the most suitable strategy to use gene transfer to genetically modify stem cells. The recent advances in induced pluripotent stem cell (iPSC) technology, where reprogramming genes are transferred to somatic cells *ex vivo* to generate embryonic or adult stem cells, broadens the *ex vivo* gene therapy approach to the promising but yet unchartered territory of iPSC therapy for human disease [17,18]. For tissue engineering applications, the *ex vivo* strategy is applicable to providing genes to enhance and/or modulate the growth of the engineered tissue, as well as to protect the engineered tissue from host responses or disease processes.

Although *ex vivo* gene therapy can be carried out using cells derived from a non-autologous source, potential immune rejection of non-matched cells generally requires that autologous or closely matched donor cells be used for the tissue-engineering strategy. However, the immune system can potentially recognize components of the vector and/or transferred gene product. For *ex vivo* strategies in general, immune recognition of the gene therapy vector is minimal, as the immune system will not be in contact with the total dose of gene therapy vector used to transfer the gene *in vitro*, but only the residue of the vector within the cells to be transferred. There is the possibility of immune recognition via MHC presentation of viral antigens inducing anti-vector immunity, and this may be responsible for shutdown of gene expression over time.

In vivo

In vivo gene transfer strategies administer the gene therapy vector either directly to the target organ or deliver it via the vascular system into vessels feeding that organ. *In vivo* gene transfer has an advantage over *ex vivo* strategies in that it avoids the cumbersome (and costly) process of removing cells from the patient, manipulating the cells *in vitro*, and returning the genetically modified cells to the patient. Challenges that need to be overcome for *in vivo* gene transfer strategies include the induction of immunity by the gene transfer vector, transport of the gene therapy vector to the targeted cells/organ, efficient binding of the vector to the cell, translocation of the genetic material to the nucleus, and toxicity and immunity induced by expression of virus and/or transgene peptides.

The retina has so far been an ideal target for the *in vivo* gene therapy approach because:

1) It is in a confined, relatively small space;
2) The gene therapy vector can be administered directly;
3) Vectors with natural tropism for retinal cells are available; and
4) It is immunoprivileged.

The *in vivo* gene therapy approach has recently achieved a significant success by achieving correction of the genetic disease Leber's congenital amaurosis [19,20].

CHROMOSOMAL VS. EXTRA-CHROMOSOMAL PLACEMENT OF THE TRANSFERRED GENE

One critical decision in strategizing gene therapy is whether the transferred gene is to be inserted into the chromosomal DNA of the targeted cells or is designed to function in an extra-chromosomal location within the nucleus. There are advantages and disadvantages to both strategies, and the choice is determined by the specific aim of the gene transfer.

As described below, some gene transfer vectors (e.g., retroviruses, lentiviruses) insert their genome, and hence the transferred gene, directly into the chromosomal DNA of the target cells. This has the advantage that it is permanent, and when the genetically modified cells divide, both daughter cells have the newly transferred sequences, as they are now part of the genome of the genetically modified cell population. This is a desirable feature for applications where persistent gene expression is required, such as for the correction of a hereditary disorder. The disadvantage is that, once inserted, the gene cannot be removed, and thus unless controls are designed into the transferred sequences, it cannot be shut down. Equally important is the issue of the randomness of where the gene is inserted. If the gene is inserted into a relatively 'silent' region of the genome, the resulting gene expression will be low, while gene expression from other regions will be high [21]. This genome regional modulation of the level of gene expression will be different for each targeted cell. While there are some more favored regions of gene insertion for given vector characteristics, the genetically modified cells essentially become a mixed population in terms of where the gene has been inserted. Thus, expression may be low for some cells, while other cells may be average or high expressors. More troubling, if the gene is inserted into a region influencing cell proliferation, the result may be uncontrolled cell growth, i.e., malignancy. This phenomenon, referred to as 'insertional mutagenesis', has been observed in experimental animal and human applications of gene transfer [21]. Several strategies have evolved out of these observations to improve the safety of integrating gene therapy vectors [22–26].

Some vectors (e.g., non-viral, adenoviruses, adeno-associated viruses) transfer the gene into the nucleus, but mostly into an extra-chromosomal location, where the gene is transcribed using the same transcriptional machinery as for genomic DNA [1,10]. The consequence of this strategy is that as long as the cells do not proliferate, and as long as host defenses do not recognize the genetically modified cell as foreign, expression of the transferred gene will persist. However, if the cell divides, the transferred gene will not be replicated, and gene expression in the daughter cells will eventually wane as proliferation continues. The consequences are transient expression of the gene, a 'pharmacokinetic' result that is ideal for some applications (e.g., angiogenesis, most cancer therapies), but not desirable for applications requiring persistent expression (e.g., correction of a genetic disease). Independent of the issue of persistence of gene expression, extra-chromosomal placement of the gene has the advantage that insertional mutagenesis and variability of gene expression secondary to variability of chromosomal insertion are not of concern.

GENE TRANSFER VECTORS

There are two general classes of commonly used vectors for gene therapy: non-viral and viral (Fig. 34.3, Table 34.1). Although a variety of vectors have been developed in both classes, the most commonly used non-viral vectors are naked plasmids and plasmids combined with liposomes, and the commonly used viral vectors are adenoviruses, adeno-associated viruses, retroviruses and lentiviruses [1,5,6,8,10,27].

Independent of the vector used, all carry an expression cassette which includes the gene to be transferred together with the relevant regulatory sequences to control the expression of the gene once it has been transferred to the target cells (Fig. 34.3a). The typical expression cassette includes (5′ to 3′) a promoter, an intron (this is not critical, but it usually enhances gene expression and enables identification of the cytoplasmic mature mRNA from the pre-mRNA and transferred expression cassette), the transgene itself (usually in the form of a cDNA, but it can contain one or more introns, or can be the generic form of the gene), and finally the polyA/stop and other 3′ regulatory sequences, if desired. In special cases, such as *trans*-splicing (discussed in the section on regulation of expression), the expression cassette may contain only a fragment of the gene together with sequences to direct splicing into an endogenous nuclear pre-mRNA.

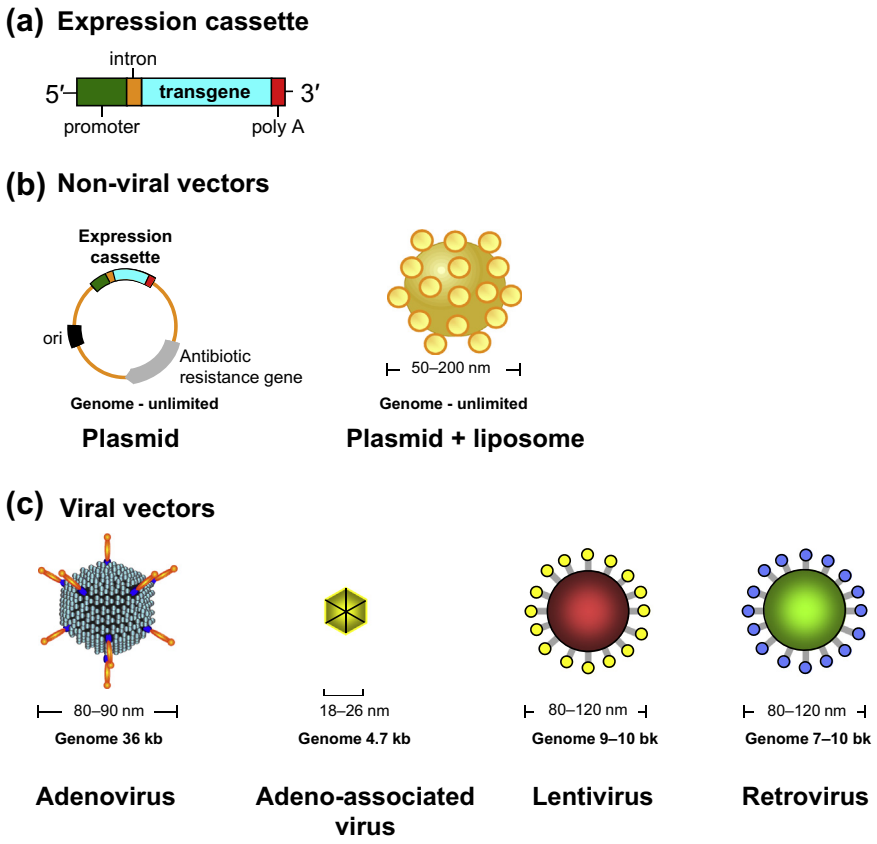

FIGURE 34.3
Commonly used gene therapy vectors. (a) All gene transfer vectors contain an expression cassette with (5′ to 3′) a promoter, usually an intron, the transgene and a polyA site/stop signal. (b) Commonly used non-viral vectors, including naked plasmids (typically comprised of an origin of replication, the expression cassette and antibiotic resistance gene), or plasmid combined with a liposome. The plasmid genome can be unlimited in size, but usually the expression cassette is <10 kb; the liposome/plasmid combination ranges from 50 to 200 nm in diameter. (c) Commonly used viral vectors, including adenovirus, adeno-associate virus, lentivirus, and retrovirus. Shown is the size of the genome of each viral vector, as well as the relative size of each vector. The size of the expression cassette depends on how much of the viral genome is included.

Non-viral vectors

The most simple gene transfer vectors are plasmids. To achieve relevant transduction of cells *in vivo*, the plasmids are usually combined with liposomes to facilitate attachment and entry into target cells [28–30]. A variety of physical methods have also been developed to promote entry of plasmids into target cells, including microinjection, hydrodynamic administration, electroporation, ultrasound and ballistic delivery (the so-called 'gene gun') [28]. Most of these physical methods of gene delivery are not applicable for *in vivo* gene transfer due to the inaccessibility of the target cells for direct manipulation.

Plasmids contain a relatively simple expression cassette, with the transgene driven by a promoter and flanked by an intron and the polyadenylation/stop site (Fig. 34.3a). Plasmid DNA has an unlimited size capacity; however, because plasmids >10 kb are potentially unstable during production, most gene transfer strategies being considered for human applications use plasmids <10 kb. Although plasmids can efficiently transduce cells *in vitro*, their efficiency *in vivo* is limited. There have been may attempts to correct hereditary disorders with plasmid gene transfer alone, but with little evidence of expression of the plasmid-directed mRNA in those trials [31,32]. Part of the reason of the inefficiency of plasmid-mediated gene transfer is that plasmids have no means of directing their traffic to the nucleus [29,30]. For gene transfer applications for tissue engineering, the most that can be envisioned for the use of plasmid-based systems is to use them in an *ex vivo* approach with possible need for selection of the transduced cells.

Adenovirus

Adenoviruses are non-enveloped viruses containing a linear, double stranded DNA genome of 36 kb. Among the 49 different Ad strains that infect humans, the subgroup C, serotype 5 and

TABLE 34.1 Characteristics of most commonly used gene transfer vectors

Vector type	Maximum expression cassette capacity (kb)	Transfers genes to non-dividing cells	Anti-vector immunogenicity	Chromosomal integration	Expression	Other characteristics
Plasmids / liposomes	< 10[1]	Yes	None	No	Transient	Poor transduction efficiency
Adenovirus	7–8[2]	Yes	High	No	Transient	Typically, mediates expression for 1 to 3 wk
Adeno-associated virus	4.5	Yes	Low	No[3]	Persistent in non-dividing cell populations	Expression usually takes 1 to 3 wk to be initiated
Retrovirus	8	No	None	Yes	Persistent	Risk for insertional mutagenesis, difficult to produce in high titer
Lentivirus	8	Yes	None	Yes	Persistent	Theoretical safety concerns regarding HIV components; risk for insertional mutagenesis, difficult to produce in high titer

[1]*Plasmid size are generally not limited, but plasmids with >10 kb expression cassette capacity are more difficult to produce with consistent fidelity and thus are generally not suitable for gene human transfer.*
[2]*Typically, Ad vectors have a 7–8 kb capacity for the expression cassette; the capacity can be increased by removing additional viral genes.*
[3]*Wild type adeno-associated virus (AAV) integrates in a site-specific fashion into chromosome 19, a process mediated by the rev gene; rev is deleted in the AAV gene transfer vectors; whether or not there is minimal integration of the genome AAV gene transfer vectors is debated (Summerford and Samulski, 1998; Qing et al., 1999; Summerford et al., 1999; McCarty et al., 2004).*

serotype 2 are widely used in gene transfer studies, and the only serotypes used in humans to date. The Ad5 genome is composed of early and late genes [33]. The E1 region controls the replication of the virus. Conventionally, the Ad gene transfer vectors have a deletion in E1 and E3 (a non-essential region). The expression cassette containing the promoter, and the gene to be transferred is usually inserted into the E1 region. The vectors are produced in the 293 embryonic kidney cell line that provides the E1 information in *trans*, enabling replication of the recombinant vector.

Ad vectors can hold 7 to 7.5 kb of exogenous sequences [33]. If more space is needed, the E2 or E4 region can also be deleted and the vector made in cell lines providing these deleted sequences. The Ad capsid is an icosahedral structure composed of 252 subunits, of which 240 are hexons and 12 are pentons. Hexon is the major structural component of the Ad capsid, forming 20 facets of the icosahedron, and is composed of three tightly associated molecules of polypeptide II forming a trimer. Polypeptide IX is associated with the hexon protein and serves to stabilize the structure. Each penton contains a base and a non-covalently projecting fiber. Sequences within the fiber are the primary means by which Ad interacts with cells, with the penton base providing secondary attachment sequences.

Several cellular receptors have been identified for Ad vectors and they differ for various serotypes (Table 34.2), see also reference [76]. The primary Ad receptor for the subgroup C Ad is the coxsackie adenovirus receptor (CAR) [34]. CAR is expressed on most cell types, and thus Ad group C vectors, but also some group A, D, E and F serotypes are capable of transferring genes to most organs [35,76]. Besides the primary CAR receptor, epitopes in the penton base of the group C Ad vectors use $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins as co-receptors for virus

TABLE 34.2 Cell receptors of commonly used viral-based gene therapy vectors

Vector	Virus group/serotype[1]	Receptor	Reference
Adenovirus	Group C, serotypes 2, 5	CAR[2]; co-receptors – $\alpha_V\beta_3$, $\alpha_V\beta_5$ integrins	[36,34]
	Group C, serotypes 2, 5 Group B, serotypes 3, 35	Heparan sulfate	[196,37,38]
	Group C, serotype 5	Vascular cell adhesion molecule I	[40]
	Group C, serotype 5	MHC class I α2	[39]
	Group D, serotype 37	Sialic acid	[197]
	Group B, serotypes 3, 11, 14, 16, 21, 35, 50	CD46	[41,42]
	Group B, serotype 3	CD 80, CD 86	[43]
Adeno-associated virus	AAV1	Sialic acid (N-linked)	[70,71]
	AAV 2	Heparan sulfate proteoglycan; co-receptors – fibroblast growth factor receptor 1, $\alpha_v\beta_5$ integrin	[66,198,75,67]
	AAV 3	Heparan sulfate proteoglycan	[67,37]
	AAV4	Sialic acid (O-linked)	[68]
	AAV5	Sialic acid (N-linked); coreceptor – platelet-derived growth factor receptor	[68,69,77]
	AAV6	Sialic acid (N-linked), heparan sulfate proteoglycan; coreceptor – epithelial growth factor receptor	[70,78,71]
	AAV8	Unknown primary receptor; coreceptor – laminin receptor	[79]
	AAV9	Galactose (N-linked); coreceptor: laminin receptor	[79,72]
Retrovirus	MoMLV[3]	Ecotropic	[83,199]
	470A MLV, VSV[4]	Amphotropic (phosphatidylserine?)	[83,200,199,85]
Lentivirus	HIV-1 (envelope protein)	CD4 – co-receptors – CCR5, CXCR4	[201]
	Pseudotyped with VSV-G	Amphotropic (phosphatidylserine?)	[202,85]

[1] *Adenoviruses are categorized in groups and serotypes; AAV is categorized in clades and serotypes, only the serotypes are listed.*
[2] *CAR – Coxsackie adenovirus receptor.*
[3] *MoMLV – Moloney murine leukemia virus.*
[4] *VSV – Vesicular stomatitis virus.*

internalization [36]. Heparan sulfate has also been identified as a receptor for Ad2, Ad3, Ad5 and Ad35 [37,38]. Ad5 has been shown to bind to vascular cell adhesion molecule 1 (VCAM 1) on endothelial cells and possibly also to MHC class I a2 on the cell surface [39,40].

The group B serotypes Ad 11, 14, 16, 21, 35 and 50 utilize CD46 instead of CAR, enabling more efficient gene transfer into hematopoietic cells, cells of the urinary tract epithelium and salivary glands [41,42]. For Ad3 of the subgroup B Ad vectors, CD80 and CD86, which are usually expressed on antigen-presenting cells, have been identified as a receptor for viral entry [43].

In addition to Ad vectors being effective in delivering genes to a wide variety of cell types for therapeutic purposes, Ad vectors interact rapidly with antigen-presenting cells such as dendritic cells, leading to the induction of immunity against the vector and potentially also against the transgene if it is foreign to the host [44,6]. When Ad are directly administered in large doses to animals and humans, there is an innate and acquired immune response against the vector, resulting in local inflammation and infiltration of CD4, CD8 and dendritic cells

[44,45]. The immune response is multifaceted, consisting of humoral and cellular immunity against both the capsid proteins and against the transgene expressed by the vector if it is foreign to the host. Intravenously administered Ad also interacts with a variety of host proteins, in particular coagulation factor X 46,47.

Several strategies have been investigated to circumvent the problem of host responses evoked against Ad gene transfer vectors, including the use of immunosuppressants administered together with the vector, or including transgenes expressing immunomodulatory factors to suppress the immune responses against the vector [44]. Considerable effort has also been expended on circumventing the host response by designing vectors with larger genomic deletions (e.g., of E1 plus E2 and/or E4 with complementing cell lines) which evoke milder immune reaction [5,44,48].

One challenge to the use of Ad vectors is pre-existing immunity against the vector, resulting from previous infection with a wild type Ad virus from the same serotype [44]. The acquired host responses to Ad vector administration generally results in the inability to re-administer a vector of the same serotype. To circumvent this issue, alternative serotypes can be administered, thus circumventing immunity against the first vector [49]. Also, serotypes to which humans are usually not exposed have been developed as gene transfer vectors. One example is Ad serotype 48, to which humans rarely have pre-existing immunity. A chimeric Ad vector, in which the hexon loops of Ad5 are replaced by those of Ad48, has been shown to be a possible strategy for an Ad-based genetic vaccine approach [50], and could also be envisioned as being useful for tissue-engineering applications.

Another strategy for circumventing pre-existing anti-Ad immunity is the use of non-human Ad serotypes [51–53]. Non-human, primate-derived Ad vectors were developed to overcome pre-existing immunity to common human Ad serotypes and to broaden the repertoire of Ad when used as vaccines [54–56]. For example, Ad vectors based on non-human primate serotypes C68, C6, C7 do not circulate in the human population and are therefore not affected by pre-existing immunity [51–53].

Adeno-associated virus

Over the past few years, AAV (adeno-associated virus) vectors have emerged as one of the most promising viral vectors for *in vivo* gene therapy in humans [8]. AAV is a single stranded DNA virus that belongs to the *Dependovirus* genus of the *Parvoviridae* family. AAVs were originally isolated as contaminants in laboratory stocks of adenoviruses [57]. Nine subtypes of AAV have been described for which humans are the primary host [57,12]. These isolates were found to be different based on the antibody response generated against them and were thus categorized as AAV serotypes 1–9. A substantial portion of humans have detectable antibodies against these serotypes. The capsid structures have so far been solved for all serotypes, except AAV3 [12]. The exact nature and sequelae of the natural infection with AAV in humans are not known and there is possibly no human disease associated with AAV.

An AAV consists of a single stranded 4.7 kb genome with characteristic termini of palindromic repeats that fold into a hairpin shape known as inverted terminal repeats (ITR) [57,12]. During replication into a double stranded form, it expresses genes involved in replication (*rep*) and genes that code for the capsid proteins (*cap*). In the absence of a helper virus such as adenovirus or herpes simplex, wild type AAV is capable of infecting non-dividing cells and integrating its genome into chromosomal DNA at a specific region on chromosome 19, persisting in a latent form. In the gene vectors, *rep* and *cap* are deleted, and most of the vector genome resides and functions in an extra-chromosomal locations [57].

In the AAV vectors, the *rep* and *cap* genes are replaced with an expression cassette. During vector production, the *rep* and *cap* gene products as well as the necessary helper virus elements

(usually Ad-derived) are supplied *in trans*. AAV vectors are commonly produced by transfecting two plasmids into the 293 human embryonic kidney packaging cell line. DNA coding for the therapeutic gene is provided by one plasmid, and the AAV *rep* and *cap* functions plus the Ad helper functions are provided by the second plasmid. Titers are generally significantly lower compared to those obtained for Ad vectors, but are sufficient to produce enough vector for clinical trials.

Numerous studies in animals have been performed to assess the safety and efficacy of AAV-based vectors, and AAV1, 2, 6, and 8 serotype-based human and chimpanzee-derived AAVrh10 vectors have been assessed in humans [58,8]. AAV vectors are capable of transducing non-dividing cells *in vitro* and *in vivo*. The exact molecular intracellular state of the vector genome has not been completely elucidated, but there is little evidence that the vectors integrate when used in gene therapy *in vivo*. Most transgene expression is thought to be derived from extra-chromosomal viral genomes which persist as double stranded circular or linear episomes [59,10]. This limits the usefulness of AAV for applications involving dividing cells such as stem cells, since only one daughter cell will receive the vector genome [240].

One disadvantage of AAV vectors is their packaging capacity, which is limited to expression cassettes of about 4.5 kb. This size limitation is a challenge for the use of AAV vectors for clinically relevant large transgenes, such as dystrophin (11 kb) for muscular dystrophy, or factor VIII (7–9 kb) for hemophilia A. Various efforts have been made to deliver larger transgenes using AAV vectors, including co-administration of two vectors each carrying one half of the transgene, leading to intermolecular recombination during concatamerization, an intermediate state of the vector genome. *Trans*-splicing may also be employed as a strategy to circumvent the need to package the full-length cDNA into the AAV vector; instead of delivering the full coding sequences, the gene is split into two pieces that then combine at the pre-mRNA level [60].

The host immune response against AAV vectors is not as strong as that observed with Ad vectors [8,44,61–63]. AAV vectors generate humoral immunity against the capsid proteins, which impairs readministration of vector of the identical serotype. Cellular immunity against AAV has been detected following administration to experimental animals and humans, but, unless high doses are used, there usually are no destructive cytotoxic T cell responses generated against the vector or the transgene [61]. However, recent experiences with liver-directed AAV delivery for hemophilia have challenged this notion [8,62,63].

There is usually persistent expression of the transgene directed by AAV vectors, particularly in organs with non-dividing (or slowly dividing) cells, such as liver, muscle, heart, retina and brain. The overall expression levels of the transgene appear to be lower with AAV compared to Ad and are dependent on the target organ. Recently novel AAVs have been identified from tissues of non-human primates and humans [10,64,65]. These AAVs have substantial heterogeneity in the capsid genes, and are useful as chimeric capsids combined with the AAV2 genome, leading to improved infection of organs or tissues previously not considered to be valuable targets for AAV gene transfer.

AAV interacts with its target cells by binding to cell surface receptors (Table 34.2). For AAV2, the primary attachment receptor is heparan sulfate proteoglycan [66]. AAV3 may share heparan sulfate proteoglycan as the primary attachment receptor. However, AAV3 may use other receptors, as AAV3 has been shown to infect hematopoietic cells, which were not effectively infected by AAV2 [67]. AAV4 and AAV5 use sialic acid as the primary attachment receptor. AAV4 uses O-linked sialic acid, whereas AAV5 uses N-linked sialic acid [68,69]. AAV1 and AAV6 use N-linked sialic acid as receptor [70,71]. Recently, N-linked galactose was identified as the receptor for AAV9 72. The primary receptors for AAV7 and 8 remain unknown. AAV 12, originally isolated from a stock of simian adenovirus 18, does not require cell surface heparan sulfate proteoglycans nor sialic acid for transduction and shows strong

tropism for nasal epithelia [73,74]. It is being developed as vaccine vector against respiratory pathogens [74].

AAV2 also uses co-receptors for efficient infection, including $\alpha_V\beta_5$ integrin [75] and fibroblast growth factor-1 [76]. Efficient infection with AAV5 appears to require a coreceptor; platelet-derived growth factor has been identified as a possible coreceptor for AAV5, but may also be able to act as the primary receptor [77]. Epithelial growth factor receptor has been identified as a coreceptor for AAV6 [78]. AAV 8 and 9 use the laminin receptor as a coreceptor [79].

Retroviruses

Retrovirus and lentivirus vectors are both RNA-based vectors belonging to the family of retroviridae. Because lentiviruses are capable of infecting non-dividing cells, they are discussed separately below. The original retroviral vectors used for gene therapy were based on endogenous murine viruses. Of these, the Moloney murine leukemia retrovirus (MMLV) was the first widely used gene transfer vector and was the first to be used to treat an hereditary disorder using an *ex vivo* strategy [80].

The genome of the retrovirus vector is a 7 to 10 kb single stranded RNA containing long terminal repeats (LTR) on both ends that flank *rev, gag, pol* and other regulatory genes which are required for viral function. The RNA genome of the replication-deficient retroviral vectors contains an expression cassette of to up to 8 kb that replaces all viral protein-coding sequences. The LTRs flank the expression cassette and allow transcription initiation by host cell factors. The vectors are rendered self-inactivating by deletion of the promoter and enhancer in the 3′ LTR to prevent LTR-driven transcription. The packaging of the genomic RNA is controlled in *cis* by the packaging signal Ψ. The production of the retrovirus vectors requires a packaging or producer cell line in which the viral *gag, pol* and *env* proteins are expressed in *trans* from separate helper products. Recombination between the helper constructs and the vector can be minimized by using non-retroviral regulatory sequences to control expression [81]. Enhancer and promoter sequences can be deleted from the 3′ LTR to create a transcriptionally silent 5′ LTR during infection of the target cells. This strategy provides the basis for self-inactivating vectors and can also be used for the substitution with tissue-specific promoters [82]. The main reason why MMLV viruses can only infect non-dividing cells is that they are unable to cross the nuclear membrane and can only achieve completion of the infection with provirus integration during cell division.

Retroviruses enter the cells via cell fusion of the envelope protein with the cell membrane. The murine viruses are able to infect only murine cells (ecotropic), whereas derivatives of MMLV or human retroviruses like vesicular stomatiitis virus (VSV) can infect both human and murine cells (amphotropic; Table 34.2). Providing the virus with a different coat, a process referred to as 'pseudotyping', can change the specificity of binding and entry into target cells. Over the past decade, there has been considerable effort in the development of pseudotyping strategies of retroviral vectors [81,83–85].

Most of the gene transfer strategies for retroviral vectors have been *ex vivo* approaches due to the difficulty of producing high titer concentrated preparations and the rapid inactivation of the retroviral vectors *in vivo* by complement. Due to their ability to infect rapidly dividing cells, retroviral vectors have been used extensively to develop gene transfer strategies to hematopoietic cells. The first clinical trial to treat a hereditary disorder was to use a retrovirus to transduce T cells of patients with adenosine deaminase – severe combined immunodeficiency (SCID) [80], and since then the technology has evolved to permit successful treatment of children with SCID [86–88].

One advantage of using retroviral vectors is the permanent integration of the vector genome into the host genome, providing long-term and stable expression of the transgene. This, however, also carries the greatest risk of retroviral vectors, in that they may induce insertional

mutagenesis with the subsequent development of malignancies. This has been observed in clinical trials using MMLV-based retroviral vectors, in which five out of 20 patients developed leukemia due to insertional mutagenesis [89–91]. To increase the safety of gene therapy, self-inactivating retrovirus and lentiviral vectors with internal promoters have been developed [23–26,92]. These theoretically reduce the risk of undesirable activation of genes adjacent to the integration site and favor integration near transcription start sites [89,93,94]. Retroviruses are more vulnerable to silencing than lentiviral vectors [93]. However, important advances have been made in understanding the integration mechanisms to retain retroviral vectors as a viable option for human gene therapy for genetic hematopoietic cell disorders [22,25,26,95,96].

Lentivirus

Based on the knowledge that the human retrovirus HIV-1 is able to infect non-dividing cells such as macrophages and neurons, replication-defective versions of HIV were developed, capable of infecting non-dividing cells and achieving stable long-term expression through integration of the provirus into chromosomal DNA [97,98]. The genome of a lentivirus vector is a 9 to 10 kb single stranded RNA genome, containing components of HIV-1, but otherwise similar to that of retroviral vectors. All viral protein-coding sequences are deleted and are replaced with an expression cassette that can be up to 8 kb in length.

Unique to lentiviruses are the central polypurine tract (cPPT) and central termination sequences (CTS), *cis*-acting sequences that coordinate the formation of a central DNA flap which improves nuclear import [99]. This may explain the increased transduction efficiency of lentiviral compared to retroviral vectors. A *rev*-responsive element can be incorporated into the vector to facilitate the nuclear export of unspliced RNA. The packaging of the genomic RNA is controlled in *cis* by the packaging signal Ψ.

Despite the greater complexity of the lentivirus genome, the basic principles of generating vectors free of replication-competent virus are similar to those for retroviral vectors. Packaging systems use the HIV *gag* and *pol* genes, with or without the *rev* gene [100,101]. The HIV virulence genes *tat*, *vif*, *vpr*, *vpu* and *nef* are completely absent from lentiviral vectors, thus making it theoretically impossible that a virus similar to HIV can be inadvertently produced [102]. The production of lentiviral vectors in high concentrated titers is still a challenge, but the production issues are slowly being solved [98].

The cellular receptors for the lentivirus vectors are the same as for HIV1-1, including CD4, together with the chemokine co-receptors CCR5 and CXCR4 (Table 34.2). However, the lentiviral gene transfer vectors are usually pseudotyped with envelopes from other viruses, such as VSV-G, mediating the binding and entry into target cells similar to retroviral vectors. Efficient gene transfer of lentiviral vectors has been reported for a variety of dividing and non-dividing cell types, including muscle, neurons, glia and hematopoietic cells. Lentivirus vectors have been successfully used to correct disease phenotypes in experimental animals for central nervous system (CNS) disorders such as metachromatic leukodystrophy using *in vivo* administration of the lentiviral vector to the brain [103] or, using an *ex vivo* strategy, by infecting hematopoietic stem cells with the β-globin gene to correct β-thalassemia [104]. There have only been a few clinical studies using lentiviral vectors, but the results for an *ex vivo* approach correcting hematopoietic stem cells with lentiviral-mediated gene transfer in patients with adrenoleukodystrophy, a severe demyelinating disease, or thalassemia have been very promising [14,16,96].

Like retroviral vectors, insertional mutagenesis is a concern for the clinical use of lentiviral vectors, and liver cancers have been observed in mice infected *in utero* or neonatally with lentiviral vectors [105]. One hypothesis for this phenomenon is based on potentially oncogenic sequences present in the woodchuck hepatitis virus-derived post-transcriptional

regulatory element (WPRE) that was included into the vector to increase mRNA stability [105]. Approaches that have been taken to decrease the risk of genotoxicity in lentiviral vectors include:

1) Self-inactivating (SIN) long terminal repeats that delete transcriptional control elements [25,106];
2) Integration systems that do not bias towards insertion near active genes or promoters [107]; and
3) Weaker internal promoters [108].

Besides being the most promising vector system for *ex vivo* gene correction strategies, lentiviral vectors have evolved as a powerful tool to silence gene expression by RNA interference [109]. Extensive human and murine siRNA-expressing lentiviral libraries are now available (RNAi consortium: http://www.broadinstitute.org/rnai/trc, accessed July 11, 2013) and lentiviral vectors are also efficient to knockdown cellular miRNA [110].

CELL-SPECIFIC TARGETING STRATEGIES

Viral gene transfer vectors use the receptors of their wild type versions to enter into cells (Table 34.2). Modification of gene transfer vectors to target specific cell types or tissues is an attractive means of increasing the specificity of the vectors to the target cell, and may also enable the vectors to infect cells which are usually not infected by unmodified vectors. In general, targeting modifications can be accomplished by either genetic modification of the vector genome to change the properties of the outer surface of the vector or by chemical modification of the vectors via the addition of ligands (Table 34.3). Based on the targeting strategy used, the range of tropism can be widened or narrowed (Fig. 34.4).

Targeting of Ad vectors

Strategies have been developed to re-direct Ad tropism and to enhance Ad tropism for cells difficult to transfer genes to because of lack of Ad receptors. In general, Ad vectors exhibit a broad tropism due to the widespread expression of the primary Ad receptor CAR and the secondary integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$. While the widespread expression of the Ad receptors enables the efficient infection of a wide range of target cells, it poses the problem of unwanted uptake and gene expression in non-target tissue when the vectors are administered *in vivo*. There are some tissues that have low expression of CAR (e.g., endothelial cells, antigen-presenting cells and some tumor cells), which limits the use of Ad vectors for these targets.

Most genetic targeting strategies for Ad vectors have been focused on ablating CAR binding and have introduced new peptides or other ligands to the fiber knob domain, the primary site for the interaction of group C Ad vectors with CAR [111]. Fiber modifications to modify the target cell binding of Ad vectors include the introduction of poly(L) lysine to allow binding to heparan-containing receptors and the integrin-binding motif RGD, which is essential for penton-mediated internalization to allow integrin-mediated binding and uptake [112]. Peptides can also be incorporated into other sites of the Ad capsid to achieve retargeting, such as incorporation of RGD into the hexon [113] and poly(L) lysine into polypeptide IX [114].

Another approach to the genetic retargeting of virus vectors is to create chimeras of different serotypes, which are known to use different cellular receptors. Replacing the fiber or fiber knob domain of the Ad5-based vector with that of Ad3 or Ad7 has been shown to achieve CAR-independent infectivity [115–117]. Replacement of the fiber of Ad2 with that of Ad17 has led to improved infectivity of airway epithelial cells [118]. Replacement with the fiber of Ad35 achieved improved infectivity of hematopoietic cells [119,120] and Ad16 enhanced infectivity of cardiovascular tissue [121]. Ad5 vectors pseudotyped with fibers of the subgroup D Ad 19 and 37 increased the infectivity of endothelial and smooth muscle cells [122]. Recognition peptides for fiber modification have been identified by phage display, [123–125] and other

TABLE 34.3 Alteration of viral gene transfer vector cell targeting by modifying capsid/envelope structure

Modification	Viral vector	Examples	Purpose/target	Reference
Genetic alteration of capsid/envelope proteins	Adenovirus	Delete CAR[1]	CAR-independent infection	[203]
		Add RGD[2] to fiber	$\alpha_V\beta_3$, $\alpha_V\beta_5$ integrins	[112]
		Add polylysine to fiber or protein IX	Broadening infectivity by targeting heparan-containing molecules	[112,114]
		Replace Ad5 fiber with Ad19 or Ad37 fiber	Improved infection of smooth muscle and endothelial cells	[122]
		Replace Ad5 fiber with Ad37 fiber	Infection of hematopoietic cells	[119]
		Replace Ad5 fiber knob with Ad3 fiber knob	CAR-independent infection	[117,204]
		Replace Ad5 fiber with Ad7 fiber	CAR-independent infection	[116]
		Replace Ad2 fiber with Ad17 fiber	Improved infection of airway epithelial cells	[118]
		Replace Ad5 fiber with Ad16 fiber	Improved infection of smooth muscle and endothelial cells	[121]
		Replace Ad5 fiber with trimerization motif of phage T4 fibritin	Chimeric fiber phage fibritin molecules targeted to artificial receptors	[126]
		Replace Ad5 fiber knob with CAV2[3] fiber knob	Target to CAR-deficient cells	[205]
		Replace Ad5 fiber with fiber of ovine AdV7	Targeting to kidney and 'detargeting' of CAR	[206]
		Add VSV[4]-G epitope to Ad5 fiber knob	Targeting tropism to CAR-deficient cell expressing phosphatidylserine	[207]
		Replace 7 hypervariable regions (HVR) of Ad5 hexon with HVR of Ad48	Circumvention of anti-Ad5 hexon immunity for Ad-based HIV vaccine	[50]
	Adeno-associated virus	Pseudotyping entire capsid with different serotype	Targeting tropism based on capsid serotype	[132]
		Pseudotyping with capsid of multiple serotypes	Generate mosaic AAV to target different serotype tropisms	[133,134]
		Addition of L14[5] to AAV2 capsid	Targeting to L14-binding integrins	[208]
		Incorporation of tumor-targeting peptide to AAV2 capsid	Targeting to CD13	[209]
		Insertion of serpin receptor ligand to AAV2 capsid	Targeting to serpin receptor	[210]
		Ad5 capsid with Ad37 fiber and partial AAV genome	Vector with increased capacity and tropism for hematopoietic cells	[119]

Continued

TABLE 34.3 Alteration of viral gene transfer vector cell targeting by modifying capsid/envelope structure—continued

Modification	Viral vector	Examples	Purpose/target	Reference
	Retrovirus	Pseudotyping entire envelope with envelope of vesicular stomatitis virus, Gibbon ape leukemia virus, murine leukemia virus	Targeting tropism of new envelope (from ecotropic to amphotropic)	[83,84,81]
		Addition of peptides: – Erythropoietin – Heregulin – Epidermal growth factor	Erythropoietin receptor Heregulin receptor Epidermal growth factor receptor	[145,146,148]
	Lentivirus	Pseudotyping entire envelope with envelope of vesicular stomatitis virus G	Improves infectivity to muscle	[139]
		Rabies 6 envelope	Improves infectivity to motor neurons	[142]
		Ebola	Improves infectivity for airway epithelial cells	[140]
		Hantavirus	Improves infectivity to endothelium	[143]
		Fowl plague hemagglutinin (with expression of influenza M2 protein)	Improves infection in airway epithelial cells,	[211]
Chimerical modifications of capsid/envelope	Adenovirus	Cationic lipids, cholesterol, polycationic polymers	Modify tropism	[129,130]
		Polyethylene glycol	Decrease immunity	
		Coupling of U7 peptide to Ad5	Targeting of urokinase plasminogen activator receptor on airway cells	[212]
		Addition of single chain antibodies	Target antibody ligand-expressing cells	[213]
	Adeno-associated virus	Chemical conjugation of avidin-linked ligands to AAV2	Targeting to ligand receptors	[214]
		Incorporation of bispecific antibody to AAV2	Targeting to human megakaryocytes	[215]
	Lentivirus	Polyethylene glycol	Prolonging half-life in serum by preventing inactivation	[150]
	Retrovirus	Addition of single chain antibodies	Target antibody ligands	[147]

[1]*CAR – Coxsackie adenovirus receptor.*
[2]*RGD – Integrin-binding peptide arginine (R), glycine (G), aspartate (D).*
[3]*CAV2 – Canine adenovirus 2 (Summerford and Samulski, 1998; Qing et al., 1999; Summerford et al., 1999; McCarty et al., 2004).*
[4]*VSV – Vesicular stomatitis virus (Summerford and Samulski, 1998; Qing et al., 1999; Summerford et al., 1999; McCarty et al., 2004).*
[5]*L14 – Integrin-binding motif.*

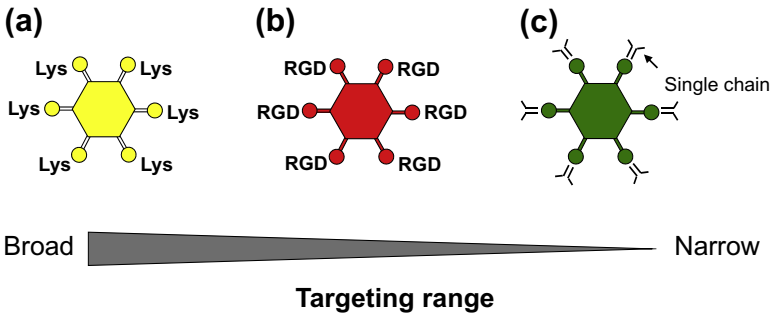

FIGURE 34.4
Examples of modifications of tropism of gene transfer vectors. Shown are three examples of modification of adenovirus vector tropism. (a) The addition of polylysine to the Ad fiber protein provides broad tropism; (b) the addition of the integrin-binding motif RGD enhances targeting of cells expressing $\alpha_v,\beta_{3,5}$ integrins; and (c) the addition of single chain antibodies to the Ad fiber targets cells expressing the antibody ligand. Strategies AC provide broad to narrow cell-specific targeting.

complex genetic modifications of the fiber to retarget the Ad vector have been reported [126,127]. These cell-targeting modifications can also be combined with the use of tissue-specific promoters to achieve selective infection and transcription in the targeted cell type. For example, inserting the RGD motif into the fiber has been combined with the use of the endothelial cell-specific Flt-1 promoter, which resulted in more specific infection and gene expression in endothelial cells [128]. Finally, a capsid-modified Ad/AAV hybrid vector was able to achieve long-term expression in human hematopoietic cells [119].

Besides genetic modification of gene therapy vectors to modify tropism and target vector uptake to a particular cell type, chemical modifications of Ad vectors have been utilized for targeting. Ad vectors have been complexed to cationic lipids, polycationic polymers or cholesterol to increase the efficiency of gene transfer *in vitro* and *in vivo* [129–131]. Bispecific monoclonal antibodies have been added to the fiber to target specific cell types expressing the ligand for that antibody.

Targeting of AAV vectors

The strategy of creating AAV chimeras by pseudotyping with capsids of different AAV serotypes has been used to broaden the tropism of AAV vectors [10,132–134]. Most of these vectors used the vector genome derived from AAV2 along with the capsid from another serotype. As an example, this strategy has enabled enhanced transduction in lung-directed gene transfer using an AAV5 pseudotype [135]. This *in vitro* process also been used *in vivo* with systemic administration of a chimeric viral library, resection of the target tissue and PCR amplification of the sequestered vector genomes [136]. This approach has led to the identification of a heart-tropic [137] and neuron-tropic [138] AAV chimeric vectors.

Targeting of retroviral and lentiviral vectors

The classic method to broaden the tropism of retroviral and lentiviral vectors is by pseudotyping, creating chimeras using envelope glycoproteins from other viruses. Most retrovirus vectors are based on MuMoLV, an ecotropic virus, which infects only murine cells. To achieve infection of human cells, the vectors are propagated in packaging cells that express the envelope of the amphotropic or non-murine viruses such as 4070A murine leukemia virus, gibbon ape leukemia virus, VSV or the feline endogenous virus RD114 [83,84]. Various envelopes have also been used for lentiviral vectors to increase infectivity of specific cell types, including VSV-G for muscle [139], Ebola for airway epithelial cells [140,141], rabies-G for motor neurons [142] and hantavirus for endothelium [143]. Hybrid proteins of the murine amphotropic envelope have been combined with the extracellular domains of GALV or RD114 envelope to enhance infection of CD34+ cells [144,143]. As with Ad vectors, strategies have been developed to target retro- and lentiviral vectors by adding ligands to the envelope

glycoprotein. Examples include peptide sequences from erythropoietin [145], heregulin [146], epithelial growth factor and ligands for the Ram-1 phosphate transporter [81] as well as the addition of single chain antibodies [147,148]. The efficiency of these modifications, however, has not been very high and the vector production yield is significantly impaired.

Another strategy has been to utilize the membrane proteins that are incorporated during the budding process for targeting. For example, incorporation of the membrane-bound stem cell factor provided not only a growth signal for the CD34+ cells expressing c-kit, the receptor for stem cell factor, but also lead to increased infection efficiency of the CD34 cells [149]. Monomethoxy poly(ethylene) glycol conjugated to VSV-G protects the vector from inactivation in the serum, leading to a prolonged half-life and increased transduction of bone marrow following intravenous administration in mice [150]. Another strategy for targeting of retro- and lentiviruses *in vivo* has been to target retrovirus producer cells [151].

REGULATED EXPRESSION OF THE TRANSFERRED GENE

A variety of strategies have been developed to regulate expression of the genes transferred by gene therapy vectors (Table 34.4). The ability to regulate gene expression is particularly important for applications where too much expression of the gene transfer product could lead to unwanted effects, e.g., sustained expression of a growth factor that could potentially be tumorigenic. For *in vivo* strategies of gene transfer, tissue-specific regulation of gene expression may be warranted to avoid expression of genes in undesired cells or tissues. In the context of the use of gene transfer vectors to genetically modify stem cells, regulation of gene expression may be critical to avoid differentiation into an unwanted tissue or cell type. The regulation of the gene expression of the transgene could also be combined with inducible systems of suicide genes or factors that could destroy the genetically modified cells, should unwanted differentiation occur.

To turn gene expression on and off at will, a number of inducible promoters and inducible regulated systems have been developed that are applicable to be used in gene transfer vectors (Table 34.4). For example, inducible gene expression in gene transfer vectors can be achieved using inducible promoters such as those responsive to glucocorticoids, cGMP, heat shock protein, radiation, and insulin. Inducible regulated systems also include systems based on response to tetracycline, antibiotic resistance, chemical-induced dimerization, steroid receptors and insect ecdysone receptors. The basic mechanism for these systems is a combination of ligand-binding synthetic inducer or repressor proteins and a promoter control system that regulates transgene expression.

The tetracycline-responsive system has been widely used to study gene function and to generate conditional mutants in cell lines and transgenic animals [239]. The transgene is placed behind a promoter that also contains binding sites for the tetracycline response element (TRE), which can act as a repressor or inducer of transgene expression. A fusion protein that binds to the inducer doxycycline needs to be present on a separate gene construct. The tetracycline transactivator binds to the TRE and activates the transcription in the absence of doxycycline. Upon addition of doxycycline, the expression is turned off (Tet-off). Another fusion protein that can be used is the reverse tetracycline transactivator, which only binds to TRE in the presence of doxycycline, causing induction of expression upon addition of doxycycline (Tet-on). Expression can be controlled in a graded manner: the more doxycycline is added, the greater the level of suppression or induction. The disadvantages of this system are the potential side effects of doxycycline. The tetracycline system has been used for regulated gene transfer with gene therapy vectors, including Ad [103,152], AAV [153–155], retroviral [156,157], and lentiviral vectors [158,159].

The steroid receptor systems use a mifepristone-binding progesterone receptor fused to the DNA-binding domain of the yeast GAL4 protein and the transactivation domain from the

TABLE 34.4 Regulation of Expression of the Transferred Gene

Category	Strategy	Note	Reference
Inducible promoter	Glucocorticoid-responsive	Multiple response elements	[216]
	cGMP[1]-responsive	Multiple response elements	[217,218]
	Heat shock protein-inducible	Hyperthermia and cellular stress induce gene expression	[219,220]
	Radiation-inducible		[221]
	Insulin-responsive		[221,222]
	Tetracycline-responsive	Repressible-TET_{off} or inducible TET_{on} systems	[103,156,153,158, 159,154,155]
	Antibiotic resistance	Streptogramin class antibiotic (e.g., pristinamycin) induces pristinamycin-induced protein preventing expression	[223]
		Erythromycin binds to prokaryotic DNA-binding protein MphR(A)[2]	[224]
	Chemical-induced dimerization (FKBP/FRAP[3])	Rapamycin induces heterodimerization of FKBP and FRAP	[225–227]
	Steroid receptor	Transactivator (GLVP[4] or Glp65) targeting genes with GAL-4-binding site in the presence of mifepristone (RU486)	[222,162]
	Insect ecdysone receptor	Ectodysone receptor ligand induces transactivation of transgene	[161,160]
Tissue-specific promoter	Liver	Albumin, α1-antitrypsin, liver- activated protein (LAP), transthyretin promoters	[228–231]
	Smooth muscle	Smooth muscle actin, SM-22, smooth muscle myosin heavy chain promoter	[232,166]
	Prostate	Prostate-specific antigen promoter	[233,234]
	Neuron	Synapsin I, neuron-specific enolase	[163]
	Dendritic cells	Dectin 2, vFLIP	[164,165]
	Vasculature	VE-cadherin, smoothelin B	[235]
	Tumor vasculature	Tie2	[236]
Trans-splicing	Therapeutic *trans*-splicing	Target pre-mRNA is trans-spliced into independent pre-mRNA	[170,237]
State of differentiation	Differentiation-specific endogenous transcriptional regulatory elements	Beta-globin gene expression by endogenous transcriptional regulatory elements or from other genes with a similar expression pattern	[171,172]
Gene disruption	Non-homologous end-joining gene disruption by zinc finger nucleases	Disruption of CCR5 gene to create resistance to HIV in T cells and hematopoietic stem cells	[173,174]
MicroRNA	Target cell-specific microRNAs	No transgene expression by incorporating a target sequence for microRNA in the 3′UTR	[238,176]

[1]*cGMP – cyclic guanosine monophosphate.*
[2]*MphR/A – a prokaryotic DNA-binding protein that binds to a 35 bp operon sequence.*
[3]*FKBP (McCarty et al., 2004) – FK506-binding protein; FRAP – FK506-binding protein rapamycin-binding).*
[4]*GLVP – a mifepristone-activated chimeric nuclear receptor.*

NfkB p65 subunit [160,161]. The fusion protein binds to a GAL4 activating sequence to regulate gene expression. Upon addition of mifepristone, gene expression is induced, and upon removal of the drug, gene expression returns to baseline within five days.

The insect ecdysone receptor system consists of a fusion protein of the transactivation domain of the glucocorticoid receptor fused to the ecdysone-binding nuclear receptor and an ecdysone-response element placed upstream of the promoter driving the transgene expression

[161,162]. Upon addition of ecdysone, an insect hormone with no mammalian homologs, the fusion protein dimerizes and induces expression. Because there are no known mammalian factors binding to the insect protein, there is very low background expression in the absence of the drug and expression can be very tightly controlled.

Another strategy to control gene expression at the desired location is the use of tissue-specific promoters (Table 34.4). The majority of tissue-specific promoters have been used to target expression to the liver and the cardiovascular system by targeting muscle cells. Recently, lentiviral vectors with tissue-specific gene expression in dendritic cells and neurons have been reported [164–166]. The challenge in the use of tissue-specific promoters is that the level of expression is usually lower than the commonly used strong viral promoters. However, viral promoters such as CMV have been shown to be subject to silencing after several weeks *in vivo*. This has been seen in airways, cardiomyocytes and smooth muscle cells, in particular with non-viral gene transfer [167].

Another modality for regulating gene expression is by *trans*-splicing at the pre-mRNA level. In therapeutic trans-splicing, the sequence of the target pre-mRNA is modified by being trans-spliced to an independent pre-mRNA, the sequences for which are delivered exogenously by a gene transfer vector [168,60]. Therapeutic *trans*-splicing can be used to alter coding domains, to create novel fusion proteins, to direct gene products to various cellular compartments and to enable gene therapy with large genes or genes coding for toxic products. *Trans*-splicing gene transfer strategies also offer the advantage that the expression of the *trans*-spliced sequence is controlled by endogenous regulation of the target pre-mRNA. Trans-splicing strategies have been used to correct animal models of hemophilia, X-linked immunodeficiency with hyper IgM and cystic fibrosis [169,170,177].

To achieve transgene expression limited to the therapeutic target cells, endogenous transcriptional regulatory elements from the endogenous locus or from other genes with a similar expression pattern can be incorporated into the vectors [171,172]. This approach will be useful, for example, in gene therapy for thalassemia or sickle cell disease.

Another approach to selective expression is T cells that are resistant to HIV infection, generated through disruption of the expression of CCR5, an essential HIV coreceptor, by a gene transfer strategy of non-homologous end-joining gene disruption by zinc finger nucleases [173,174].

Another strategy to regulate transgene expression is to take advantage of the differential expression of microRNAs between mature cell lineages and stem cells [175,176]. Transgene expression can be suppressed in cells expressing a specific microRNA by incorporating a target sequence for that microRNA in the 3′ untranslated region [110,176].

COMBINING GENE TRANSFER WITH STEM CELL STRATEGIES

In general, stem cells are categorized into embryonal stem cells, the bone marrow-derived stem/progenitor cells, including mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and the tissue-derived stem cell populations [178]. Stem cells offer the potential for tissue regeneration, and the combination of gene therapy and stem cell approaches is a promising strategy for directing the differentiation of stem cells into the desired cell type, and for regulating and controlling the growth and differentiation of the stem cells. Stem cells have the potential for both self-renewal and differentiation, and are dependent on signals from their microenvironment to direct stem cell maintenance or differentiation. The precise spatial and temporal presentation of these signals is critically important in development and will most likely be the decisive factor in the success of stem cell therapies [179]. As the potential of stem cells to be used clinically becomes more realistic, gene transfer strategies may play a pivotal role in controlling stem cell growth, by either preventing uncontrolled growth and/or directing differentiation into specific cell types, and regulating gene expression. In addition,

as more of the microenvironmental cues that control stem cell differentiation into the desired phenotypes become known, the potential to use gene transfer strategies to express or inhibit these signals becomes a viable strategy to support stem cell therapy. The use of gene transfer for reprogramming somatic cells to pluripotency has been a rapidly growing field over the past years [180]. Genetic modification of these cells using gene therapy holds immense promise for cell-based therapies and tissue engineering, once safe and robust methods to generate these cells and their differentiation have been established. Three general areas can be currently envisioned for gene transfer to be helpful in stem cell therapies:

1) To control unwanted stem cell growth or differentiation;
2) To provide environmental cues for stem cell differentiation; and
3) To regulate gene expression.

In addition, gene transfer may also be useful for marking stem cells as a modality to track the cells and their progeny *in vivo* [181].

Gene transfer to stem cells

The challenges in developing gene transfer strategies for stem cells are:

1) How to most efficiently infect the cells;
2) Gene silencing during differentiation;
3) If gene expression should be transient or persistent; and
4) If gene expression is persistent, should it terminate once the cell has differentiated into the desired phenotype?

Gene transfer has been accomplished into a variety of stem cell types. Human and murine embryonal stem cells have been successfully infected with subsequent gene expression with adenovirus, AAV and lentiviral vectors [182]. The reported efficiency of each of these vector systems is limited, however, requiring selection to obtain pure populations of genetically modified cells. Gene expression can be affected by subsequent differentiation of the cells, and it is not clear how the stem cell properties are affected by the various gene therapy modalities themselves without transgene expression. Bone marrow-derived endothelial progenitor and MSCs have been successfully transduced with the most commonly used gene therapy vectors [4].

Gene transfer to control uncontrolled stem cell growth

Gene transfer strategies developed to control the growth of cells for cancer gene therapy applications can be applied to controlling the unwanted growth of stem cells, in particular, the risk of the development of teratomas with embryonic stem [183]. Some of the suicide gene transfer strategies, including the prodrug strategies using herpes simplex thymidine kinase (HSV-TK) (ganciclovir) and cytosine-deaminase (5-fluorocytosine) that lead to activation of cell death following administration of the prodrug may prove useful. Other suicide genes used with regulatable gene expression systems (as outlined above, Table 34.4) may be useful for controlling potential malignant stem cell growth, as long as there is no 'leak' of baseline gene expression of the suicide gene.

Gene transfer to instruct stem cell differentiation

Gene transfer strategies may prove useful in providing the necessary environmental factors necessary to develop specific phenotypes from stem cells. For example, expression of growth factors or other known differentiation signals secreted by the stem cell should aid in mediating differentiation, i.e., the genetically modified stem cell would create its own microenvironment, favorable for differentiation. Expression of these factors could be regulated by inducible gene expression systems. For example, the cytokines required for the differentiation in the respective lineage have been identified for muscle-derived MSCs, with their potential for the treatment of skeletal, cardiac and smooth muscle injuries and disease [184]. The technology of

induced pluripotent stem cells has been possible by this approach. Differentiated pluripotent cells can be either generated from embryonic stem cells (donor oocytes) or from reprogrammed somatic cells, induced pluripotent stem cells (iPSCs) [18]. Induced pluripotent stem cell technology has emerged as one of the most promising strategies to generate patient-specific embryonic and adult stem cells [17,18]. IPSCs were initially developed from murine somatic cells by expressing reprogramming transcription factors implicated in pluripotency [185]. The human version was developed shortly thereafter [186–189]. The factors required for reprogramming somatic cells have been introduced by retroviruses and lentiviruses. Because of the risks with integration and that low-level residual expression of reprogramming factors may alter the differentiation potential of the human iPSCs, non-integrating vectors, plasmids and Ad vectors have also been used for transient expression of reprogramming factors in mice to generate iPSCs [190,191], although with much lower efficiency. Episomal vectors, based on the Epstein-Bar virus, have been used successfully [193]. Non-viral methods also used include expression plasmids, piggyback transposone, Cre/loxP excisable polycistronic constructs, CPP-tagged reprogramming proteins and synthetic modified RNA [189,17,18].

Gene transfer to regulate gene expression

Regulation of gene expression is critically important in the maintenance and differentiation of stem cells, and the transfer of genes coding for factors that regulate endogenous gene expression in response to specific stimuli could prove very helpful for stem cell therapies. This concept has been used in gene transfer strategy to regulate angiogenesis in the ischemic myocardium. AAV vector-mediated transfer of a hypoxia, a responsive element to ischemic myocardium, resulted in endogenous expression of the vascular endothelial growth factor (VEGF) [193]. Similarly engineered transcription factors capable of activating endogenous VEGF expression have been successfully transferred with adenoviral vectors [194]. As more of the gene expression and regulation patterns critical for stem cell differentiation and maintenance are known, the possibilities to direct or aid in gene expression using gene transfer are significant.

One potential barrier to using embryonic stem cells in humans is rejection of the transplanted cell by the immune system [196]. This could theoretically be circumvented by using gene transfer with the relevant gene to autologous stem cells. For example, transfer of genetically modified iPSCs (Fig. 34.5a) or transfer of a genetically corrected nucleus to an enucleated egg from an unrelated donor would result in the generation of genetically modified embryonic stem cells that could be then differentiated, and the corrected, differentiated cells transplanted to the same patient (Fig. 34.5b).

CHALLENGES TO GENE THERAPY FOR TISSUE ENGINEERING

Although proven to be very effective in a variety of model systems, the major challenges for gene therapy to cure human diseases include circumvention of immune responses against viral vectors, transferring the genes to a sufficient number of cells to change the phenotype and controlling the expression of the gene. The main hurdle for successful gene therapy to compensate for a missing or defective protein has been the host response to the gene therapy vector, the lack of long-term gene expression and problems related to integration into the host genome. However, short-term expression of transgenes has been feasible in humans and has been shown to be efficient in a variety of cell types and tissues. Immunogenicity against a non-self transgene, as well as vector-derived proteins, may be an issue if gene transfer is being used for permanent expression of a transgene. Controlling the gene expression is a challenge that needs to be addressed, especially if gene transfer strategies are combined with stem cell strategies. Most regulatable gene expression systems show some baseline expression, which may be problematic if gene transfer is used to regulate gene expression for stem cell differentiation.

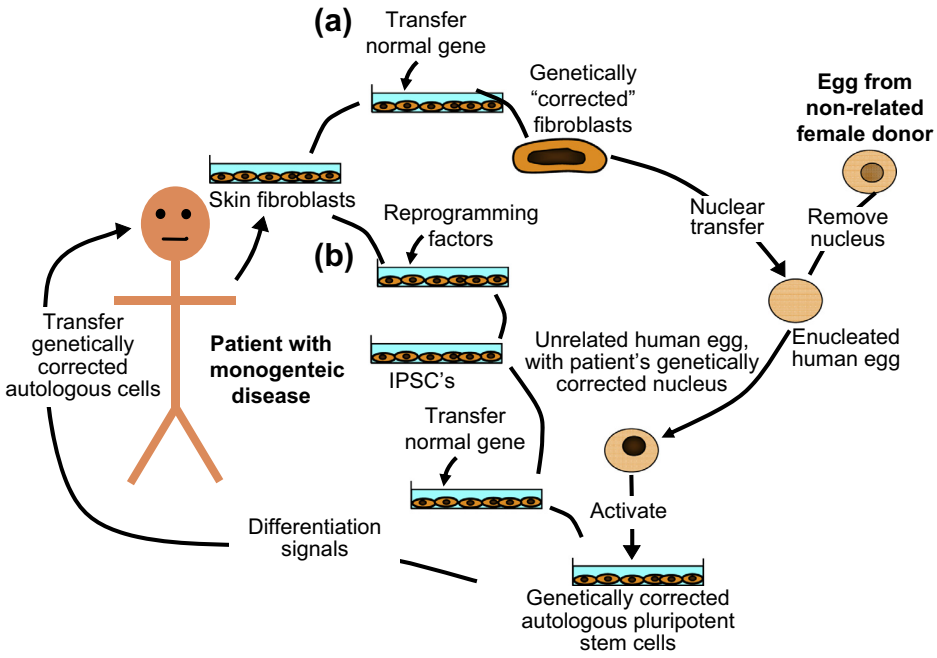

FIGURE 34.5

Strategies to combine gene therapy with nuclear transfer and stem cell therapy. Shown are two examples to genetically modify skin fibroblast or skin-fibroblast-derived induced pluripotent stem cells of an individual with a monogenetic disease to correct the abnormality. (a) The nucleus of the genetically corrected fibroblast is transferred to an enucleated egg of an unrelated donor to generate corrected autologous pluripotent stem cells. (b) Induced pluripotent stem cells are genetically corrected. Both genetically corrected pluripotent stem cells can then be differentiated and transferred back to patient.

Over the past decade, progress has been made in addressing many of these challenges. Gene therapy in humans has led to promising results for *ex vivo* hematopoietic stem cell gene transfer with retroviral and lentiviral vectors and for *in vivo* gene therapy with AAV vectors. As the focus continues on solving the remaining issues, together with gene therapy, the knowledge gained from successes and setbacks will prove beneficial in its use for tissue engineering and stem cell applications.

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CHAPTER 35

Gene Delivery into Cells and Tissues

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INTRODUCTION

This chapter provides an overview of intracellular gene and nucleic acid delivery by engineered nanoparticles (NPs) and viral vectors, including particle and vector design, strategies for overcoming cellular barriers, and methods for delivery to cells that are particularly relevant to tissue engineering and regenerative medicine. NPs include a variety of engineered, chemically defined, colloidal objects capable of entering cells and tissues and delivering cargo intracellularly. Viral vectors broadly include a variety of infectious particles with diverse properties that can be adapted to tissue engineering and other applications. This chapter focuses on nonviral and viral design approaches for safe and efficacious intracellular delivery of nucleic acids, including design of gene-functionalized tissue-engineering scaffolds for local delivery to pathological sites.

FUNDAMENTALS OF GENE DELIVERY

The engineering of nonviral and viral nucleic acid delivery systems is a valuable approach for enhancing tissue engineering and regenerative medicine. Nucleic acid-based drugs are broadly sought for the replacement of missing, mutated, or deficient genes, for augmentation of cell function by gene addition or overexpression, and for gene suppression by RNA interference (RNAi) to silence aberrant or pathologic genes. Gene replacement or addition is a more mature but still challenging field that involves transfer and chromosomal integration of DNA that encodes the desired gene through nonviral or viral delivery.

Because of the potency, specificity, and transient activity of RNAi, engineering vectors for delivery of this class of gene therapy has become a major focus of the tissue engineering and medical communities, and significant progress has been made toward technologies that enable therapeutic gene silencing *in vivo*. Gene expression knockdown can be achieved through several different strategies, the earliest of which was utilization of antisense oligodeoxynucleotides (ODNs), which are single-stranded DNAs that bind to complementary mRNAs and yield a relatively modest reduction in gene expression [1]. The mechanism for endogenous post-transcriptional regulation through microRNA (miRNA) was subsequently elucidated, and it was discovered that delivery of double-stranded RNA (dsRNA) achieved more potent gene

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silencing than ODNs [2,3]. Short hairpin RNA (shRNA), which more closely mimics the structure of endogenous miRNA, has also been delivered directly or via encoding plasmids [4]. In both cases, the enzyme Dicer cleaves the larger precursor/transcript to form small interfering RNA (siRNA), which is a 19−21 base pair, double-stranded RNA [5]. siRNA can also be delivered directly to cells, a strategy that circumvents the need for Dicer processing and allows direct loading into the RNA-induced silencing complex (RISC). The activated RISC mediates recognition and enzymatic degradation of mRNA that is complementary to the siRNA antisense/guide strand [3]. Due to the greater instability of RNA, the therapeutic application of RNAi is more susceptible to degradation and poses unique hurdles, compared to DNA delivery.

Gene augmentation/replacement and RNAi therapies face both common and unique delivery barriers. Both approaches require intracellular delivery across the plasma membrane. Therapies for stable gene expression, including shRNA, must localize to the cell nucleus, while RNAi activity of siRNA depends upon delivery to the cytoplasm where the RISC machinery is located. In both cases, a variety of viral and nonviral delivery approaches can accomplish these tasks. Nonviral approaches, which offer a potential safety margin, typically involve formulation with synthetic reagents such as polymers or lipids; however, these synthetic formulations are typically less efficient at triggering cell uptake and navigating subsequent intracellular delivery barriers. Recent advances in viral technologies have made them safer, and there are numerous ongoing trials for clinical applications of viral technologies. At the same time, high throughput screening and other advanced synthetic approaches are yielding more efficient nonviral carriers than ever before, and recently, the results have been released from the first successful RNAi clinical trials involving delivery with synthetic nanovectors. The sections 'Viral Nucleic Acid Delivery' and 'Nonviral Nucleic Acid Delivery' below survey the latest technologies for both nonviral and viral approaches to nucleic acid-based drug delivery, and subsequent sections address tissue engineering applications and recent clinical trial results.

BIODISTRIBUTION, TARGETING, UPTAKE, AND TRAFFICKING

For successful gene therapy *in vivo*, viruses or nonviral plasmid/siRNA carriers have three main delivery barriers that must be overcome:

1) Efficient biodistribution to the desired site of action (i.e., tumor or site of tissue damage/dysfunction),
2) Internalization of the cargo by the targeted cell type, and
3) Trafficking to the subcellular compartment where the cargo will be active (i.e., cytoplasm for siRNA or nucleus for plasmid).

Viruses have evolved to efficiently overcome the latter two barriers, but achieving efficient access to the desired target cells is still a challenge. The successful design of synthetic delivery systems requires consideration of all three of these barriers and a thorough understanding of the mechanisms that dictate the interactions of nonviral delivery systems with target cells.

Tissue biodistribution/targeting

The most straightforward approach to site-specific delivery is local application, such as inhalation of dispersed solutions into the lungs to treat cystic fibrosis, or direct injection into the affected tissue, such as muscle for muscular dystrophy. Local, sustained delivery from biomaterial scaffolds is another approach that is especially popular for tissue-engineering, and because this is the unifying topic of this book, we have dedicated the section 'Engineering Tissue Scaffolds for Viral and Nonviral Nucleic Acid Delivery' to tissue-engineering-specific gene therapy technologies. The current section discusses methods for enhancing target site-specific bioactivity of nucleic acid-based drugs delivered intravenously. Systemic delivery systems should be designed with functionalities that enhance the deposition or accumulation (biodistribution) of the therapeutic in a particular tissue, prolong the association of the

therapeutic within the target site, mediate specific binding to and uptake by a target cell population, and increase trafficking of the cargo to the desired intracellular compartment. Typically these functions are achieved through optimization of physicochemical properties to preferentially enhance non-specific accumulation at defined sites and/or inclusion of ligands that target cell receptors uniquely expressed or overexpressed by the target cell type.

Key characteristics for intravenously delivered drug carriers are avoidance of rapid blood clearance by the reticuloendothelial system and preferential accumulation and retention at the site of disease. Long blood half-lives are vital for optimal performance of delivery systems designed to passively accumulate in target tissues. For example, drug carriers with long circulation times can extravasate through abnormally large fenestrations of the cancer vasculature and non-specifically accumulate into tumor tissues through the enhanced permeation and retention (EPR) effect [6]. The EPR effect is believed to occur more in tumors than healthy tissues because tumor blood vessels are characterized by poorly adherent endothelial cells with wide fenestrations and the lack of a smooth muscle cell layer. Therefore, there is easier passage of larger biomacromolecules or NPs from the vasculature. Furthermore, poorly formed lymphatic drainage in tumors is believed to increase retention at these sites. Utilization of EPR-like vascular leakiness to achieve non-specific local accumulation is also potentially a generalized approach for targeting sites of tissue inflammation. For example, in osteoarthritis, synovial inflammation increases local vascular permeability, and long-circulating delivery systems can more readily accumulate at these sites relative to healthy tissues [7].

Optimization of physicochemical properties such as surface charge, size, shape, and mechanical properties are major design considerations for achieving the desired biodistribution of nonviral delivery systems (see recent reviews [8,9]). Traditionally, nanocarriers have been synthesized using bottom-up synthetic approaches to form NPs such as micelles, dendrimers, and liposomes (examples discussed in more detail below in the section 'Nonviral Nucleic Acid Delivery') that have been predominantly limited to formation of structures spherical in shape. For these spherical structures, it has been hypothesized that NPs in the size range from approximately 10—200 nm may be ideal. Structures smaller than 10 nm suffer from rapid renal filtration and systemic clearance. It has been reported that the upper limit for escape from tumor leaky vasculature may be as high as 1.2 μm, though it varies based on tumor location and type [10,11]. In a study of 80, 170, and 240 nm spherical NPs, the smallest NPs had longer circulation times, decreased hepatic clearance, and increased tumor accumulation relative to larger particles [12]. Furthermore, smaller particles would be more desirable in terms of more rapid diffusion through the tissue matrix and potentially achieving more homogenous tissue distribution following vascular extravasation [13]. Thus, it may be desirable to design gene delivery vectors to be as small as possible, as long as they are above the size cutoff for renal filtration.

In addition to size, the shape of nonviral vectors has a significant effect on behavior for *in vivo* systemic delivery applications; for example, rod-shaped filomicelles have ten times longer circulation times relative to spherical micelles [14]. Recently, innovative techniques have been developed in labs such as those of Mitrogotri [15], DeSimone [16], and Roy [17] that enable nanofabrication of particles with arbitrarily-defined size and shape. Decuzzi and Ferrari have contributed computational modeling and *in vivo* experimental data on this topic, and they have uncovered a significant effect of particle shape on biodistribution and ability to achieve vascular targeting [18]. In recent studies, these authors showed that shape-defined, discoidal porous silicon nanovectors accumulate up to five times more in tumor tissues than spherical particles with similar diameters, and these shape-defined particles have also been effectively utilized for two-stage delivery of siRNA [19]. In the case of micron sized particles, stiffness of particles has been shown to have a significant impact, with stiffer particles rapidly distributed to the lungs, and those with a lower elastic modulus preferentially sequestered in the spleen [20].

Surface chemistry, in particular surface charge, can be tuned to act synergistically with physical properties such as size and shape in order to engineer optimal delivery systems. For example,

neutrally-charged NPs generally have lower rates of opsonization [21], and cationic NPs with superficial amines produce the highest levels of complement activation [22]. Xiao et al. recently utilized a micellar NP (~20 nM in size) to systematically study the effect of surface charge on biodistribution of a series of seven NPs with zeta potential ranging from −27 to +37 mV in tumor-bearing mice [23]. Using a ratio to represent the preferential accumulation in the tumor relative to the liver, NPs with a zeta potential of −27, −18, −9, +4, +19, +30, and +37 mV produced tumor:liver ratios of 0.82, 1.28, 1.75, 1.18, 0.84, 0.60 and 0.40, respectively. The authors determined that a 'slight' negative charge (in their case, −8.5 mV zeta potential) was ideal for tumor accumulation and that 'highly' positively or negatively charged NPs were preferentially cleared by macrophages (Kupffer cells) in the liver. Arvizo et al. [24] produced similar results using gold NPs, and they found that neutral or zwitterionic surfaces (−1.1 or −2 mV, respectively) produced longer circulation times and better tumor biodistribution than highly negative (−38 mV) or positive (+24 mV) NPs.

As discussed in more detail in the section 'Nonviral Nucleic Acid Delivery', most nonviral nucleic acid delivery systems involve formulation with an excess of cationic biomaterials in order to tightly package the polyanionic nucleic acid cargo into nano-sized particles. Unfortunately, the presence of surface-exposed cationic materials is not amenable to achieving the optimal, 'slightly' negative surface charge desirable for systemically delivered nonviral vectors. Attachment of poly(ethylene glycol) (PEG), or PEGylation, is a popular approach for giving cationic carriers more stealth by shielding non-specific cell interactions, reducing reticuloendothelial system clearance, and extending the blood circulation half-lives [25]. There are several fundamental properties of PEG that make it desirable for systemic delivery applications. It is a flexible, hydrophilic, and non-toxic polymer that reduces opsonization and non-specific interaction with blood cells. It can diminish the access of degradative enzymes to drug cargo and provide increased steric bulk, which minimizes rapid renal clearance. The desirable performance of PEG is well documented in these regards, and PEG polymers are incorporated into therapeutic proteins and liposomal drug nanocarriers currently in the clinic.

For gene therapy, one risk is that the inertness achieved by PEGylation can mask underlying functionalities necessary to trigger cell uptake and/or endosomolytic activity of nonviral delivery systems. The most common strategy for increasing internalization of PEGylated nanocarriers is incorporation of ligands, antibodies, or peptides that bind to specific cell receptors that internalize drug carriers and ferry them into the cell, and this approach will be discussed in more detail below. Several promising approaches are also under development for attaching PEG by linkages that are reversible when exposed to signals uniquely present in the target tissue. For example, one can use attachment chemistries that are labile when exposed to tumor microenvironmental hallmarks such as mild acidity and high matrix metalloproteinase (MMP) activity in order to trigger shedding of PEG from the nanocarrier surface [26–28].

Conjugation of cell receptor ligands to the surface of (PEGylated) nonviral carriers is one of the most studied preclinical approaches for enhancing localization, retention, and bioactivity in specific target tissues. Targeted delivery can potentially increase bioavailability of the therapeutic agent at its site of action and concurrently reduce off-site effects. Significant progress has been recently made in actively targeting unique markers on the vascular endothelium in specific tissues. For example, phage display technology has been instrumental in identifying targeting peptides that bind to specific 'vascular zip codes', and peptides identified using this technique present a potentially promising strategy for enhancing tissue-specific accumulation of nonviral delivery systems [29]. In order to achieve delivery into the tissue, transcytosis must occur across the vascular endothelium or, for the brain, the blood-brain barrier (BBB). Recently, systemic strategies to increase drug penetration into the brain have been developed, including temporary disruption of the blood brain barrier, chemical modification of available therapeutic substances, utilization of endogenous transport systems, and employment of lipid-based delivery systems [30].

Many other targeting approaches increase binding to cells at the target site once extravasation from the vasculature has occurred, i.e., following tissue accumulation through the EPR effect. For these approaches, biodistribution is primarily dictated by the physicochemical properties of the vector, and the targeting moiety affects retention and/or bioactivity at the site. Active binding to extravascular cells in the target site can enhance local retention through decreased diffusion or clearance from the tissue, and this prolonged residence time and/or targeting to actively internalized receptors can improve bioactivity. For example receptors for ligands such as transferrin [31] and folate [32] are commonly targeted because they have increased expression on a variety of cancer types and because targeting these internalizing receptors can be used to deliver cargo intracellularly through receptor-mediated endocytosis. The ligands themselves (i.e., folate and transferrin) can be used to functionalize nonviral vectors or antibodies against these receptors can be utilized. While there has been limited clinical success of targeted nonviral vectors to this point, a variety of techniques are being actively pursued to develop targeting moieties based on natural ligands, antibodies or their fragments, peptides, aptamers, sugars, and small molecules, and screening techniques such as peptide phage display are aiding in the discovery of new tissue-specific drug targets. Though they will not be reviewed in detail here, it should also be mentioned that several methods have also been studied for externally-stimulated tissue targeting and drug release in response to an externally applied stimulus, such as light, ultrasound, ionizing radiation, or magnetic force [33].

Cellular uptake and intracellular trafficking

Once gene delivery systems have reached the target tissue, their route of internalization and trafficking to the desired subcellular compartment is paramount to achieving optimal bioactivity. To a large degree the mode of internalization dictates the subsequent route of trafficking within the cell. Thus, an understanding of intracellular uptake and trafficking facilitates the design of better and more efficient gene delivery systems. For additional information, the reader is referred to recent reviews on nanovehicular uptake and trafficking [34,35].

Endocytosis is a generalized terminology used to describe cellular internalization. Endocytosis of nonviral vectors is the result of co-localization with the external side of the cell membrane (via receptors or non-specifically) followed by formation of invaginations in the cell's plasma membrane that bud off inside the cell, forming lipid bilayer-enclosed vesicles that sequester the internalized cargo. Three kinetic modes of endocytosis can be defined: fluid-phase, adsorptive, and receptor-mediated endocytosis [36]. Fluid-phase endocytosis refers to the bulk uptake of solutes in exact proportion to their concentration in the extracellular fluid. This is a low-efficiency, non-specific process. In contrast, receptor-mediated endocytosis involves internalization of macromolecules that are bound and concentrated at the cell surface before internalization. In adsorptive endocytosis, molecules preferentially interact with non-specific binding sites (e.g., electrostatic interaction of cationic vectors with anionic heparin sulfate proteoglycans). Internalized endocytotic vesicles subsequently undergo a complex series of fusion events that direct the internalized cargo to other intracellular compartments.

Endocytosis occurs by multiple mechanisms that fall into two broad categories: phagocytosis, or cell eating (of large particles), and pinocytosis, or cell drinking (of fluid and solutes) [37]. Phagocytosis is typically restricted to specialized mammalian cells (dendritic, macrophages, monocytes, and neutrophils). Distinct subcategories of pinocytosis will be discussed further below, including macropinocytosis (MPC), clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CavME), and lipid raft-mediated endocytosis (LRME). Transcytosis and exocytosis, though they will not be discussed at length here, are 'competing' cellular pathways that involve passage directly through the cell and expulsion of intracellular material into the external environment, respectively. A summary of different mechanisms is presented in Table 35.1, and schematic illustrations of different uptake/exocytosis mechanisms and their possible intracellular fates are depicted in Fig. 35.1.

TABLE 35.1 Efficiency of nanoparticle entry via multiple portals as differentiated by cargo chemistry/size[a] and cell type

Phagocytosis	Pinocytosis					
		Macropinocytosis (fluid phase)	Clathrin-mesiated (CME)	Caveolae-mediated rafts	Lipid rafts	Clathrin- and Caveolin-independent
Vehicle size	1–10 μm[b]	1–5 μm[b]	<150 nm	<60 nm	40–60 nm	200–300 nm
Cell types	Dendritic, macrophages, monocytes	Many cells	Many cells	Differentiated endothelial cells, apipocytes, epithelial, and muscle cells	Lymphocytes, cancer cells, rodent macrophages	Specialized cells, endothelial cells, and others
Efficiency of uptake[c]	+++++ for specialized cells	+++	+++++	+	+	++++

[a]*Typically, cargo chemistry determines a size range due to technological limitations (e.g., dendrimer-based and micellar-based nano-vehicles are limited to rather small sizes).*
[b]*Phagocytosis and macropinocytosis most likely do not contribute to the nanovehicular uptake.*
[c]*The efficiency of uptake may depend on nanoparticle type and chemistry. The proportion between different uptake mechanisms may vary. Estimated on scale of 1–5, 5 being the highest.*

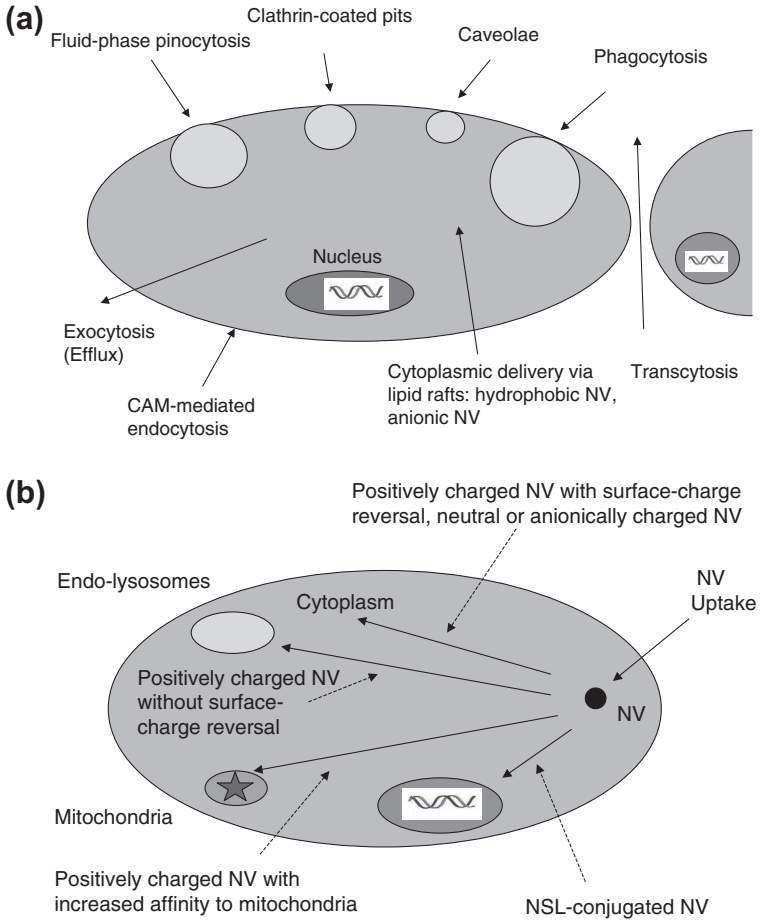

FIGURE 35.1
Cellular uptake and transport. (a) Schematic representation of the uptake and exocytosis routes of nanovehicles. (b) Schematic representation of intracellular trafficking of nanovehicles. Although charge-based phenomena are shown to have a controlling role, a targeting ligand, attached to the nanovehicle periphery, may also contribute to the organelle distribution. Note that the endoplasmic reticulum (ER) has been eliminated from this drawing for the sake of simplicity. NSL — nuclear signaling ligand. *Adapted from Panyam and Labhasetwar ([255]). Not drawn to scale. Courtesy of Springer Verlag.*

Macropinocytosis is generally considered to be a non-specific process where cells take up large volumes of extracellular fluids and solutes. Macropinocytosis is characterized by membrane ruffling and formation of protrusions that ultimately produce large endocytic vesicles called macropinosomes (>1 μm in size, up to 5 μm), which sample large volumes of the extracellular milieu. The macropinocytosis process is constitutive in specialized cells (macrophages and dendritic cells) and in some tumors, and greater internalization may be achieved by non-specific binding of solutes to the cell membrane (adsorptive pinocytosis). Although the pH of macropinosomes decreases, they do not fuse into lysosomes. This pathway provides some advantageous aspects, such as the increased uptake of particles and macromolecules, the avoidance of lysosomal degradation, and the ease of escape from macropinosomes because of their relatively leaky nature.

CME constitutively internalizes cell membrane sites with concentrated receptor–ligand complexes to form clathrin-coated vesicles (size ~100–150 nm), and it functions for efficient cell uptake of essential nutrients, antigens, growth factors, and pathogens. Since receptors are differentially expressed in various cells types and tissues, receptor-mediated endocytosis via clathrin-coated pits provides a potential strategy for cell- and tissue-specific delivery of nonviral gene vectors. Molecules entering via this pathway experience a drop to approximately pH 6 in the early endosomes, with a further reduction to approximately pH 5 during progression to late endosomes and lysosomes [38]. While the initial events in the endocytosis of the receptor–ligand complex are similar for most systems, the processing of the ligand can differ, depending on both receptor and cell type. Following CME, ligands and receptors are sorted to their appropriate cellular destinations, such as lysosomes, the Golgi apparatus, the nucleus, or back to the cell surface membrane. In the conventional model, the internalized ligands are degraded in the acidic endo-lysosomal compartments while the receptor is recycled back to the plasma membrane.

CavME is mediated by flask-shaped invaginations in the cell membrane called caveolae (50–60 nm in size) that are particularly abundant on endothelial cells. Caveolae are cholesterol and sphingolipid-rich microdomains of the plasma membrane, and they are concentrated 'hot spots' for a diversity of signaling molecules and membrane transporters. A unique aspect of CavME is that it is a non-acidic and non-digestive route of internalization that bypasses lysosomes and thus may be an advantageous route for drug delivery. However, caveolae are internalized slowly and their fluid-phase volume is small; this results in low capacity for uptake of external cargo. Thus, it is unlikely that they contribute significantly to constitutive endocytosis, although in endothelial cells, caveolae constitute 10–20% of the cell surface [37].

Lipid raft-mediated, endocytosis-independent uptake is an alternative pathway that can be operative for anionic and neutral-lipid liposomes, solid-lipid NPs, and hydrophobic (i.e., polystyrene) NPs. Lipid rafts are cholesterol and sphingolipid-rich 40–60 nm microdomains and are characteristic of cells that lack caveolin and caveolae. This uptake mechanism is likely mediated through scavenger receptors that are involved in the uptake of both lipophilic and anionic cargo [39]. For example, scavenger receptor class B (SRB1; CD36 superfamily of proteins), which is expressed on mature macrophages, binds to a diversity of protein, polyribonucleotide, polysaccharide, and lipid ligands that are 'polyanionic'. The involvement of SRB1 in liposome uptake was further supported by the competitive inhibition of hepatic cell uptake of neutral phospholipid/cholesterol liposomes by the strong polyanion polyinosinic acid [40]. Lipid raft-mediated endocytosis bypasses lysosomes and is facilitated by a transmembrane potential that exists across cell membranes. This is a unique feature relative to caveolae-mediated endocytosis, which requires an energy input.

Physicochemical properties of NPs play a significant role on the level of uptake and the mode of transport into cells. Numerous studies have shown that surface charge has a significant impact on cellular internalization of nanocarriers [41]. Size has also long been known to affect uptake, with several studies concluding that NPs in the 100–200 nm range possess the best

properties for cellular uptake [42]. Great strides have been made in the last decade in terms of understanding the interplay between size and shape on cell internalization. Using polystyrene particles of various sizes and shapes, Champion and Mitragotri studied phagocytosis by alveolar macrophages, and they were some of the first to report on the importance of shape rather than just size [43]. In particular, the local particle shape at the cell-particle interface was found to dictate macrophage phagocytosis versus spreading on particles (Fig. 35.2a). Recently, DeSimone et al. tested uptake in non-phagocytic HeLa cells for a series of particles with varying sizes and shapes that were fabricated using a lithographic method called PRINT (Particle Replication In Non-wetting Templates; Fig 35.2b) [44]. They found that the internalization kinetics of the NPs by HeLa cells depended on aspect ratio and particle size and/or volume. The most striking finding was that rod-like particles were internalized more rapidly, with cylindrical particles of 150 nm diameter and a 3:1 aspect ratio showing the most rapid uptake in their study. Their data also indicated that the PRINT particles utilized a combination of the different internalization pathways described above to enter the cells, suggesting that size and shape alone cannot be utilized to fully define the mechanism of uptake and subsequent intracellular trafficking.

In addition to optimizing their physical properties, nanovectors can be endowed with a variety of other specialized constituents that dictate their mode of cellular internalization, either by binding to specific internalizing receptors or by directing traffic to specific intracellular compartments such as the cytoplasm and nucleus. Several examples of amine-containing polymers exist that buffer endosome acidification and lead to endosome disruption through the osmotic proton sponge mechanism, which is discussed in more detail in the section 'Nonviral Nucleic

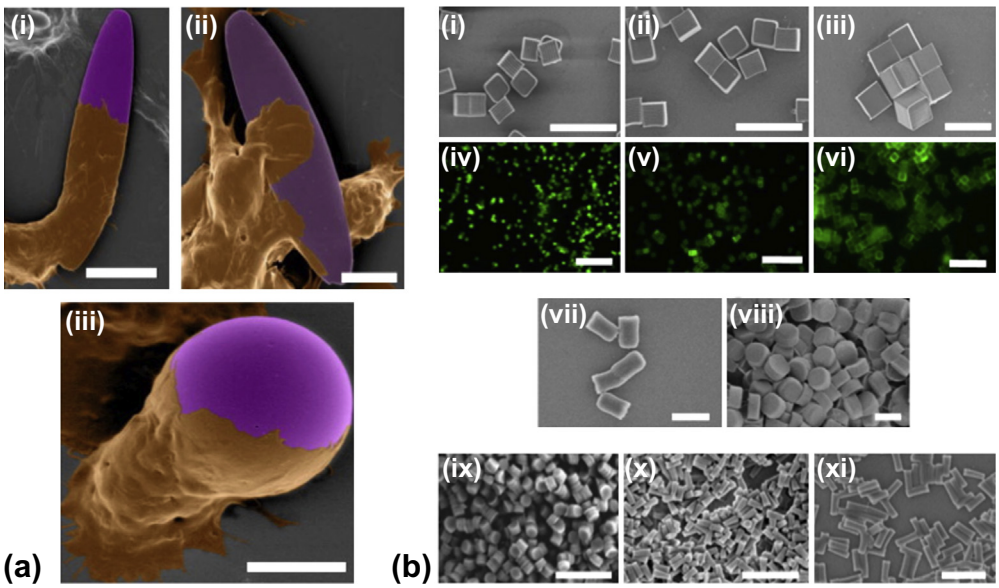

FIGURE 35.2

Nanoparticulate shape variation. (a) Scanning electron microscopy images provided evidence that particle internalization is dependent on local particle shape from the perspective of the phagocyte (cells and particles were colored brown and purple, respectively). Phagocytosis progressed further when the cell approached the end of the opsonized elliptical particle relative to when contact was initiated on the flatter, more elongated side of the particle. Phagocytosis progressed further when a smaller particle dimension was approached by the cells, i.e., as shown for the smaller spherical particle in panel (iii). Scale bar = 10 μm is sub-panel (i) and 5 μm in sub-panels (ii)—(iii) (43). (b) Panel of micrographs demonstrating PRINT particles varying in both size and shape. Sub-panels (i)—(iii) show scanning electron micrographs of cubic particles of size 2, 3, and 5 μm, respectively, and sub-panels (iv)—(vi) show fluorescence micrographs of the same cubic particles. Sub-panels (vii) and (viii) show scanning electron micrographs of cylindrical microparticles of 1 μm in height with diameters of 0.5 and 1 μm, respectively. Sub-panels (ix)—(xi) show scanning electron micrographs of (ix) 200 nm diameter and 200 nm height (x) 100 nm diameter and 300 nm height and (xi) 150 nm diameter and 450 nm height. Scale bars = 20 μm for (i)—(vi) and 1 μm for (vii)—(xi) (44).

Acid Delivery' below. Poly(alkylacrylic acids) are another class of polymers that can mediate endosomolysis, but they act through an active membrane disruption mechanism. In this case, protonation of carboxylates within acidic environments transitions the polymers from an anionic, hydrophilic state into an uncharged, hydrophobic, and more compact state that is membrane interactive/disruptive [45]. NPs that show a transition in their surface charge from anionic (at pH 7) to cationic in the acidic endosomal pH (pH 4–5) have also been shown to enable escape from endosomal compartments [46].

For plasmid gene delivery, trafficking to the nucleus is of particular significance. As discussed below in the section 'Nonviral Nucleic Acid Delivery', polymers and lipids are the two main classes of biomaterials used to formulate nonviral vectors, and choice of vector can affect, among other things, the mode of cell internalization. For example, the uptake of cationic lipids proceeds mainly by the clathrin-dependent pathway, but internalization of polymer-based systems is more dependent on the polymer and the cell type. Polymer-based delivery systems can proceed by both clathrin-dependent and clathrin-independent pathways simultaneously in the same cells [47]. Independently blocking either the clathrin- or caveolae-dependent pathway does not dramatically affect quantity of uptake, but blockage of caveolae-mediated uptake significantly abrogates gene expression, suggesting this route of uptake plays a significant role in intracellular trafficking to the nucleus [47,48]. In agreement with this result, Sullivan and authors have shown that use of histone H3 tail peptides that predisposes trafficking of polyplexes to caveolar pathways can be used to enhance plasmid transfection [49]. Their follow-up work has provided mechanistic evidence that caveolar polyplexes are trafficked to cell nuclei through a retrograde Golgi-to-ER (endoplasmic reticulum) pathway [49]. Other mediators of intracellular trafficking have also been successfully co-delivered to modulate NP intracellular trafficking. For example, fusogenic endosomolytic peptides or nuclear localization signals (NLS) can be attached to NPs to enhance targeting to the nucleus. Recently, it has also been shown that treatment with histone deacetylase (HDAC) inhibitors can be used to liberate polyplexes from sequestration at the perinuclear recycling compartment/microtubule organizing center (PNRC/MTOC) and to increase transgene expression by up to forty-fold [50].

VIRAL NUCLEIC ACID DELIVERY

Introduction to viral gene therapy

A virus is a NP consisting exclusively of biological components, including proteins, lipids, carbohydrates, and nucleic acids. Each virus carries a viral genome that encodes the structural and functional elements of the infectious particle, but does not encode the necessary machinery for transcription, translation, and genome replication. Therefore viruses infect living cells and can only replicate inside these hosts by hijacking native cellular transcription and translation processes.

Viruses have evolved over billions of years to be extremely effective at infecting and replicating within host cells. Therefore, many of the challenges of nonviral gene delivery, such as cellular uptake, trafficking, and nuclear import, are readily addressed by the innate biological properties of viral vectors. To take advantage of these properties, researchers have re-engineered natural viral vectors by removing all pathogenic components and signals necessary for virus replication within infected cells, and replacing them with therapeutic genetic sequences. This approach has been widely successful in engineering vectors for efficient gene delivery in research but has encountered severe hurdles en route to clinical efficacy.

The first gene therapy clinical trial occurred in 1990 and used a retroviral vector for gene delivery to T cells of patients with severe combined immunodeficiency [51]. Over the past decade, there have been roughly 100 new clinical gene therapy clinical trials each year worldwide [52]. Over 66% of these trials have used viral vectors for gene delivery. The first gene therapy approved for clinical use was an adenoviral p53 vector for cancer treatment in China in

2003 [53]. More recently, a second gene therapy was approved in Europe consisting of an adeno-associated virus (AAV) encoding a gene to treat a rare genetic disorder known as lipoprotein lipase deficiency [54]. These are the only two approved gene therapies, which is a reflection of both the potential for viral gene therapy, but also of the challenges in engineering either viral or nonviral vectors with the ideal combination of efficacy and safety necessary for broader clinical adoption.

The recent approval of an AAV-based gene therapy in Europe, as well as many other clinical successes of viral gene therapy in recent years, are anticipated to pave the way for other new gene and cell therapies [55]. However, the viral gene therapy field encountered severe complications before reaching this more recent sequence of positive results. In 1999, a patient named Jesse Gelsinger was treated in a clinical trial with an adenoviral vector as a therapy for a genetic metabolic disease. He died four days after vector administration due to a severe inflammatory episode and multi-organ failure, which was later linked to an immune response against protein components of the virus [56]. One year later, another landmark event occurred when the first reports of successful gene therapy for curing a genetic disease, in this case a fatal immunodeficiency disorder, were reported [57]. In all, 20 children were treated with autologous hematopoietic stem cells transduced with retroviral vectors carrying a therapeutic transgene, and ultimately 17 of these patients were cured of the disease [58]. However, five of these cured patients later developed leukemia as a result of integration of the viral vector into the genome near to an oncogene, leading to oncogene activation [59]. One of these five patients died as a result of the leukemia. Due to these adverse events, viral gene therapy was the subject of social and scientific stigma in the first part of the twenty first century. However, scientists have been able to identify and address the mechanistic causes of these adverse events, leading to improved viral vectors and numerous ongoing and successful clinical trials as described above. Nonetheless, the fundamental source of these challenges is the complexity of viral vectors, each of which contains hundreds to thousands of copies of dozens of distinct biological macromolecules that perform complex functions. This is a primary motivating factor for engineering nonviral delivery vehicles comprised of defined chemical components to achieve precise control over all elements of gene transfer and expression.

Although the clinical trial results discussed above are largely focused on rare hereditary disorders, the history and critical concerns of viral vector development are essential to understanding the potential of viral nucleic acid delivery for tissue engineering and regenerative medicine. Furthermore, new medical technologies such as gene delivery are often first developed for fatal diseases for which there is no other option, such as genetic disorders and cancer. The technology development and demonstration of safety and efficacy of gene transfer in these areas is directly translatable to applications in other fields, including tissue engineering.

Types of viral vectors

A variety of viral vectors have been employed to deliver genes to cells. Each approach has its own advantages and disadvantages, including packaging capacity, persistence of expression, immunogenicity, and non-specific effects on the cellular genome [60,61].

Adenoviruses are the most commonly used therapeutic viral vector, largely due to applications in cancer therapy. An adenovirus consists of a double-stranded DNA genome and a protein shell, known as a capsid (Fig. 35.3). The adenoviral particle binds to cells through fiber proteins in the capsid that recognize specific receptors on the cell surface, followed by endocytosis of the virus. The adenoviral vectors exist extrachromosomally in the nucleus and therefore they are not copied with the cellular genome during cell division. As a result, gene expression is transient as the vector is degraded or diluted out during cell replication. The adenovirus has an outstanding ability to escape endosomes, enter the cytoplasm, and pass through the nuclear membrane pore. Despite extensive investigation of dozens of viral vectors for cancer treatment and promising results in clinical trials, the FDA (Food and Drug

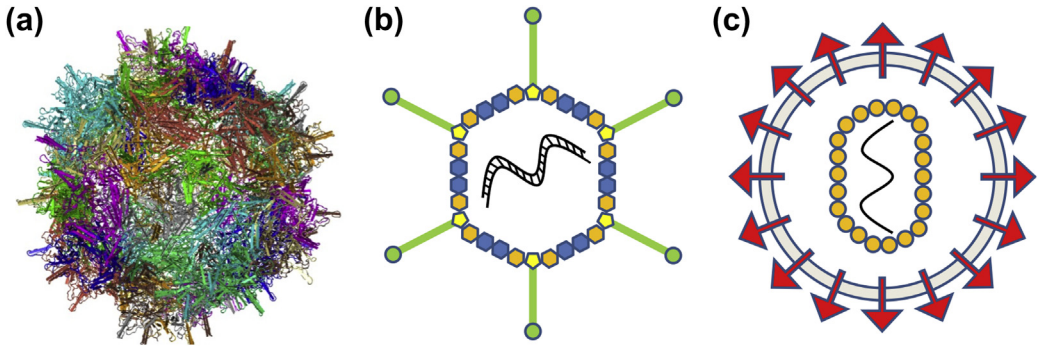

FIGURE 35.3

Representative schematics of viral structures. (a) Adeno-associated virus. The crystal structure of the AAV capsid proteins that encompass the vector and interact with cellular receptors is shown (MMDB ID 20256). Capsid monomers are represented by different colors. (b) Adenovirus. Adenoviral vectors consist of a capsid shell made up of proteins known as hexon (blue), penton (orange), and the penton base (yellow), to which the fiber protein (green) is attached. The double-stranded DNA viral genome is packaged inside the capsid. Other accessory proteins are not shown. (c) Retrovirus/Lentivirus. Retroviral and lentiviral vectors are encompassed with a lipid bilayer envelope (gray), which contains proteins that interact with cell surface receptors (red). Within the envelope, a capsid shell (orange) surrounds the single-stranded RNA genome.

Administration) has not approved any virus-based therapeutics because of widely reported toxicities and immunogenicity of the capsid proteins [62]. Because of this significant immunogenicity, any adenoviral vector can only be delivered once to a patient. Therefore, adenoviruses are not an optimal choice for chronic conditions but can be very useful for modifying cells *ex vivo*. The primary advantage of the adenovirus is its ability to achieve very high concentrations of viral preps ($>10^{12}$/ml). Unlike unstable RNA viruses with lipid bilayers, including retroviruses and lentiviruses, adenoviruses can easily be concentrated to high viral titers and can infect dividing as well as non-dividing cells with high efficiency. This is a major advantage for some applications, including *in vivo* gene transfer to tissues such as the lung, where cell division is infrequent.

Adeno-associated viruses (AAV) are small viruses with single-stranded DNA genomes and a protein capsid (Fig. 35.3). Unlike other viral vectors developed for gene therapy, wild-type AAV is not known to cause any human disease. The main disadvantages of AAV are that the vector can efficiently package only small transgene cassettes (up to 4.7 kb), the capsid proteins can be immunogenic, and the preparation of the recombinant vector is complex. Nonetheless, AAV is quickly becoming the preferred vector in the gene therapy field [63,64]. This is because like adenovirus, AAV can be prepared to high concentrations and is relatively stable in storage. Additionally, recombinant AAV does not efficiently integrate into the genome and, therefore, there is little risk of disruption of native genes. In contrast to adenoviruses, the AAV vector is particularly stable within the nucleus and can exist with high levels of gene expression for months or years [65,66]. As discussed in more detail below, many serotypes of AAV exist and the capsid proteins are exceptionally flexible for being re-engineered to target new cell types. AAV can also be engineered for tissue-specific targeting following systemic delivery [67].

Retroviral and lentiviral vectors contain a single-stranded RNA genome, a protein capsid, and a lipid bilayer, known as the envelope, that also contains transmembrane proteins (Fig. 35.3). The vectors are able to efficiently carry about 8–10 kb of transgenic material. A disadvantage of these vectors relative to adenoviruses and AAVs is that the RNA genome and lipid bilayer make them comparably unstable, with a half-life of less than 12 hours at 37°C. This also creates challenges for storage, and the titers of these viruses are relatively low (10^6–10^7 particles per ml), although they can be concentrated to $>10^9$/ml. The major advantages of retroviral- and lentiviral-mediated gene transfer are that recombinant viruses are capable of transferring genes to a wide range of different primary cell types, in contrast to nonviral systems, and the genes

are stably integrated into the chromosomal DNA. Therefore these vectors are ideal for applications requiring long-term gene expression. However, the integration of these viral vectors into the genome also has the potential to disrupt endogenous genes, such as critical oncogenes and tumor suppressors, as discussed above [68]. There are two ways in which this has been addressed. First, self-inactivating (SIN) vectors have been developed in which the strong viral promoters and viral splice sites have been disabled, and therefore, the virus is less likely to alter the expression of nearby genes [69]. Second, integrase-deficient lentiviral vectors have been engineered that have the advantages of high-efficiency lentiviral transduction. However, these virions do not integrate into the genome, and they result in only transient gene expression [70]. It is becoming more common that lentivirus is preferred over retrovirus, as retroviral transduction is dependent on cell division whereas lentiviral transduction is not [71].

Herpes simplex virus type-1 (HSV-1) is a human neurotropic virus used primarily as a vector for gene transfer to the nervous system, although the wild-type HSV-1 can infect and lyse other non-neuronal epithelial cells [72]. Because of its large genome size, up to 30–50 kbp of transgenic material can be packaged into recombinant HSV-1 vectors. At present, two major classes of HSV-1 vectors have been developed: replication-defective viruses and replication-conditional mutants. However, the ability of HSV and these mutant recombinants to establish lifelong latent infections raises concerns about the use of these vectors in humans. Efforts have been made to create HSV-1 amplicon vectors essentially free of HSV-1 helper virus, which might be a promising genetic vehicle for *in vivo* gene delivery.

Most commonly, viral vectors are used to deliver cDNAs of transgenes encoding proteins that will confer some therapeutic effect. In the context of tissue engineering, these transgenes may facilitate progenitor cell expansion, cell differentiation, tissue formation, and/or wound healing. However, viruses can also be used to deliver small interfering RNAs to silence target gene expression [73]. In order to take advantage of the RNAi mechanisms of processing short double-stranded RNAs by the RISC complex, viral vectors are designed to express shRNAs that mimic the structure of the naturally occurring miRNAs. Both AAV and lentiviral vectors are routinely used for shRNA-mediated gene knockdown. Other possible cargo for viral vectors includes aptamers and ribozymes [74]. Viral particles have also been engineered for the transfer of proteins across the cell membrane [75].

Engineering viral vectors

The natural evolution of viruses has produced a wide variety of gene transfer vectors with innate capabilities for overcoming many of the challenges of gene delivery to living cells, including recognition of cell surface proteins, internalization, endosomal escape, trafficking to the nucleus and transfer across the nuclear membrane, and ultimately transcription of viral genes. However, these natural properties do not necessarily address all of the challenges for many applications of gene delivery for gene therapy, regenerative medicine, and tissue engineering. Additional challenges include virus purification, immunogenicity of viral proteins, proper virus localization following systemic delivery, targeting the virus to specific cell types, tissue-specific gene expression, and controlled release of viral vectors from biomaterials. To address these challenges, the biological properties of these vectors can often be re-engineered via rational design. Alternatively, the complex properties of these systems may be more easily altered by high throughput selection of large libraries of variants of viral particles [76].

Viral particles predominantly interact with cells via the proteins on the surface of the virus. In the case of retroviruses and lentiviruses, these interactions occur through the proteins in the lipid envelope that comprise the outer barrier of the particle, whereas for adenoviruses and AAVs, the capsid proteins encompass the particle [76]. Therefore, exchanging these proteins with those of other serotypes, in a process known as pseudotyping, provides a means of changing the tropism of the virus, which is defined by the types of cells the virus will transduce. Modifying the capsid and envelope proteins can also be used to alter the recognition of the

viral particles by the immune system. For example, pseudotyping adenoviral vectors with alternative capsid proteins led to increased transduction of human cell lines and primary cells, including transduction of intact human saphenous veins and human bone marrow stroma cells for seeding onto scaffolds for bone tissue engineering [77]. Adenoviral pseudotyping has also been used to evade antivector immunity [78]. The pseudotyping of retroviruses and lentiviruses is particularly straightforward since the envelope proteins exist in the lipid bilayer and do not serve important structural roles, as the capsid proteins do [79]. This approach has been used to broaden virus tropism to facilitate transduction of many cell types [80] or to narrow tropism to target transduction to a specific cell type, such as neural stem cells in the brain [81]. Pseudotyping these vectors can also enable the transduction of tissues that are otherwise resistant to gene transfer, such as the airway epithelium [82] and spinal cord [83]. AAV has been pseudotyped both by using capsid proteins from alternative serotypes, mixing capsids from two serotypes, and generating single chimeric capsid proteins with elements from multiple serotypes [76].

As an alternative to pseudotyping, which uses surface proteins from other virus serotypes, grafting targeting peptides into their genetic coding sequence can also functionalize the capsid and envelope proteins. For example, insertion of the Arg-Gly-Asp integrin-targeting peptide into adenoviral vectors has directed viral transduction to cells expressing high levels of integrin receptors [84,85]. This approach has also been used to preferentially direct viral transduction to endothelial cells [86]. Alternatively, biotin-binding peptides [87], single chain antibodies [88], and genetically-encoded imaging agents [89,90] have been incorporated into adenoviral capsid proteins as general approaches for redirecting cell targeting or tracking viral localization. Similar strategies are being explored for AAV [91], although they are in general more challenging due to the structural complexity of the capsid [76]. Single chain antibodies have also been grafted into retroviral and lentiviral vectors to control targeting [92], though typically with a decrease in overall transduction efficiency [93]. Alternatively, growth factors and cytokines have been inserted into the envelope to target transduction to cells bearing the complementary receptor [94,95].

In many cases, our understanding of the structure-function relationships of the viral proteins is insufficient to allow rational design of highly active engineered proteins with new functions. In these circumstances, a directed molecular evolution strategy may be useful. In this approach, large libraries of viral variants are created and library members with desirable properties are selected [96]. For example, display of random peptide libraries on AAV and adenovirus have led to vectors with increased selectivity and/or transduction efficiency of endothelial cells [97] and myoblasts [98]. Random mutagenesis or DNA shuffling and selection of novel AAV capsids has led to new variants with beneficial immune evasion and purification properties [99,100] and enhanced the transduction of human airway models [101,102], specific neural cell types [103,104], and induced pluripotent stem cells [105]. Collectively these technologies provide a means to design improved viral vectors for a variety of applications.

In contrast to genetic modification of the viral proteins, adaptors or chemical modifications can be used to redirect viral transduction. PEGylation can be used to mask viral vectors from the immune system, but in many cases this approach will decrease overall transduction efficiencies [106]. Conjugation of the PEG group to a functional targeting moiety, such as an antibody, can lead to simultaneous protection of the vector and delivery to specific cell types *in vivo*, such as endothelial cells [107]. Other work has used bispecific adaptors that bind both the viral surface proteins and a particular cell surface receptor [108,109]. The incorporation of antibody-binding domains or biotin molecules into viral capsids has led to a general approach in which the virus can be retargeted to any cell type via an antibody linker targeted to a cell surface epitope [110] or a fusion protein of streptavidin and a targeting ligand [111].

The methods described above are predominantly designed to control which cells a virus will transduce. Another approach to controlling gene expression is to use an engineered promoter

that can be regulated in magnitude, time, space, and/or tissue type [112]. These approaches are applicable to both viral and nonviral systems. The most commonly used inducible gene expression system is based on the small molecule antibiotic tetracycline [113]. Examples of this system that are relevant to tissue engineering include its use to control gene expression for bone tissue engineering [114,115], treatment of arthritis [116], and control of drug or hormone production by engineered tissues or implanted cells *in vivo* [117], among others. Systems have also been developed to control gene expression in response to a variety of other chemical inducers, including ecdysone [118], rapamycin [119], and other antibiotics. Other systems to control gene expression of implanted cells have been based on skin lotions [120] and inhaled gases [121]. In addition to chemical inducers, gene expression can also be controlled from promoters that are sensitive to hypoxia and temperature, which have been used to trigger gene-mediated cardioprotection and angiogenesis [122,123]. More recently, optogenetic approaches have emerged in which genes are placed under the control of light-inducible proteins from plants or the eye [124–126]. Consequently, the magnitude, dynamics, and spatial patterning of gene expression can be controlled with illumination *in vitro* and *in vivo*. In the future, this approach may be used to control tissue formation as part of a regenerative medicine strategy. Finally, gene expression can be restricted to certain cell types by regulating transgenes with promoters that correspond to genes only expressed in particular tissues. This approach has been widely successfully employed to create promoters that are specific to bone, cartilage, skeletal muscle, cardiac muscle, and liver.

NONVIRAL NUCLEIC ACID DELIVERY

Introduction to nonviral nucleic acid delivery

While viral delivery systems are equipped with inherent, evolved mechanisms for efficient intracellular delivery, engineered, nonviral nucleic acid delivery systems can offer a well-defined and potentially safer alternative that can be cheaply made on large scales. However, optimizing these systems to approach the efficiency of intracellular delivery achievable with viral vectors has remained problematic. A variety of synthetic systems have been sought for nonviral nucleic acid delivery, primarily based on polymers (synthetic and natural), lipids/liposomes, and inorganic NPs. Significant progress has been made in the development of nonviral carriers that can overcome the primary intracellular delivery barriers such as cellular internalization, escape from endo-lysosomal pathways, and trafficking to the desired intracellular space (i.e., cytoplasm for siRNA and nucleus for plasmid).

Although there are a host of delivery systems that have shown efficacious results *in vitro*, only a fraction have succeeded *in vivo* in preclinical models, and promising clinical data have only recently started to emerge. The remainder of this section will provide a more thorough overview of polymer, lipid, and inorganic NP technologies that have been recently developed for delivery of nucleic acids.

Synthetic polymers

A variety of natural and synthetic polymers have been developed for nucleic acid delivery systems. The literature on the design and utilization of standard synthetic polycations, such as polyethylenimene (PEI), poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), and cationic dendrimers, biodegradable synthetic polycations such as poly(beta-amino esters) (PBAE), glycopolymers, and peptide/protein-based polymers is briefly surveyed herein.

The earliest polymeric approaches to nonviral gene therapy employed cationic, amine-rich polymers such as PEI, PDMAEMA, and the cationic poly(amido amine) (PAMAM) dendrimers (Fig. 35.4). PEI is commonly used in both in its linear and branched forms for DNA plasmid delivery, whereas PDMAEMA is typically utilized as a linear homopolymer or as a diblock polymer with PEG or other compositions (e.g., see discussion below on pH-responsive,

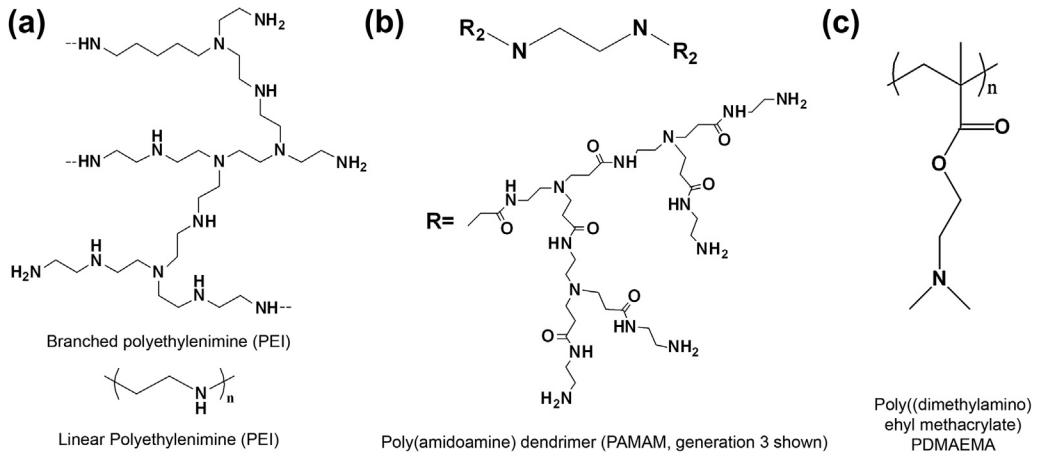

FIGURE 35.4

Polycationic polymers for nanoparticle production. (a) PEI (branched and linear), (b) PAMAM dendrimers and (c) PDMAEMA are representative polycationic polymers that have been thoroughly studied for polyplex formation and delivery of nucleic acids.

endosomolytic micelles with a PDMAEMA corona). Dendrimers such as PAMAM have more complex 'tree-like' architectures that form spherical, monodisperse macromolecules. These branched structures are synthesized either from the central core towards the periphery (divergent synthesis) or starting from the outermost residues (convergent synthesis). Commonly used, commercial PAMAM forms spherical polymers with good aqueous solubility because of its highly charged, exposed surface groups, which include abundant primary amines for convenient functionalization [127].

The amines can serve three primary functions in these systems: nucleic acid packaging, enhanced cell uptake, and endosome escape. By mixing with polycations in aqueous solutions, DNA and siRNA, with their negatively charged, phosphate-containing backbone, can be electrostatically condensed into particles, termed polyplexes. Typically, an excess of the polycation is used during polyplex formation, yielding particles with an overall net positive charge. The positive surface charge of the polyplexes increases interaction with negatively charged cell membranes, a process that is likely mediated through anionic, heparan sulfate proteoglycans anchored on the cell surface [128]. This binding enhances their endocytotic cell uptake. Following endocytosis, these polyplexes are capable of mediating endosomal escape through the osmotic disruption (e.g., the hypothesized 'proton sponge effect') [129].

Cationic polymers composed of secondary and tertiary amines, which enable endolysosomal escape through the proton sponge mechanism, have been shown to efficiently transfect nucleic acids into cells [130]. Although these net cationic polyplexes can effectively delivery nucleic acids *in vitro*, they can cause cytotoxicity, and they have a limited biodistribution profile if delivered intravenously. This is because the cationic surface charge of these polyplexes causes aggregation with serum proteins and red blood cells. These non-specific interactions can cause disproportionate biodistribution to the capillary beds of the lungs, very short circulation times, and acute toxicity [131]. Thus, many recent strategies have focused on decreasing the cytotoxicity and improving steric stabilization of cationic polyplexes. As discussed above, the incorporation of poly(ethylene glycol) (PEG) onto the polyplex surface is an important design aspect for reducing the positive surface charge, improving biodistribution, and decreasing acute toxicity [132,133]. More recently, a number of groups have also pursued bioreducible polycation variants that are degraded by the reducing environment in the cell, and consideration of this design parameter has yielded polyplexes with lower cytotoxicity and higher transfection efficiency [134,135].

Micelles formed from synthetic polymers represent another promising category of nucleic acid carriers [136]. In the area of nucleic acid delivery, the Kataoka group has focused on micelles

driven by electrostatic interactions, or polyion complex (PIC) micelles [137]. These nanovehicles typically self-assemble after mixing of nucleic acids with diblock polymers, consisting of PEG and a polycationic segment such as poly(Lys). This class of carriers has been shown to achieve gene silencing *in vitro*, and recently, a variant composed of a diblock polymer consisting of PEG, poly(Lys) and cyclic Arg-Gly-Asp (RGD) targeting peptides was found to efficiently achieve gene silencing and reduced tumor mass following delivery of anti-angiogenic siRNA [138].

More traditionally, micelles are defined as core-shell self-assemblies of amphiphilic diblock polymers into morphologies where a more hydrophobic block forms the micelle core, and a more hydrophilic block forms the corona. This class of micelles, which self-assemble into NPs in aqueous solutions even in the absence of added nucleic acid, has recently shown great promise [139–141]. One recent example of a micelle-forming polymer is composed of a block of poly(DMAEMA) and a second block that consists of a random terpolymer of 50% butyl methacrylate (BMA), 25% DMAEMA, and 25% propylacrylic acid (PAA) (Fig 35.4). When a concentrated stock solution of this polymer in ethanol is added dropwise into an excess of phosphate-buffered saline (PBS), the polymer self-assembles into micellar NPs of approximately 50 nm in diameter, with the poly(BMA-co-DMAEMA-co-PAA) terpolymer block in the particle core. The 50 mol% of the hydrophobic BMA drives self-assembly, and electrostatic interactions between PAA and DMAEMA also help to stabilize the micelles near physiologic pH. The homopolymer block of DMAEMA forms the micelle corona and provides a cationic surface that can be used to electrostatically condense siRNA into serum stable siRNA-NPs, sometimes referred to as micelleplexes (Fig. 35.5a). DMAEMA and PAA monomers both contain protonatable groups (tertiary amine and carboxylic acid, respectively) with pK values approximately equal to physiologic pH. The net charge of the anionic PAA and cationic DMAEMA is relatively balanced at physiologic pH, which stabilizes the micelle core. However, due to concurrent protonation of DMAEMA and PAA, the core-forming terpolymer block acquires a net positive charge when it enters the acidic endo-lysosomal pathway. This causes a shift to a net cationic state that electrostatically destabilizes the micelle core, exposing the terpolymer block, and activating its membrane disruptive activity (Fig. 35.5b). This terpolymer composition has been fine-tuned for intracellular delivery based on the BMA content [140] so that this pH-driven transition occurs in environments representative of the early and late endosomal compartments.

The micelles in Fig. 35.5a have a cationic surface charge that is not amenable to effective intravenous delivery because, as described for PEI and PDMAEMA homopolymers, particles with cationic surfaces have short circulation times. As discussed above, reversible PEGylation can be utilized to create more stealthy cationic carriers in the circulation while maintaining

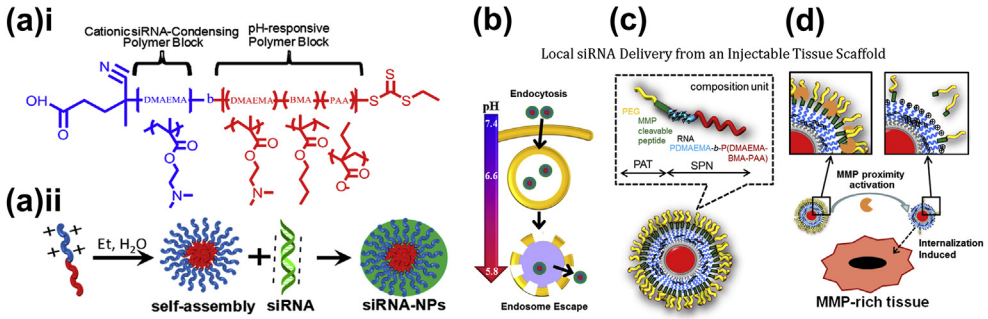

FIGURE 35.5

siRNA-NPs. (a) Diblock copolymer (i) composition and (ii) scheme for self-assembly/siRNA loading. (b) These siRNA-NPs are designed to respond to the more acidic pH in the endo-lysosomal pathways following endocytotic uptake. This triggers endosomal membrane disruptive activity and mediates siRNA delivery into the cytosol. (c) Environmentally activated siRNA-NP with MMP-removable PEG surface layer and (d) concept for MMP-dependent PEG removal and cell uptake.

key, underlying functionalities. For example, a variant of this micelle has been created with a PEG corona that can be removed by naturally occurring proteases for 'proximity activated targeting' (PAT) [27,142–145] (Fig. 35.5c). The intact PEG corona reduces non-specific binding and uptake in the circulation, while high MMP activity at the pathological site releases the corona and triggers activation of the underlying siRNA-NP (Fig. 35.5d).

Evidence suggesting that toxicity profiles are improved with biodegradable versions of traditional polycations has spurred the development of new and better optimized biodegradable polymer chemistries. PBAEs are one very promising class of biodegradable polycationic nucleic acid carriers that have been shown to be superior to *in vitro* transfection reagents such as Lipofectamine 2000 [146,147]. A key characteristic of PBAEs is that they are amenable to parallel, high throughput synthesis for simultaneous screening of large numbers of polymer variants. Using this approach, PBAE compositions have been identified with transfection activity superior to other nonviral agents and that rival the performance of viral vectors in some applications [148,149]. Conveniently, poly(β-amino ester)-based carriers rapidly hydrolyze and degrade into low molecular weight diols and bis(β-amino acids) in response to the pH drop that occurs during endosomal/lysosomal trafficking, which both facilitates nucleic acid release and makes them less cytotoxic than polymers like PEI [146]. Other promising, new biodegradable, polycationic materials in development have shown very promising preclinical data (i.e., poly(amine-co-esters) [150]), supporting the concept that polymeric nonviral vectors will continue to advance nearer to clinical reality.

Polymers derived from natural sources or monomers

Amino acids and saccharide-based materials have been extensively explored for nucleic acid delivery, as more thoroughly reviewed elsewhere [151,152]. These natural building blocks have the potential to have reduced cytotoxicity and if optimized, superior overall function relative to fully synthetic biomaterials. Here, peptide-based and carbohydrate-based nucleic acid delivery polymers will be surveyed.

The first amino acid-based polymer to be pursued was poly(L-lysine) (poly(Lys, Fig. 35.6a) [153], and many iterations of this cationic polymer have been extensively studied in a fashion similar to PEI and the other synthetic polycations described above. Poly(Lys) can efficiently complex nucleic acids, but its transfection efficiency is low, and it requires cell treatment with

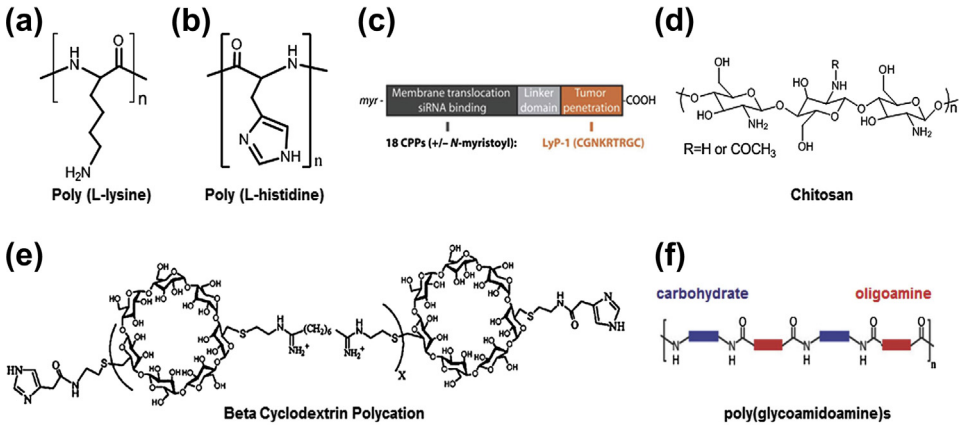

FIGURE 35.6

Examples of polymers derived from natural components that are used for nucleic acid delivery. Amino acid-based polymers such as (a) poly(Lys), (b) poly(His), and (c) CPP-containing peptides have been actively tested for delivery applications. Likewise, (d) chitosan and polymers containing (e) β-cylcodextrins and (f) other carbohydrate-containing polymers such as PGAAs have also shown tremendous potential. *Illustrations in panels c, e, and f are respectively based on references [173,185,255].*

endosomolytic agents such as chloroquine to enhance gene expression. Nonetheless, the early studies on poly(Lys) yielded important mechanistic insights related to polyplex formulation, intracellular trafficking, and endosome escape [153]. While the clinical promise of poly(Lys) is limited, these early studies have had a significant impact on the field.

Poly(histidine) (poly(His)) (Fig. 35.6b) is another amino acid-based polymer that has shown some usefulness for gene therapy. The amino acid His has an imidazole R-group containing a secondary amine that endows poly(His) with proton sponge activity for endosome escape [154]. Polymers containing both Lys and His have also been utilized successfully in combinations. In these hybrid polymers, the primary amines on Lys are fully protonated and cationic at physiologic pH, enabling efficient electrostatic complexation with DNA. The lower pKb of the secondary amines from His provide complementary proton sponge activity for endosome escape [155]. However, the transfection efficiency of polymers with poly(His) grafted to poly(Lys) is significantly improved by the addition of the endosomal disruption agent chloroquine, indicating that the polymers alone are still partially prone to endosomal entrapment [156]. Highly branched architectures of His/Lys polymers and His-containing reducible polycations have also been found to efficiently deliver siRNA [157,158]. There is also a precedent for incorporation of amino acid-based subunits into other gene therapy systems in order to produce 'hybrids' with enhanced delivery functionality. For example, His has also been used to modify chitosan (discussed more below) to enhance its endosomal escape and transfection efficiency [159].

Cell-penetrating peptides (CPPs) and pH-responsive fusogenic peptides are two other classes of peptides that have been rigorously explored to trigger cell uptake and endosomal escape, respectively. These peptide classes have been used both in unison and as components of multifunctional polymer and liposomal delivery systems. Most CPP and fusogenic peptides are derived from bacterial toxins and viral vectors, or they are synthetic analogs of the naturally occurring peptides. The trans-activating transcriptional factor (TAT) of HIV-1 [160] and the antennapedia peptide derived from *Drosophila* [161] are two examples of well-studied CPPs. These peptides are typically rich in cationic amino acids, and as a result, synthetic, arginine-rich CPPs of various types have also been found to mediate biomacromolecular cargo cell uptake. The ability of CPPs to trigger cell internalization has been leveraged for delivery of several classes of therapeutic cargo including plasmid DNA and siRNA (see additional information in recent CPP reviews [162,163]).

Another class of peptides useful for nucleic acid delivery is the fusogenic peptides, which are pH-responsive peptides that can fuse with or form pores through the endosomal membrane. An example is the diphtheria toxin, which has a subunit that forms transmembrane pores in endosomes that enable entry of a disulfide-linked toxin fragment into the cytosol [164]. Another example is hemagglutinin, an influenza protein that creates pH-dependent endosomal membrane fusion to deliver the viral genetic material into the cytoplasm [165]. The peptide GALA is a synthetic, pH-dependent, fusogenic peptide that has been extensively characterized [166]. GALA self-assembles and inserts into lipid bilayers at acidic pH, forming a pore that allows traversal of the membrane [167]. For example, GALA has been successfully applied to enhance efficiency of cytosolic delivery of nucleic acid cargo packaged in PAMAM and liposomes [168–170].

Bhatia and authors recently reported one of the most promising peptide-based nucleic acid delivery approaches to date [171,172]. In their work, they have designed and screened a library of peptides that were made from the fusion of two peptide sequences known to have tumor penetration and CPP activities. A small linker sequence consisting of a 4-glycine spacer was incorporated between the two functional aspects of the peptide, and the CPP N-terminus was modified with by myristylation in order to incorporate more hydrophobicity and to enhance particle stability and the potential for membrane interactions (Fig. 35.6c). In this study they screened a variety of candidate CPPs, including a total of 18 different peptide forms, in order to

optimize performance in terms of uptake, endosomal escape, and cytosolic release/bioactivity [171]. In their follow-up work, they showed that silencing of the oncogene inhibitor of DNA binding 4 (ID4) using this class of carriers suppressed growth of established tumors and significantly improved survival in ovarian tumor-bearing mice [172]. CPPs are generally thought to act through a non-specific cell uptake mechanism that is not desirable for achieving site-specific effects *in vivo*, and this study represents a breakthrough in harnessing the power of CPPs with preferential activity at a specific tissue site.

The polysaccharide chitosan, oligosaccharides like cyclodextrins, and a variety of other saccharide-containing glycopolymers represent another class of polymers for nucleic acid delivery. For example, natural anionic saccharide-based polymers can be fabricated into thermodynamically stable, polyelectrolyte complex (PEC) NPs through spontaneous association triggered via mixing of polyelectrolytes of opposite charge, as reported by Prokop et al. [173,174]. Typically, PEC NPs are made by mixing polyanionic core polymers, such as alginate or chondroitin sulfate with corona polycations such as spermine hydrochloride or poly(methylene-co-guanidine) hydrochloride (PMCG). This multipolymeric nanoparticulate approach has been shown to be effective for gene transfer *in vitro* [175], particularly in cell systems that are normally refractory to gene transfer, such as pancreatic islets and antigen-presenting cells (Fig. 35.7). In addition, PEC coronal surfaces can be decorated with PEG-ligand complexes to increase cell targeting and reduce non-specific uptake.

Chitosan, which is a polysaccharide composed of glucosamine and N-acetyl glucosamine units bonded via β(1→4) glycosidic bonds is one of the most thoroughly studied saccharide polymers (Fig. 35.6d). Chitosan benefits from being a 'green' approach because it is a renewable resource derived from chitin. This natural polymer is also biodegradable and not toxic.

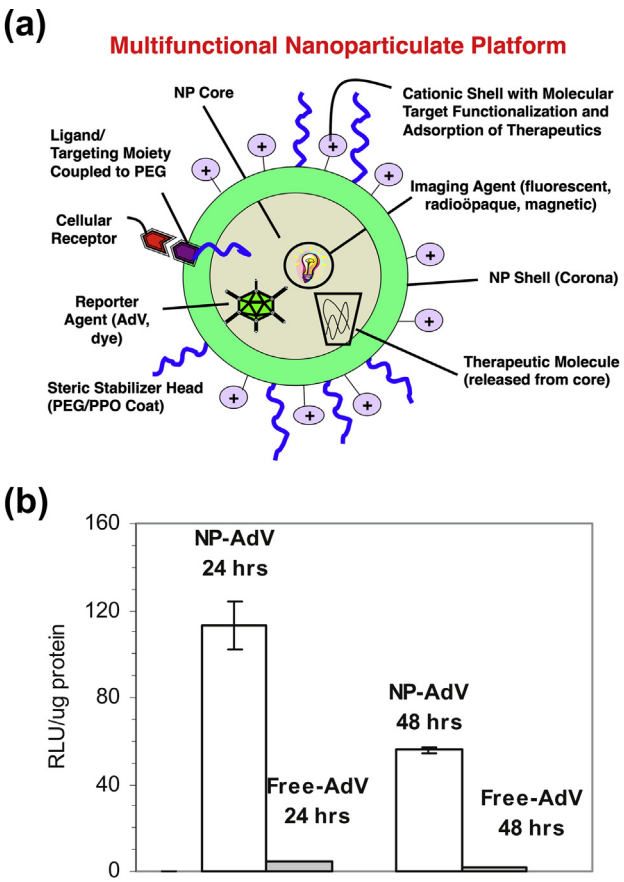

FIGURE 35.7
PEC NPs: Engineering for Enhanced Adenoviral Gene Delivery. (a) Schematic illustration of the construction of a multifunctional, nanoparticulate PEC. The PEC core can serve as a carrier for optical and chemical reporters, viral vectors, imaging agents, and pharmacological agents. The cationic nanoparticle (NP) shell can anchor both stabilizers and targeting ligands. (b) Delivery to pancreatic islets *in vitro*. NP were formed by ultrasonic dispergation of a polyanionic (core) solution of sodium alginate, cellulose sulfate, and a luciferase-expressing adenovirus (AdV) into a cationic (corona-forming) bath containing spermine HCl, PMCG, $CaCl_2$, and pluronic F-68. Particles were then purified by centrifugation. Freshly isolated murine islet cells were transduced with either adenovirus-containing NP or free AdV. Luciferase activity was markedly elevated by nanoparticulate encapsulation of AdV at both time points [175]. *Courtesy of ACS.*

The Alonso laboratory introduced chitosan-based NPs [176] made via ionotropic gelation, based on the interaction between the negative groups of pentasodium tripolyphosphate (TPP) and the positively charged amino groups on chitosan. The chemistry of chitosan is also adaptable to nonviral gene therapy, since it contains several primary and secondary amines capable of endosomolysis via the proton sponge effect. Therefore, chitosan has been examined as a pH-responsive polymer for nucleic acid delivery. Howard et al. employed chitosan NPs containing siRNA to knock down enhanced green fluorescent protein (eGFP) in both H1299 human lung carcinoma cells and murine peritoneal macrophages (77.9% and 89.3% reduction in eGFP fluorescence, respectively) [177]. The chitosan NP has a high potential for transmucosal delivery. Effective *in vivo* RNA interference was achieved in bronchiolar epithelial cells of transgenic eGFP mice after nasal administration of chitosan/siRNA formulations (37% and 43% reduction as compared to mismatch and untreated control, respectively). The principal drawbacks of chitosan are poor solubility in physiological buffers and lower endosomolytic activity compared to some stronger proton sponge polymers. As a result, several variants of chitosan have been made with modifications to increase endosomal escape and solubility. For example, PEI and imidazoles have both been conjugated to chitosan to enhance its performance in gene therapies [178].

Cationic polymers containing beta-cyclodextrins (β-CD) have arguably been one of the most promising polymeric carriers tested to date. Cationic β-cyclodextrin-based polymers (βCDPs, Fig. 35.6e) synthesized by the condensation of a diamino-cyclodextrin monomer with a diimidate comonomer are capable of forming polyplexes with nucleic acids, and their transfection performance depends on βCDP structure [179]. The β-CD-containing polycations are especially unique because cyclodextrins contain an interior cavity that can be used to form inclusion complexes with hydrophobic moieties. For example, β-CD binds tightly to the hydrophobic molecule adamantine, and this provides a convenient 'handle' from which to functionalize the surface polyplexes made from βCDPs with PEG or targeting ligands [180,181]. The Davis laboratory has translated this concept from benchtop to clinical trials [182]. This carrier was the basis for a recent report demonstrating RNA interference in man for the first time using targeted polymeric NPs. This carrier was composed of βCDPs functionalized with both PEG and the cancer-targeting ligand transferrin [183].

A variety of other novel, synthetic cationic glycopolymers are also in the developmental pipeline for clinical applications of nucleic acid delivery [152,184]. The Reineke lab has made key contributions in this area, and an example class of glycopolycations developed by this group are the poly(glycoamidoamine)s (PGAAs, Fig. 35.6f) [184]. A library of PGAAs was made through the condensation reaction between carbohydrate and oligoamine comonomers (Fig. 35.6f). These PGAAs were varied based on a variety of parameters including the carbohydrate size, the hydroxyl number and stereochemistry, the amine number, and whether or not heterocyclic groups were present. These polymers have been screened for gene delivery, and optimized formulations have been identified that facilitate efficient DNA packaging and intracellular delivery properties. The Reineke group has also sought a variety of trehalose-based polymers, and promising results continue to suggest the potential for clinical translatability of this safe and efficient class of polymers [185].

Lipid-based delivery systems

Lipids are one of the most commonly used approaches for nucleic acid transfection. Lipid agents can form small, artificial, spherical liposomal vesicles with a lipid bilayer membrane surrounding an aqueous interior. Liposomes can be produced from natural non-toxic phospholipids and cholesterol. Liposome properties vary substantially with lipid composition, size, surface charge, and the method of preparation. Because of their size, hydrophobic and hydrophilic compartments, as well as biocompatibility, liposomes are promising systems for drug delivery. Hydrophilic drugs can be encapsulated, or cationic lipids can be used to form

lipoplexes with anionic nucleic acids. Like other nucleic delivery systems discussed, PEGylation can be used to shield liposomal surfaces to reduce non-specific protein and cell interactions and to improve circulation time [186]. Targeting of liposomes has also been accomplished by anchoring a variety of targeting ligands, such as antibodies, to the liposomal surface [187]. The earliest approaches to lipid-based nucleic acid delivery focused on cationic lipids such as N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA, Fig. 35.8a) and were initiated over 20 years ago [188].

The majority of IV-delivered siRNA drugs that have advanced into clinical testing to date employ lipid-based delivery systems, with the lipid NP (LNP) delivery technology serving as the centerpiece for numerous trials on candidate drugs developed by Tekmira and Alnylam. Langer and Anderson have been significant contributors to the recent literature on optimization of lipid-like materials for delivery of siRNA through a series of high throughput combinatorial approaches seeking to identify optimized lipid-like materials, or lipidoids, for siRNA delivery. In some of the first published work using this approach, they synthesized nearly 700 lipidoids based on the conjugate addition of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines [189]. This library featured compounds with varied alkyl chain length, linker degradability, amine R-groups, and post-synthesis quaternization of the amine to induce stable cationic charge (Fig. 35.8b). The resulting lipidoids were used to package siRNA and screened for optimal transfection efficiency *in vitro* and *in vivo*. In these studies, efficient gene silencing activity was shown in both rodents and non-human primates at doses of 2.5 mg/kg and greater. In a follow-up paper, they synthesized a library of 126 lipid-like compounds using a unique synthetic strategy based on epoxide chemistry [190]. Their screen

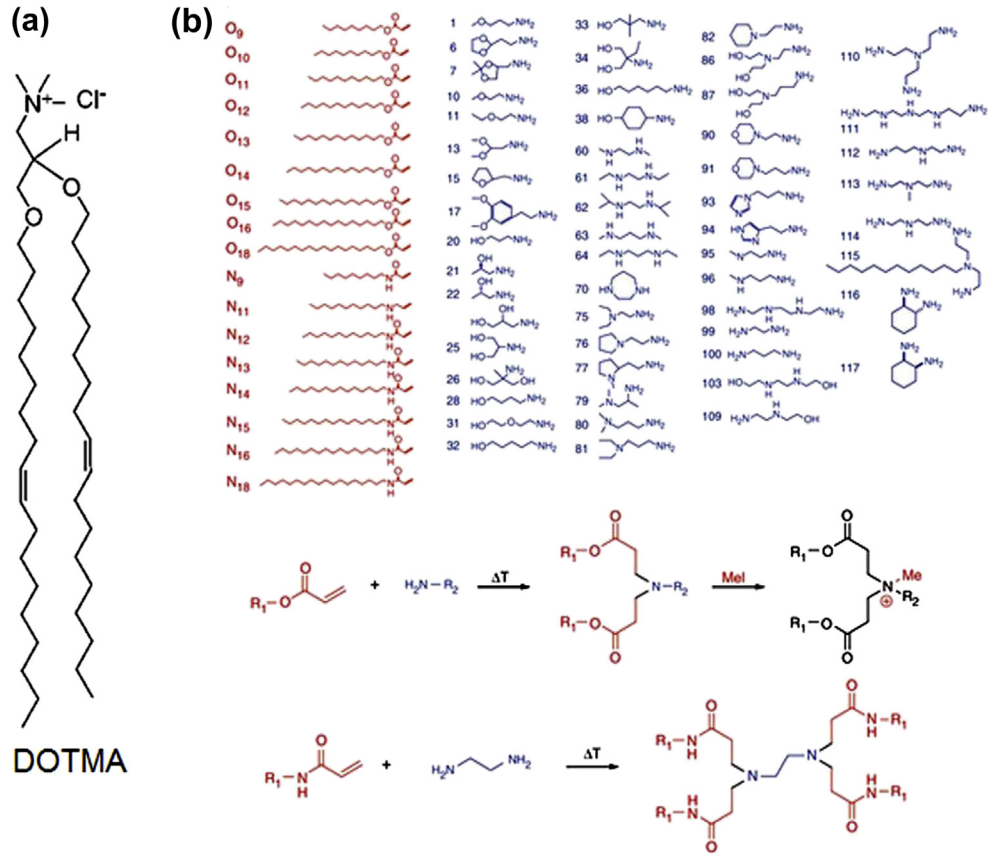

FIGURE 35.8
(a) Lipids traditionally studied for nucleic acid delivery include DOTMA and DOTAP. (b) Recent progress has been made using high throughput combinatorial approaches for identifying optimal siRNA delivery systems [189].

in this study identified some of the most potent RNAi delivery compounds produced to date, and their best lipidoids produced liver-specific silencing at 0.01 mg/kg in mice and at 0.03 mg/kg in non-human primates. Similar work has also been utilized recently to identify lipidoids for transfection of pDNA, and compounds were identified that were superior to Lipofectamine 2000™, which is a gold standard of commercially available *in vitro* transfection reagents [191], One of the limitations to much of the lipidoid work is that it has been primarily limited to liver targets. The promising current data suggest that clinical success will be established first in the liver and that subsequent work will optimize this class of carriers for additional clinical applications.

Inorganic NPs

In some applications, especially with inorganic NPs, 'theranostic' technological advances have been pursued that combine both image contrast and therapeutic functionalities. For example, quantum dot NPs, renowned for their optical properties, have been functionalized to successfully deliver siRNA [192,193]. NPs that provide MRI contrast have also been successfully utilized to simultaneously provide image contrast and siRNA delivery [194]. Recently, gold NPs have come to the forefront of inorganic NP approaches for siRNA delivery. Mirkin has been a leader in this field through development of polyvalent siRNA conjugates on the surface of gold NPs [195]. These particles are efficiently internalized by pattern-recognition scavenger receptors and can mediate gene silencing without addition of any additional transfection reagents [196]. Interestingly, these gold NPs surrounded by a dense shell of covalently bound siRNA have been recently found to efficiently penetrate the epidermal layer of the skin, indicating that they may be especially useful for clinical applications where topical delivery is a logical approach [197].

These skin-penetrating nanoparticles may obviate the need for the previously popular biolistic particle delivery systems. This older method uses a handheld gene gun with a pulse of helium to fire gold particles coated with DNA into target cells. This method of transfection is an effective physical means of rapid plasmid delivery into mammalian tissue. It is generally restricted to local expression in the dermis, muscle, or mucosal tissue since the gold particles are shot into confined tissue sites. Transfection depth is limited to about 1 mm, and about 10% of the cells in the tissue (skin) can be transfected. From a translational perspective, the DNA-loaded particles have a relatively long shelf life, and they have been successfully used for cutaneous administration of growth factor/receptor constructs [198–200].

ENGINEERING TISSUE SCAFFOLDS FOR VIRAL AND NONVIRAL NUCLEIC ACID DELIVERY

Nonviral delivery from scaffolds

Local, nonviral delivery of nucleic acids from biomaterial scaffolds presents a promising approach for tissue regeneration. Scaffold-based delivery can provide an efficient means to stimulate local, site-specific effects without the need for targeting and other challenges presented by systemic, intravenous delivery. These approaches can be designed for both delivery of gene-encoding plasmids and RNAi gene silencing through delivery of siRNA or plasmids that encode shRNA. These local, nonviral gene therapies have applicability for improving repair of a variety of tissues such as skin wounds, bone defects, and myocardial infarcts.

There is a relatively established precedent for scaffold-mediated plasmid delivery for increasing expression of growth factors such as platelet-derived growth factor (PDGF) to induce blood vessel and tissue formation within the scaffold [201]. It has been shown that embedding plasmid DNA within a biodegradable tissue scaffold or immobilizing it on a biomaterial surface can provide a 10–100 fold increase in transfection efficiency because of increased local concentration and extended retention of plasmid at the cell-biomaterial interface [201–203]

relative to a local injection of plasmid-containing solution. Mechanistically, 'substrate-mediated delivery' of nucleic acids is believed to operate through multiple modes of endocytosis, though caveolae-mediated uptake may play the largest role [204]. This bioinspired approach to tissue regeneration is also analogous to the pathways that viruses 'hijack' when they attach to extracellular matrix as a strategy to increase their cell internalization and infection [205]. The seminal work on scaffold-based plasmid delivery by Mooney and Shea has been subsequently applied in a diversity of applications, including delivery from both natural [206] and synthetic biomaterials [207,208]. Through more complex approaches to surface immobilization, plasmid delivery systems can also be engineered to control spatial patterning, concentration gradients, and temporal profiles in 2D and 3D [209]. Work is also underway to optimize the therapeutic benefits of substrate-mediated delivery by using enzymatically labile tethers [210], cell-specific targeting proteins [211], and other means of cell-specific targeting and intracellular release.

Scaffold-based local RNAi through delivery of siRNA is a more recent development and was initially approached primarily using natural biomaterials such as alginate, collagen, and agarose [212–214]. The first *in vivo* studies recently demonstrated gene silencing in skin wounds using Lipofectamine 2000 siRNA lipoplexes embedded in agarose gels. These commercially available cationic lipoplexes were released in a rapid burst and were effective for short-lived, topical siRNA application [214,215]. Pre-fabricated, electrospun scaffolds made from ε-caprolactone and ethyl ethylene phosphate copolymer (PCLEEP) nanofibers have also been pursued for the release of siRNA/transfection reagent (TransIT-TKO) complexes and have been shown to achieve more sustained delivery of bioactive siRNA [216].

A polyester urethane PE-UR scaffold-based platform has been recently developed for local delivery of siRNA-NPs [140,141] in regenerative applications [217]. These siRNA-NPs are composed of a diblock copolymer (see composition Fig. 35.5a). The PE-URs can be fabricated using a two-component foaming process that allows injection into a defect, followed by rapid curing *in situ* [218]. The siRNA-NPs can be embedded within the matrix as the porous, biocompatible, and biodegradable scaffold cures. The formed scaffolding can provide mechanical support, a template for tissue in-growth, and controlled drug release to regenerating tissues [218]. PE-URs adhere to tissue [212] and biodegrade both hydrolytically and oxidatively into biocompatible side products at rates that can be tuned based on the polyester triol and isocyanate precursor compositions [219].

In proof-of-concept studies [217], siRNA-NPs have been delivered from PE-UR scaffolds made from polyester triol and lysine triisocyanate (LTI) precursors that form a 3D polyurethane network when mixed (Fig. 35.9a). Water is added to this reaction as a blowing agent to create an interconnected porous network. Lyophilized siRNA-NPs can be simply added to the polyester triol prior to mixing with LTI, leading to siRNA-NP incorporation into the PE-UR matrix as it cures (Fig. 35.9b). siRNA-NP incorporation into the porous PE-UR scaffold is homogenous and results in sustained release of the siRNA-NPs from the scaffold (Fig. 35.9c). The released siRNA-NPs are stable during incorporation and subsequent release (Fig. 35.9d), and their gene silencing bioactivity is maintained post-release (Fig. 35.9e). Ongoing studies have confirmed the ability to achieve efficient local silencing (> 80% knockdown within the scaffold) of a target gene for over 1 month *in vivo*.

Viral delivery from scaffolds

The most common approach to viral gene delivery for tissue engineering is to genetically modify the cells in standard cell culture, and then subsequently seed them onto a three-dimensional scaffold [61,220]. However, this approach involves extra procedures and manipulation of the cells that adds significant complexity to an expensive and work-intensive process. Therefore, there has been considerable effort in functionalizing biomaterial scaffolds with viral vectors, such that cell seeding onto the scaffold and cell transduction by gene

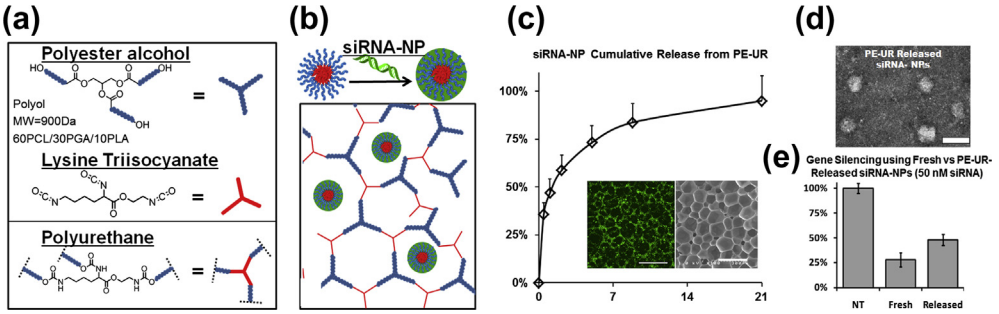

FIGURE 35.9

PE-UR scaffold delivery of siRNA-NPs. (a) Polyester triols and lysine triisocyanate are the primary precursors of the PE-UR network formed via hydroxyl-isocyanate reaction. (b) Lyophilized siRNA-NPs can be incorporated into the scaffold during curing. (c) Sustained *in vitro* release kinetics of siRNA-NPs incorporated into biomaterial scaffolds (insets show dispersion of fluorescent siRNA-NPs in the scaffold and a scanning electron microscopy (SEM) image of the porous scaffold. (d) TEM images confirmed release of intact siRNA-NPs from the scaffold (scale = 100 nm) and (e) qRT-PCR confirmed maintenance of gene silencing bioactivity of siRNA-NPs post-release.

delivery vehicles becomes a single procedure [221]. This approach may also enhance transduction efficiency by co-localizing the cells and virus and also protect them from the immune system. Patterns or gradients of viral vectors can be generated to spatially control gene transfer and tissue development. Material-mediated gene delivery also preserves and protects the vector from the immune system and controls the regional localization of the virus. These approaches typically involve either encapsulation of the virus within a hydrogel, immobilization of the virus to a surface, or a combination of both of these strategies. The unique biological properties of each virus require distinct techniques for each vector.

The incorporation of adenovirus into hydrogels or protein matrices has led to control over the spatial and temporal delivery to surrounding tissue while also protecting the virus from loss of activity [222,223]. Delivery of therapeutic genes from hydrogels can also enhance the wound healing properties of materials such as fibrin [224] and collagen [225]. This approach has been particularly successful in promoting the healing and integration of dental implants in animal models, suggesting a promising application in oral defects and disease [226]. Adenovirus may also be bound to exposed surfaces of polymer scaffolds via conjugation of anti-adenovirus antibodies to chemically reactive surfaces [227] or the use of biotinylated adenovirus [228]. This strategy can be used to spatially organize gene transfer by controlling chemical functionalization [229]. Adenovirus has also been widely used in gene-eluting stents and heart valves to prevent restenosis and valve disease [230–232].

Schwarz and colleagues have shown that the enhanced stability of AAV vectors can be exploited to create biomaterials, allografts, and other tissue substitutes with freeze-dried AAV coatings that are taken up by cells after implantation. The coating of bone allografts with osteogenic and angiogenic factors encoded by freeze-dried AAV vectors led to increased bone healing [233]. The same approach has also been used to engineer bone tissues *in vitro* by coating allografts [234] or polymer scaffolds [235].

The electrostatic properties of the retroviral and lentiviral lipid bilayer can modulate virus interactions with biomaterials, leading to enhanced and controlled transduction of cells [236]. This approach has been used to create gradients of retroviral particles on three-dimensional scaffolds that encode genes that direct tissue formation, providing a means for more complex tissue formation that better mimics natural development [237]. Ionic interactions can also be used to load lentivirus onto hydroxyapatite NPs that protect the virus to enable incorporation into hydrogels [238,239]. Lentivirus has also been immobilized onto a variety of other materials with various properties that can be tailored for controlled gene transfer *in vitro* and *in vivo* [240,241].

CLINICAL APPLICATIONS OF TISSUE ENGINEERING TO NUCLEIC ACID DELIVERY

There are now a wide variety of promising strategies and preclinical studies that have demonstrated effective delivery of plasmid, viral, and oligonucleotide agents, and there has been some translation to the clinic. Viral vectors (adenovirus and adeno-associated virus) require extensive safety testing prior to large-scale studies, and the adverse reactions to systemic administrations in early clinical studies discussed above cast a shadow over any new clinical trial. To avoid adverse, off-target effects, many current trials that involve transient gene transfer *in vivo* utilize direct injection of viral particles into the target tissue: myocardium, skeletal muscle, vitreous humor, and synovium [242]. This form of administration limits systemic exposure, immune response, and potential hepatotoxicity. Hence the development of safe and effective nucleic acid delivery systems remains an important medical objective.

A current compilation of gene therapy clinical trials gives some insight into strategies and targets [52]. There are a wide variety of classes of delivered transgenes among which antigens (20.5%), cytokines (18.4%), receptors (7.2%), tumor suppressors (8.3%), and growth factors (7.5%) predominate. Correction of deficiencies is another significant category (8%), while siRNA currently comprises a small fraction (0.6%) of current trials. Cancer therapy is the overwhelming therapeutic target (64.4%). The dominant viral vectors in current clinical trials are adenovirus (23.3%) and retrovirus (19.7%), and newer vectors like AAV (5%) and lentivirus (3%) have produced exciting preclinical data and are expanding. Naked (plasmid) DNA, which involves the carrier systems discussed in this chapter, is also a significant approach (18.3%). At this point, only 3.6% of current trials have advanced to Phase III, indicating the challenging, uphill path to drug development and nucleic acid delivery.

As discussed above, there have been many recent examples of clinical efficacy of viral gene therapies for rare genetic disorders and cancer, including approval of commercial products [53,54]. However, only a few clinical approaches have utilized biomaterials or tissue scaffolds to date. The only clinical approach that has combined a therapeutic adenoviral vector with a scaffold is the GAM501 formulation, which is a collagen matrix containing adenovirus that expresses rhPDGF-BB. Although early trials certified the safety of this formulation for both bone and skin applications [225], a Phase IIb trial comparing the vehicle with vehicle + vector showed that the collagen scaffold alone was as effective in healing diabetic foot ulcers as the combination product when compared to standard of care [243]. Another example of a vector combined with a biomaterial is the liposome-encapsulated AAV that has been administered intranasally for possible therapy in cystic fibrosis and AAT deficiency, but no conclusive efficacy has been demonstrated (www.clinicaltrials.gov). Similarly, (DOTAP):cholesterol NPs have been employed in recent clinical trials to transfer the TUSC2(FUS1) tumor suppressor gene to patients with recurrent and/or metastatic lung cancer [244].

In addition to viral DNA delivery, several recent clinical studies have focused on RNAi delivery strategies [74,245]. Recent clinical trials include the intravenous administration of I5NPQPI-1002 (siRNA targeted at p53) for acute renal failure in a proprietary, stable nucleic acid-lipid particle technology developed by Alnylam/Tekmira, the use of siG12D LODER (sustained polymeric release of siRNA targeted at the K-RasG12D mutation) for direct injection into advanced pancreatic adenocarcinoma, Atu027 (siRNA-lipoplex directed against protein kinase N3) for suppression of solid tumors, and CALAA-01 (siRNA in a cyclodextrin NP decorated with PEG and transferrin directed against the M2 subunit of ribonucloetide reductase) for head and neck tumor suppression [246,247]. At this point, lipid-based systems have advanced further than polymers in terms of clinical application, and of the nearly twenty total siRNA-based clinical candidates under active testing, seven are based on lipid NP technology [248]. Many of these trials are primarily the result of the combined work of Alnylam and Tekmira, and promising results are coming out from a variety of programs for treatment of

transthyretin-mediated amyloidosis (TTR), hemophilia, hypercholesterolemia, and liver cancer. More recently, inorganic NPs have also come into focus as a promising clinical prospect, especially for delivery of siRNA [197].

OUTLOOK

Two decades of laboratory-based gene and nucleic acid delivery have provided compelling arguments for continuing the refinement of vehicles and mechanisms for improving the safety and efficacy of gene manipulation. Recent advances have begun to yield the production of both viral and nonviral vectors that offer improved spatiotemporal control over expression kinetics and distribution. There are sharp contrasts among the design features for systemic vs. local delivery, acute vs. sustained activity, regulated vs. constitutive expression, and extrachromosomal vs. chromosomal localization. For systemic or local delivery applications, the proportion of viral or nonviral payload that reaches its intracellular target is governed by the biodistribution or local release to the target tissue site, the clearance rate of particles, the efficiency of intracellular uptake, and trafficking to the optimal intracellular compartment. For systemic applications, one should maximize the circulation time, minimize the non-specific cellular uptake, and maximize retention and/or uptake once the target cell has been reached. In contrast, local, scaffold-based delivery applications are more simply dependent on rapid cell recognition and intracellular delivery. Thus, the delivery criteria vary by applications, and there is a compelling need for safer and mode-specific therapies.

Numerous challenges must be overcome to optimize the therapeutic potential of nanovehicular delivery of nucleic acids and genes. Even with local release, better targeting and uptake strategies are important to raising the therapeutic index of genetic agents. In the coming years, we expect vigorous progress from both the cell and molecular perspective that will further enhance our understanding of the basic phenomena involved in drug/gene/vehicle uptake. Internalization of the delivery systems leads to a poorly defined trafficking process that is difficult to manipulate. Elucidation of the non-endocytic pathway(s) mechanism may enable the creation of rationally designed vehicles. Currently, it is not fully understood how the nanovector particle size, steric stabilization, targeting ligands, and cargo chemistry can be collectively optimized to positively influence the mechanism of uptake. However, the diversity of nanovehicle uptake mechanisms are constantly becoming better defined for different cell types, offering the possibility that genetic engineers can successfully manipulate or leverage uptake pathways to influence uptake to predominantly occur by the desired cell type and via the desired internalization route [249].

Engineering viral vectors with new properties, including enhanced targeting abilities and resistance to immune responses, is a growing area of research, particularly in the area of generating viral vectors with novel gene delivery capabilities. Rational design of viral vectors has yielded successful advances *in vitro* and *in vivo*. However, there is often insufficient knowledge of viral structure-function relationships to re-engineer existing functions or create new capabilities, such as virus-cell interactions, whose molecular basis is distributed throughout the primary sequence of the viral proteins. High throughput library screening and directed evolution methods offer alternative approaches to engineer viral vectors with desired properties. Parallel and integrated efforts in rational and library-based design promise to aid the translation of engineered viral vectors toward the clinic [76].

Newly emerging fields are likely to have a significant impact on gene delivery and tissue engineering in the near future. For example, a primary goal of synthetic biology is to engineer novel gene regulation systems with enhanced control and precision [250,251]. This has led to the development of various complex systems such as genetic logic gates and autonomous oscillators for greater control of gene expression in mammalian cells. Similarly, the field of genome editing with engineered nucleases, recombinases, and transposases is revolutionizing

gene therapy by enabling the targeted addition of genes to specific sites in the genome and the controlled alteration of genome sequences, in contrast to the addition of transgenes [252,253]. Many of these approaches incorporate the advantages of both conventional viral and nonviral strategies and are beginning to enter clinical trials.

As with other areas of drug development, the expense of preclinical toxicology testing of novel delivery vehicles can slow the pace of implementation. Clinical development can only proceed after safety has been established, and the bar will inevitably be higher for systemic formulations.

Further investigation is necessary for rational selection of suitable nano delivery vehicles for a specific application. Investigators tend to focus preferentially on one class of vehicles, and few generalized design rules are available to guide the optimal selection for a specific application. The desirable delivery route, localization, target cell, and biocompatibility of nanovehicles will, in the end, dictate the utility of individual constructs for each application. For both viral and nonviral approaches, the continuous efforts to improve currently available systems and to develop new methods of gene delivery are bringing us closer to more widespread adoption of safer and more efficient gene delivery systems in the clinic.

Acknowledgments

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PART 8

Breast

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CHAPTER 36

Breast Tissue Engineering: Reconstruction Implants and Three-Dimensional Tissue Test Systems

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INTRODUCTION

The chance of a woman developing breast cancer in her life time is approximately 12% [1]. It was estimated that in 2012 there would be approximately 230,000 new breast cancer cases and 40,000 breast cancer related deaths in the United States [1]. The chance of breast cancer related death in women is approximately 3%, second only to lung cancer. Fear of disfigurement due to mastectomy or lumpectomy and limitations of breast conserving options have precipitated interest in tissue-engineering breast reconstruction options. Similarly, the impact of the disease, both psychological and physical [2,3], and the limitations of current two-dimensional (2D) bioassays used to understand, combat, or prevent the disease have driven the interest in engineering 3D tissue test systems.

This chapter summarizes the various cell types that may be useful for breast reconstruction, the polymers that are being used or that are being explored for use in breast reconstruction, and the limitations/advantages of specific animal models that allow one to test new tissue-engineering approaches. Avenues to potentially promote the vascularization of engineered tissues are discussed, as the major limitation in engineering large tissue volumes is the inability to deliver nutrients and remove waste products once the tissue is implanted. Finally, the chapter overviews the concept of tissue engineering to create benchtop breast tissue test systems that may be able to aid in developing breast cancer therapies and, eventually, vaccines.

BREAST ANATOMY AND DEVELOPMENT

The breast is a dynamic organ that evolves constantly throughout a woman's lifetime. This tissue comprises multiple cell types that actively interact with each other [4]. Microenvironmental signals are key to the developmental processes of the breast throughout maturation [1].

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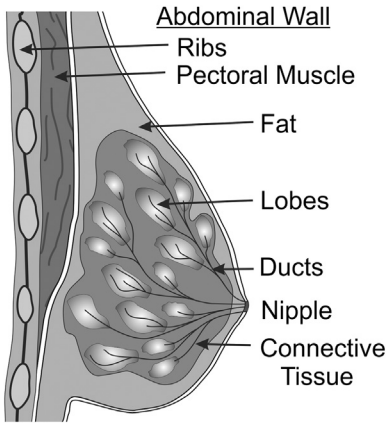

FIGURE 36.1
Breast physiology.

The complexity of the breast must be considered when investigating strategies for engineering breast tissue; however, understanding of the many interactions that occur between the stromal cells and epithelial cells in the breast tissue remains limited. Evidence indicates that paracrine molecules produced by stromal cells likely determine the success or failure of tissue-engineered solutions for breast tissue repair [5]. To better understand the development of breast cancer, it is important to first review the anatomy of the breast and the structures and functions of the tissues (Fig. 36.1). The breast structure is located on top of the pectoralis muscle, which is located on top of the rib cage [6]. Each breast contains 15 to 20 lobes which comprise 20 to 40 lobules. It is inside these lobules that the mammary glands responsible for milk production are found [7]. The lobules are connected together through ducts; the milk is collected in the ducts and then flows out through the nipple. The space between these structures is filled with fat and fibrous tissue, the ratio of which determines breast density. A high concentration of lymphatic vessels and lymph nodes is found throughout breast tissue; these structures facilitate the flow of lymph, which comprises white blood vessels called lymphocytes and a fluid from the intestines called chyle, which contains proteins and fats [8]. The lymph flows to the nearby lymph nodes located in the underarm, above the collar bone, and behind the breast bone [7]. Blood vessels are also present to carry blood around the tissues to provide nutrients to the cells. The size and shape of the breast is determined by the skin envelope and the adipose tissue surrounding the connective and glandular tissues. The firmness of the breast mound is dependent on the number of adipose clusters located within the breast, with higher adipose content resulting in a softer breast mound. The deep fascia and a thin layer of loose connective tissue are located between the breast and the pectoralis muscle; the connective tissue allows the breast to move freely over the deep fascia. The breast is attached to the skin through suspensory ligaments, also termed Cooper's Ligaments, which provide additional support and contribute to the shape of the breast mound [9].

Several changes occur in the breast as a women goes through puberty and menopause. During puberty, hormones released by the ovaries and pituitary gland cause the tissue to grow and the ducts to expand, forming mature ductal structures. While the structures are completely formed, they do not become fully active until pregnancy, when the lobules grow and begin producing milk. During menopause, when hormones are no longer produced by the ovaries, the lobule count in the breast decreases and those lobules that remain shrink in size. This change leads to a lower breast density, since the ratio of dense, fibrous tissue to adipose tissue decreases. For this reason, a woman's breast is typically denser before menopause than after [9].

BREAST CANCER DIAGNOSIS AND TREATMENTS

Historically, breast cancer was not detected at a very early stage and therefore was treated with radical mastectomy, or removal of the entire breast, underlying pectoral muscles, and axillary lymph nodes. As breast cancer detection methods improved, however, breast cancer was discovered at earlier stages, allowing modified radical mastectomies or removal of small tumors. Randomized prospective clinical trials, conducted over 18 years comparing less deformative techniques, demonstrated equivalent survival rates to modified radical mastectomy [10]. These studies demonstrated that, for most women with small breast tumors, simple excision (lumpectomy) of the breast cancer, sampling of the axillary lymph nodes, followed by radiation provides a similar outcome to a radical mastectomy.

Different treatment options can be used in combination to maximize the desired outcome. Breast cancer patients have therapeutic options, such as chemotherapy, radiation, and hormone therapy, and/or surgical options. Most often the treatment will be a combination of surgical and therapeutic options; treatment is determined on a patient by patient basis since individual cases of breast cancer have distinct characteristics. The treatment option is chosen based on stage of the cancer (e.g., *in situ* or invasive), size of the tumor, health condition of the patient, and several other factors.

There are two main surgical procedures, mastectomy and lumpectomy. The progression of the breast cancer is the main determinant of procedure. If the cancer has spread beyond the tumor mass formation, then a mastectomy, or removal of the entire breast, is recommended. If the cancer has not progressed outside the initial tumor mass, then a lumpectomy, or removal of the cancerous mass, can be performed followed by the use of an adjuvant therapy, most often radiation [11].

A mastectomy involves the removal of the interior of the breast mound, the nipple, the areola, as well as a wide margin of tissue around the incision. Prior to surgery, the extent of the axilla tissue that must be removed in order to remove the cancerous tissue is determined. Cancer cells most easily spread through the lymphatic system, so the progression of the cancer can be determined by examining the lymph nodes around the cancerous breast [11]. Because of the ease of travel through the lymphatic system, it has become common practice to remove some of the surrounding lymphatic vessels and nodes.

BREAST RECONSTRUCTION

There are no cosmetic surgical procedures available for lumpectomy patients. Following a mastectomy, patients are given the option of undergoing breast reconstruction surgery. The type and timing of reconstruction is determined first by the physical limitations of the patient and then by preference. An option that has grown in popularity is breast reconstruction immediately following mastectomy [12]. Previously, it was thought that reconstruction should be delayed to prevent any possible interference with an adjuvant therapy. However, benefits to immediate reconstruction include one surgery and hospital stay, better psychological outcomes, and improved aesthetic results. Risks involved with this combination surgery include extended surgical time and an increase in complexity of the procedure [12]. The risk and rewards of undergoing such a procedure are evaluated on a patient by patient basis. There are two primary types of tissue implants for breast reconstruction; synthetic and autologous.

Synthetic implants

Generally, synthetic implants are simpler and require less surgical time, but the results are not as aesthetically satisfactory. The simplest reconstruction is a silicone breast implant, which is a silicone gel- or saline-filled silicone bag that is implanted in the submuscular position beneath the removed breast mound. In some instances, the void volume may be increased by the progressive inflation of a tissue expander [12] prior to placement of a silicone breast

implant. Because an implant is a foreign body, it may trigger a substantial inflammatory response, resulting in fibrosis, thickening, capsular contraction and an unnatural shape and tactile quality. Also, implants may leak and require replacement. Silicone-filled implants are now rarely used due to fears of possible complications caused by the leakage of silicone.

Tissue flaps

Autologous breast reconstruction relies on the use of the patient's own tissue, is more complex, requiring more extensive surgery and a longer recovery time but the results are much more natural and aesthetically pleasing than those of prosthetic implants. There are several primary tissue retrieval sites, including the abdomen, the back, and the buttocks or thigh. The transverse rectus abdominis myocutaneous (TRAM) flap, located on the abdomen, is surgically excised, including fatty tissue, abdominal wall skin, often with the blood supply network intact, and is molded into the breast mound. A TRAM flap procedure requires that an additional surgery be performed to reconstruct the nipple-areola and to improve the shape of the reconstructed breast mound; a weakening of the abdomen as well as contour abnormalities of the abdomen can occur after this procedure [9]. The latissimus dorsi flap is removed from the back in a similar but less involved manner than removal of the TRAM flap. Disadvantages of this flap procedure include susceptibility to atrophy and lack of patient-to-patient tissue volume consistency. Muscle-free flaps from the buttock (gluteal) or thigh (tensor fascia lata) may also be used; in some instances a muscle sparing procedure may be used, wherein only fat/skin is transplanted. Adipose tissue matrices, devoid of lipids and cells, have been investigated for use in soft tissue defect repair [13].

Cell transplants

Some of the very first studies performed regarding adipose tissue replacement used a method called autologous fat transplantation. This methodology simply involved harvesting adipose tissue from a location on the patient and transplanting that tissue to the breast tissue void. This procedure was completed without a scaffold in place to guide the shape of the tissue replacement. The results of this type of procedure were very poor, with 50–70% reduction in volume due to the resorption of the grafted tissue. Resorption occurred because adipocytes are anchorage dependent and require a scaffold to survive. Additionally, the adipocytes found in the tissue graft were terminally differentiated and, therefore, could not proliferate [9]. After several failed attempts, it was determined that a scaffold was required for proper breast tissue replacement.

The cellular material used for transplantation is obtained from lipoaspiration, a process that can damage and lyse the cells. Researchers are developing means with which to treat lipoaspirate, for example, the lipoaspirate may be washed in a polyoxamer, which is thought to stabilize the cellular membranes of damaged adipocytes, and lend greater stability for implantation and graft survival. Preliminary studies suggest that the treatment can increase implant viability post implantation [14].

Cellular scaffolds

Breast tissue engineering is another reconstructive option, beyond that of synthetic or autologous implants. Breast tissue engineering may involve cellular or acellular scaffolds. Much of the current research is still focused on traditional approaches of *ex vivo* cell expansion; however, due to financial, regulatory and logistical obstacles, more focus must be placed on 'just-in-time' delivery options that do not incorporate cell expansion *ex vivo*. Currently, viable breast tissue-engineering technologies have not been translated from the laboratory to the clinic due to several existing challenges related to cell culture, scaffold type, and animal model selection that affect our ability to build a vascularized tissue that maintains volume in the long-term.

CELL TYPES AND RELATED CHALLENGES

Many issues must be considered when choosing which cell type(s) should be used in human breast tissue engineering. The first consideration is that there is substantial variability in the size, shape and consistency of the breast. The breast changes over time, with a tendency for breast parenchyma (glands and ducts) to involute or regress as a woman ages, particularly after menopause, and be replaced by fat [15]. Also, comparing breast tissue among women of any given age, there is considerable variability in the size, shape, tactile, elastic and tensile characteristics of the tissue. The tensile and elastic characteristics of the breast are influenced by three major factors:

1) The amount and quality of fat within the breast;
2) The amount and quality of glandular and ductal tissue in the breast;
3) The mechanical characteristics of the fibrous support structures of the breast (Cooper's ligaments).

The creation of a functional breast with lactational ability is not needed and, in fact, may add to a woman's breast cancer risk by introducing mammary epithelial cells that may be predisposed to cancer development. The major, immediate goal of breast reconstruction is to produce a breast mound with all of the aesthetic properties of a normal breast.

Normal breast tissue comprises adipocytes; however, these lipid-laden fat cells (adipocytes) are terminally differentiated and will not divide further *in vivo* or *in vitro*. Indeed, studies have shown that if not maintained in a 3D culture environment, these differentiated cells will likely de-differentiate and become fibroblastic [5]. Hence, use of adipocytes requires harvesting fat in the exact volume required for the construct [16–18]. Additionally, the majority of mature fat cells in a lipoaspirate sample rupture [8,9,18]; accordingly, measures must be taken to compensate for or prevent this loss.

Pre-adipocytes may be used for engineering soft tissue [19,20] as these cells are not as susceptible to retrieval damage and can potentially be expanded in culture. Pre-adipocytes are similar to fibroblasts in structure and possess the ability to expand in culture [21]. Several studies have shown ways of inducing differentiation into adipocytes. These cells can be successfully harvested and isolated from sites such as the subcutaneous tissues or the omentum. Investigations of autologous pre-adipocyte implantation with a sheep model have been promising [22]. Technologies are being developed to allow simple harvesting and fast isolation of pre-adipocyte cells for immediate re-implantation into the patient [23]. Studies in mice have shown that the incorporation of 'adipose-derived regenerative cells' in fat grafts decreases cell apoptosis and increases expression of growth factors [24].

Possible other cell types include smooth muscle cells, fibroblasts, skeletal muscle and elastic cartilage. Fibroblasts contribute greatly to the support structure of the breast by laying down bands of collagen that connect the breast tissue to the skin and to the pectoral muscle, as well as helping maintain the overall shape. The density and firmness of the breast is determined primarily by the glandular epithelium and ductal structures, where tissues that have similar tactile and elastic properties are almost exclusively muscle. Smooth muscle cells can be readily isolated from a number of organs and expanded in culture; implantation of smooth muscle-containing polymers can lead to the reformation of significant tissue masses, with reorganization of the smooth muscle tissue into appropriate 3D structures [25]. Muscle myocytes can also be greatly expanded *in vitro*, and have been demonstrated to reform functional tissue masses under appropriate conditions [26,27]. However, it remains to be demonstrated that smooth or skeletal muscle myocytes will maintain a tissue mass over long periods of time without neural stimulation. An alternate approach might even be to incorporate chondrocytes in an engineered breast tissue. Elastic cartilage has many of the mechanical properties of glandular breast tissue that are potentially important for tissue engineering of breast (e.g., elasticity). Chondrocytes can be expanded in culture, and are able to survive in low

oxygen tensions. Chondrocytes have been used extensively in tissue engineering to engineer a variety of tissue constructs both *in vitro* and *in vivo* [28–30].

However, the more commonly studied cell types for breast tissue engineering include human bone marrow-derived mesenchymal stem cells (hBMSCs) and human adipose-derived mesenchymal stem cells (hASCs) [31]. Both cell types have the ability to differentiate into adipocytes when introduced into an adipogenic differentiation medium. The hBMSCs are harvested from the bone marrow, through a relatively painful procedure, which lessens their clinical relevance, while hASCs can be harvested in large volume from adipose tissue obtained via biopsy or liposuction [31]. Recognizing that regeneration may most effectively occur *in situ* with cues from the native tissue, cell types such as mesenchymal stem cells and embryonic stem cells have rapidly become the most popular cells for breast tissue-engineering research. Human embryonic stem cells, hESCs, are readily able to differentiate into adipocytes [31]; however, a great deal of work will need to be done to ensure terminally differentiated cells, that once transplanted, do not have the potential to form a tumor mass. The implantation of embryonic stem cells has the potential to result in teratoma formation *in vivo* [32,33]. Studies in mice have shown that the formation may be site dependent and may be exacerbated in the presence of specific biomaterials [34].

Standard cell isolation and expansion protocols must also be developed; critical steps influencing successful outcomes will include aseptic technique in the harvest, routine quality control testing of all cultures, long-term cell storage, and on-site operating room handling. It may also be possible to isolate all the cell types required for breast tissue engineering from a single tissue source; furthermore, it may be advantageous to retrieve tissue isolates rather than cellular isolates [35]. For example, fat, in addition to adipocytes, contains a large vascular network, composed primarily of capillary endothelial cells and some vascular smooth muscle cells as well as a collagen stromal structure produced by fibroblasts [36]. Hence, multiple cell types or cellular aggregates (tissue isolates) can potentially be obtained from this tissue. In attempting to produce fat tissue *in vivo*, it may be beneficial to expand the cellular components of fat without isolating each component, as the complexity of the mechanisms may be crucial and impossible to rebuild from cellular blocks. Given the complexity of tissue, it is most likely that a mix of cells, with respect to maturity and type, will result in the most promising tissue-engineering solution.

SCAFFOLDS

An important consideration in engineering breast tissue is the scaffold selection. Selection of a material chemistry and form to use as a scaffold depends on the purpose and characteristics of the tissue that is being replaced as well as the physical characteristics and health of the patient. Scaffolds may be fabricated to induce tissue integration or they may be developed to house or attract cells which, in turn, assist in inducing tissue integration. Several key characteristics make a biomaterial suitable for tissue-engineering application. First, with *in vivo* endpoint, favorable biomaterials must be absorbable or degradable, and therefore facilitate new tissue integration with native tissue over time. The shape and texture of the material does not have to resemble that of natural tissue but it should induce growth of new breast tissue resembling native tissue, i.e., be soft and pliable. Cellular affinity is another important aspect of a biomaterial, whether considering *in vivo* or *in vitro* endpoint. The biomaterial must interact favorably with cellular components without negative impact (e.g., the material cannot be tumorigenic or toxic). Some element of porosity or surface texture is helpful and potentially allows cellular ingrowth into the material and/or transfer of nutrients and waste products, the establishment of a vascular network into the biomaterial scaffold [37], and/or differentiation of multipotent cells [38].

Synthetic materials

Aliphatic polyesters of polyglycolide (PG) and polylactide (PL) are well characterized synthetic biodegradable polymers which are clinically familiar to biomedicine and are familiar also to

tissue-engineering researchers [39]. PG is highly crystalline, has a high melting temperature, and has low solubility in organic solvents. PL is more hydrophobic than PG due to the presence of a methyl group. PL has a low water uptake and its ester bond is less susceptible to hydrolysis, due to steric hindrance by the methyl group. Therefore, PL degrades more slowly and has higher solubility in organic solvents than PG. Copolymers of PL and PG can be readily synthesized; their physical properties are modulated by the ratio of glycolic acid to lactic acid. Often incorrectly named in the literature, polylactide and polyglycolide are synthesized by ring opening polymerization, while the lower molecular weight polylactic acid and polyglycolic acid are synthesized by step growth. The latter are on the order of thousands of Daltons and are limited to use in structures with low mechanical demands, such as films and spheres [40]. Aliphatic polyesters can be readily processed into various physical forms appropriate for tissue-engineering applications. A number of techniques have been proposed to generate highly porous scaffolds, including solvent casting/particulate leaching [41], phase separation [42], emulsion freeze-drying [43], fiber extrusion and fabric formation [44], and gas foaming [45,46].

Polyethylene glycol (PEG) scaffolds [47] are readily modified by degradation and adhesion peptides and thus have been the subject of pre-adipocyte studies. The combination of adhesion and degradation features appears to allow the highest adhesion and proliferation of pre-adipocytes. Non-degradable polymers have also been examined for tissue reconstruction applications. For example, *in vitro* studies have been conducted with fibronectin-coated polytetrafluoroethylene scaffolds (PTFE). Human pre-adipocytes have been shown to successfully attach, proliferate, and differentiate into adipocytes on the PTFE scaffolds [48]; however, these non-degradable scaffolds are likely better suited for 3D *in vitro* tissue test systems.

A number of other synthetic polymers could be used to fabricate scaffolds for breast tissue reconstruction, including polycaprolactones, polyanhydrides, poly(amino acid)s, and poly (ortho ester)s [49]. Polycaprolactone (PCL) is an aliphatic polyester, a semi-crystalline polymer with high solubility in organic solvents, a low melting temperature, and a low glass transition temperature (T_g). The degradation rate of PCL is much slower than PG or PL; because of the low T_g, PCL has a flexible, sticky quality which can be advantageous in a scaffold. PCL has been tested in scaffold form in animals [50,51] and is used clinically in orthopedic applications. Polyanhydrides are usually copolymers of aromatic diacids and aliphatic diacids. These materials degrade by surface erosion, the rate of which can be controlled depending on the choice of diacids [52]. Poly(amino acid)s have been studied due to their similarity to proteins, and have been widely investigated for use in biomedical applications such as sutures and artificial skin [53]. Poly(amino acid)s are usually polymerized by ring opening of N-carboxyanhydrides; versatile copolymers can be prepared from various combinations of amino acids. However, due to the low solubility and limited processability of poly(amino acid)s, 'pseudo'-poly(amino acid)s were developed [54]. It has also been reported that poly(amino acid)s containing L-arginine, L-lysine or L-ornithine cause endothelium-dependent relaxation of bovine intrapulmonary artery and vein, and stimulate the formation and/or release of an endothelium-derived relaxing factor identified as nitric oxide [55]. Poly(ortho ester)s are biodegradable polymers, which degrade by gradual surface erosion and have been investigated for controlled drug delivery.

Naturally-derived materials

Naturally-derived polymers have been used for adipose tissue engineering; scaffolds produced from these materials typically are hydrogels or structural forms like mesh, sponges [9], or beads. Investigations have been conducted, for example, using Matrigel™ (reconstituted basement membrane of mouse tumor) and fibroblast growth factor 2 (FGF-2) to induce *in situ* adipogenesis. Matrigel™ consists largely of type IV collagen, laminin, and perclan and, although a highly variable material with many ill-defined components, is considered the gold standard in cancer cell biology benchtop studies. Preliminary small animal studies have

demonstrated the migration of native pre-adipocytes as well as endothelial cells into Matrigel™ when this material is injected into subcutaneous tissue [9,37,56].

Hyaluronic acid (HA) is a natural component of the extracellular matrix of many tissues. HA comprises repeated sequences of glucuronic acid and acetylglucosamine; this material is susceptible to enzymatic degradation via hyaluronidase. Simple modifications have been made, such as cross linking the chains to form insoluble hydrogels [57]. In its natural form, HA plays a role in enriching wound healing by promoting early inflammation and stimulating angiogenesis [58]. Hyaluronan benzyl ester (HYAFF® 11) scaffolds are derived from hyaluronic acid that is esterified with benzyl groups at the glucuronic acid monomer. Researchers experimented with these sponges, seeding them with human pre-adipocytes and surgically implanting them into subcutaneous tissue of athymic nude mice. The sponges allowed good cellular penetration as well as the development of new vascular networks within the sponges. However, adipose tissue development remained sparse [59]. The researchers also compared collagen scaffolds to HYAFF® 11 scaffolds *in vivo*, and concluded there was increased implant weight, adipose tissue formation and distribution of cells in the HYAFF® 11 scaffolds [60]. Hydrogels of hyaluronic acid have been prepared by covalent cross linking with various kinds of hydrazides [61] and have been used in drug delivery [62].

Collagen is the best-known tissue-derived natural polymer and is the main component of all mammalian tissues, including skin, bone, cartilage, tendon, and ligament. Collagen has been used as a tissue culture or artificial skin scaffold due to its high cell affinity. However, collagen offers a limited range of physical properties, can be expensive [63], is highly variable, and can elicit a strong immunologic response. Chemical modification and incorporation of fibronectin, chondroitin sulfate, or low levels of hyaluronic acid into the collagen matrix can change cell adhesion [64]. *In vivo* comparisons of freeze-dried collagen scaffolds with hyaluronic acid sponges and non-woven mesh, implanted in mice for 8 months, revealed a greater number of adipocytes in the hyaluronic sponges than in the non-woven mesh. This difference was mainly attributed to the porous nature of the sponge, which allowed greater surface area for adipocyte cell distribution and growth [60,65]. Recent studies also have assessed the idea of creating a fibrovascular tissue bed or natural scaffold, using a pre-expansion vacuum into which fat cells are transplanted [66].

Alginate is a naturally occurring hydrogel that can be easily formed into an injectable gel or beads, but must be modified with a peptide sequence to allow cell attachment [22,67,68]. Interestingly, the human body does not contain alginase, the enzyme that breaks down the alginate chain, hence molecular weight is a crucial consideration for implantation as large molecular weight alginate chains will not be eliminated from the body. That is, the molecular weight of many alginates is typically above the renal clearance threshold of the kidney [69]. Alginate chains are bound together with divalent ions that migrate in areas of divalent ion deficiency, causing uncontrolled dissolution. To address this point, hydrolytically degradable, covalently cross linked hydrogels derived from alginate were developed [70]. Specifically, polyguluronate blocks with molecular weight of 6,000 Da were isolated from alginate, oxidized, and covalently cross linked with adipic dihydrazide. The gelling of these polymers could be readily controlled, and their mechanical properties depended on the cross linking density. It was also demonstrated that alginate gel degradation can be readily regulated by controlling the molecular weight distribution of the polymer chains in the gels, and their susceptibility to hydrolytic scission by partial oxidation [71].

Other materials have found limited, preliminary use in breast tissue engineering. Chitosan is relatively biocompatible and biodegradable [72,73], making it useful for breast tissue engineering [74] and wound healing [75]. Chitosan is abundant and easily derivatized by coupling molecules to the amino groups [75,76] and has shown early success in murine studies focused on injectable breast tissue-engineering systems [35]. Fibrin glue has been used as an adipocyte scaffold and, in small animal studies, has facilitated maintenance of adipose tissue up to

one year after implantation [78]. Fibrin is a blood based product, a characteristic which has slowed its translational appeal in the United States.

Therapeutic scaffolds

Some scaffolds provide more than a simple matrix on which cells will grow. Some scaffolds have an incorporated therapeutic agent such as a drug or growth factor, and are termed therapeutic scaffolds. The concept behind these scaffolds is that the therapeutic agent incorporated in the scaffold is released as the cells remodel the scaffold during cell growth and proliferation. The therapeutic agent therefore has a direct effect on the surrounding tissue where the scaffold is placed; accordingly, the use of growth factors and the stimulation of cell growth must be carefully evaluated in breast cancer related reconstruction, to avoid facilitating the cancer process.

Indeed therapeutic agents can affect the tissue surrounding the implanted scaffold. An example can be seen in a study where the therapeutic agent, angiogenin, was incorporated into a scaffold [79]. Angiogenin is a drug that has been shown to promote neovascularization, so the intent of its incorporation was to help promote the growth of new vasculature throughout the scaffold, increase the chances for the success of an implanted tissue replacement, and improve the overall outcome of the procedure. The investigators subcutaneously implanted these scaffolds into rabbits and, after 28 days, the scaffolds were excised. The study showed that scaffolds with incorporated angiogenin had increased neovascularization. Other therapeutic scaffolds have been designed; for example, a collagen/chitosan/glycosaminoglycan scaffold was assessed [80]. The agent for this therapeutic scaffold, transforming growth factor-beta 1 (TGF-β1) targeted cells grown on the scaffolds rather than the surrounding tissue. Accordingly, TGF-β1 was incorporated into chitosan microspheres that were embedded into the scaffold.

A final example of a therapeutic scaffold is one in which the agent targets and neutralizes a specific type of cell found in the surrounding tissue, such as a cancer cell. Researchers developed, for example, scaffolds with nanoparticles containing emodin, an anti-cancer drug [81]. These scaffolds were intended to fill a site where a cancerous tumor was removed from the breast. The concept of this scaffold is that as cells proliferate on the scaffold and remodel it, the emodin contained within the nanoparticles is released and neutralizes cancerous cells in the surrounding tissue. These scaffolds were implanted next to the mammary fat pads of nude mice in which cancerous cells had been injected. The results of this study indicated that the size and number of the tumors next to emodin-loaded scaffolds were reduced compared to those next to scaffolds without emodin.

Injectable scaffolds

Early scaffolds took forms such as foams or mesh; they required implantation via an open surgical procedure. However, the reality of the transport and surgical limitations of large volume implants led to the development of alternate scaffolds [82]. Injectable scaffolds can be combined with cells and surgically implanted in a minimally invasive manner, including by syringe, catheter or endoscopic needles [83,84]. These injectable materials take forms such as gels, beads, or composite gels. Examples of injectable scaffold chemistries that have been assessed for breast tissue engineering include alginate, chitosan, hyaluronic acid, collagen, polyanhydride, degradable PEGs, decellularized adipose tissue, small intestinal submucosa, and blends thereof [13,35,82,85,86]. While gels support cell growth and readily conform to a defect, they typically do not support the necessary functions of anchorage dependent cells and are therefore useful as carriers but not as scaffolds in breast tissue engineering. Beads and composites (which contain beads or other filler) in contrast, are amenable to breast tissue engineering.

Combination scaffolds

It is likely that a combination of biomaterials, with respect to chemistry and form, will result in the most promising tissue-engineering solution. Scaffold combinations that use two or

more types of materials can be used to help combat and overcome the weaknesses and shortcomings of one material. For instance, a material that has excellent cell attachment characteristics but is not very durable can be combined with a more durable material, hopefully resulting in a scaffold that is both durable and able to support cell growth.

One of the first combination breast tissue-engineering scaffolds was an injectable composite comprising beads in a delivery gel [82]. Indeed, cellular constructs that are approximately 500 μm in thickness or less, once implanted, may optimize diffusional transport of nutrients to the cells while each small cell-polymer unit becomes vascularized. Accordingly, these injectable composites were developed specifically to allow trafficking and infiltration of blood vessels, nutrients, waste products, other factors and cell types, within the discrete portions of the scaffold (i.e., between beads) [35,82]. The beads, or a fraction of the beads, may be selectively loaded with appropriate factors to induce tissue growth or prevent abnormal tissue growth. The gel is degradable and facilitates delivery of the composite through a needle, and also allows the composite to conform to and fill an irregular defect site. The gel may be loaded with factors for release on degradation. A variety of studies have been conducted to demonstrate the modularity of an injectable composite approach [35,68,82,87,88].

It is unlikely that there is a one-size-fits-all biomaterial chemistry or form; accordingly, there is interest in a wide range of combination scaffolds. In one study, gelatin sponges, polyglycolide mesh, and monofilament polypropylene mesh were used to construct three-dimensional scaffolds of predefined shapes on which human adipose-derived mesenchymal stem cells (hAD-MSCs) were grown [89]. The scaffolds were made of an outer polypropylene mesh pocket that contained gelatin sponge cubes and polyglycolide mesh. Gelatin is an attractive scaffold for cell growth and attachment and can be molded into a desired shape but rapidly loses its dimensional stability over time. The polyglycolide mesh was used to increase the surface area available for cell attachment. Polypropylene mesh was used because of its ability to maintain dimensional stability after being implanted into the body. These scaffolds were seeded with a high density hAD-MSC suspension and cultured for 2 weeks. Subsequently, the scaffolds were implanted into the backs of nude mice for 2 months and then excised for analysis. Analysis showed that the scaffolds contained new adipose tissue as well as neovascular structures. The gelatin cubes, as well as the polyglycolide mesh, were completely absorbed by the body but the outer polypropylene mesh retained the predefined dimensions; the neovascular structures may simply be the transient part of the normal foreign body response.

STRATEGIES TO ENHANCE THE VASCULARIZATION OF ENGINEERED TISSUE

A critical challenge to engineer breast tissue, or any tissue of significant thickness, remains the development of a vascular network to support the metabolic needs of the engineered tissue and integrate it with the rest of the body. Adipose tissue is highly vascularized, with a resting blood flow two to three times higher than that of skeletal muscle. The presence of vascularized networks in natural metabolic organs results in short diffusion distances between the nutrient source and the cells [90], and these vascular networks must be created in engineered tissues as well. Nutrient diffusion *in vivo* is constrained to a distance of approximately 150 μm. Most metabolically active cells that are located further than this distance from a nearby capillary are subject to hypoxia. Thus the success of any large engineered tissue hinges on its blood supply. The important interrelationship between pre-adipocytes and endothelial cells was demonstrated in hypoxic culture experiments. Frye and coworkers [91] exposed cell cultures of pure pre-adipocytes, as well as mixed cultures of pre-adipocytes and microvascular endothelial cells, to hypoxic conditions (5 to 2% O_2) and found that pre-adipocytes co-cultured with microvascular endothelial cells had higher viability than cultures consisting of pre-adipocytes alone.

Several general approaches have been taken to date to promote angiogenesis in engineered tissues; however, none provide a robust, consistent solution to this complex problem. Bland and colleagues [92] provide a detailed review of approaches to combat hypoxia in

tissue-engineered systems. In short, this problem has been unsuccessfully addressed in the long-term by incorporating endothelial cells or angiogenic factors in tissue-engineered implants. Concerns about blood vessel promoting factors are high when targeting solutions for breast cancer patients. The composite injectable systems do provide the option of gradually building smaller volumes of tissue over time to collectively build a large volume [93]. Many studies highlight angiogenic response at short time points, suggesting that the foreign body response provides the necessary vascularity [94,95]. Indeed, while this is the case in the short term (and at later defined intervals as the material is further degraded or absorbed and elicits further response), the vascularity is temporary only. Blood vessel ingrowth occurs slowly with this approach, will likely not be sufficient to engineer large tissue volumes, and will likely subside with termination of the foreign body response. Other approaches attempt to actively modulate the vascularization process by either delivering angiogenic molecules or blood vessel forming cells (e.g., endothelial cells) to the site at which the tissue is being engineered. Prevascularization, either *in vitro* or *in vivo*, has also been proposed and investigated by numerous groups, but the problem of integrating newly developed vasculature with host tissue remains unsolved.

Microstamping using nano-fluid chambers is one research approach of interest in liver tissue engineering [96], where the long-term goal is to develop a 3D vascular bed *ex vivo* that could be anastamosed to the host vasculature to support cellular engraftment. This approach could also be developed for breast tissue engineering. Another approach involves the direct association of an implant with a pre-existing blood supply. Experiments with nude mice were performed in which silicone molds packed with polyglycolide fibers were sewn to the inferior epigastric blood vessels. These silicone molds were injected with a combination of Matrigel and FGF-2. *In situ* adipogenesis was demonstrated over a 4 to 20 week time course. Direct application of a vascular pedicle to a construct is another promising approach. Prefabricated flaps have been created with vascular pedicles since the 1960s; vascular pedicles can be supplied in many different configurations. In general, however, conduits comprising an intact artery and vein fare better than those comprising a single vein alone [97,98].

Special considerations

The need for nipple reconstruction occurs after mastectomy. Traditional implants include free composite grafts, local tissue transfer, and prosthetic devices. Free composite grafts were initially used and were created from autologous tissues such as the labia, inner thigh, cartilage (auricular or costal), the contralateral nipple, or the toe. Complications at the graft site made this technique less desirable. Local tissue flaps are the most popular option for nipple reconstruction. Commonly used techniques include the bell flap, the modified star flap, and skate flap [99–102]. Unfortunately these techniques can be hindered by flap necrosis and poor aesthetic results, including loss of nipple projection. Additionally, the presence of underlying subcutaneous fat is important for bulking; this layer is not always sufficient. Tissue-engineering approaches include the use of tissue flaps coupled with acellular, naturally-derived (collagen, extracellular matrix, etc.) matrices [103] and/or fat grafts.

BREAST CANCER MODELING

As Savage stated, 'Cancer is not a simple disease' [104]. A significant challenge to both the development of breast cancer treatments and fundamental understanding of breast cancer processes is the development of a tool, a model, which allows isolation and control of specific parameters of interest. The study of basic disease processes and treatments in a patient is generally very inefficient because of the number of confounding biological (and ethical) issues. In response to this challenge, researchers have proposed animal models, mathematical models, and benchtop test systems as the means to more effectively study breast cancer.

Animal models

It is important to first consider the application for which an animal study is planned before the selection of the animal model itself. Either the nude (nu/nu) or severe combined immunodeficiency (SCID) mouse model is used for the transplantation of human-derived cells [105]. These animals have compromised immune systems to the extent that they will often accept xenogeneic transplanted tissues, and these mice have been particularly useful for transplantation and immunologic studies of human tumors, bone marrow, skin and other tissues [93,106,107]. SCID and nude mice are used to evaluate various polymer constructs with and without human cells *in vivo*, without the adverse effects of a major immunologic reaction. Furthermore, basic questions about human cells and polymers *in vivo* may be answered without launching human trials prematurely. Development of these models may not be straightforward since there are subtle differences between strains with respect to the acceptance of various tissues and growth of the implanted tissues in different sites (e.g., a tissue may grow in one mouse strain but not in another, or may grow in a subcutaneous site but not in an internal location). Even though mice are genetically altered for immunodeficiency, the particular genetic modifications can vary. These varying genetic modifications can cause varying levels of different hormones throughout the host body. Hormonal differences will result in adipose tissue development differences that will likely result in different biological reactions to the same implant. Several different genetic varieties of laboratory rats exist, which poses complications when attempting to compare studies [105]. Biologically, males and females of the same species have different hormone levels due to differences in development and maturation. Additionally, gender-induced differences in hormones cause a difference in adipose tissue development which may result in different biological reactions to similar scaffold constructs [105].

Nude mice lack a T-lymphocyte response, while SCID mice lack both a T- and B-lymphocyte response. However, both types have natural killer cells that may interact with some transplanted materials. These mouse colonies must be monitored closely for changes in the immune status of the mice that, at times, occurs spontaneously. In addition, the human cells must be routinely screened for the human immunodeficiency virus, hepatitis and mycoplasma before being transplanted into immunosuppressed mice to assure the safety of the animals and workers, and the validity of the experiments.

One other complicating factor is the size of the actual animal in question. Small size animals include animals such as mice and rats, while larger sized animals include sheep, domestic pigs, and cows [105]. When considering mammary tissue, the larger sized animals are more physiologically relevant to humans. This issue raises the question of applicability of relating animal model results to an expected human clinical trial outcome. Large animals can accommodate the same size implant as one that would be used in a human. Also, the internal anatomy of larger animals, such as the domestic pig, is much more similar to the human anatomy in regard to organ size and heart rate. It has also been shown that the histoarchitecture and hormonal control of the mammary glands of larger animal models is much more applicable to humans than those of rodents [108]. Studies have shown that bovine cells as well as human cells do not grow in the mammary fat pads of immune suppressed mice. This observation suggests that the fat pads of rodents do not provide an environment suitable for the proper growth of human or bovine mammary epithelium. It has also been shown that progesterone has a very different effect on the mammary tissue of mice when compared to that in larger animals, including humans. In mice, progesterone stimulates epithelial proliferation and ductal side branching, whereas progesterone has a limited effect on the mammary epithelium of larger animals and humans.

There has been excellent success using inbred female Lewis rats as a small animal model for the development of transplantable tissues with absorbable polymers such as PG, PL, poly(lactide-co-glycolide), and hydrogels such as alginate [109]. This model allows transplantation of cells

between individuals without concern for immunologic rejection, which parallels the likely autologous nature of cell transplantation for breast engineering. In addition, the Lewis rat is larger than many other strains, allowing the testing of larger (1−2 cm) or multiple constructs in the same animal.

In evaluating larger animals as models for breast tissue engineering, animals with skin and subcutaneous tissues that are similar to humans are required in order to evaluate larger constructs subcutaneously. For this reason, the same animal must be used as a tissue donor and recipient. Porcine skin and subcutaneous tissues are very similar to that in humans, but most pigs continue to rapidly gain weight throughout their lives, which makes monitoring implants very difficult. Sheep have very little subcutaneous fat and, depending on the location, have well-defined subcutaneous space for cellular engraftment. Hence, if new adipose tissue is formed there is a high probability that this is developed from the implanted cells. One of the few locations in the sheep where there are significant fat deposits is the omentum. Researchers have shown that pre-adipocytes isolated from the omentum can be expanded in culture, seeded onto porous alginate-RGD fragments [22], where they attach, proliferate, and spread onto the biomaterial surface. Cellular alginate-RGD fragments were subsequently injected into the nape of the neck of sheep to determine if new adipose tissue would form. Although the cells were autologous and not labeled with a tracking marker, there appeared to be *de novo* adipose tissue formation in the cell implant sites compared to the acellular biomaterial control sites.

Indeed, further investigation of breast tissue-engineering options within a large animal that has biological characteristics comparable to that of humans is required. The bovine mammary gland consists of the same anatomical structures and tissue types as that of the normal human breast [110]. Histological evaluation reveals that bovine mammary tissue is more similar to that of humans than is mammary tissue of traditional animal models such as mice and rats. The ductal structures in the human and cow are surrounded by relatively dense stromal tissue [111,112] unlike the ducts in the mouse which are almost completely enclosed by adipocytes [113].

Table 36.1 summarizes the similarities and differences seen in mammary gland development in mice, humans, pigs, and ruminant animals like sheep, goats, and cows. However, larger sized animals present logistical and financial issues. Large animals are much more expensive than smaller animals and fewer researchers are trained in, or have the facilities for, the proper care of these larger animals [108].

Breast tissue test systems

To better mimic aspects of the *in vivo* setting, three-dimensional tissue test systems are used to model experimental clinical conditions. Events that are notable in a 2D setting may not be present in a 3D setting, or vice versa [5]. Breast tissue test systems can be used to inform tissue-engineering reconstructive techniques, or to better understand and prevent the disease process. Many studies have been conducted to assess breast cell co-cultures in 3D. The 3D material, i.e., the scaffold, has enormous influence on cellular behavior, and can be selected according to the biological aspect of interest. Obviously, 2D cultures are not amenable to the study of ductal structures or questions regarding spatial co-location [114].

In order to distinguish tissue test systems from *in vivo* human or animal models and refine the expectations of an *in vitro* model, a tissue test system can be defined as a modular unit of 'useful' biology. A tissue test system is depicted in Fig. 36.2 as a tissue model built using a Lego™-like approach with biological and non-biological components that can then be used to answer biological questions. The three primary questions that must be considered in designing a test system are:

1) What is the biological question of interest and how can this question be answered with an approximate biological model?

TABLE 36.1 Comparative aspects of mammary gland development [108]

Attribute	Mice	Humans	Pigs	Ruminaants
Morphology	Sparse ducts: alveolar	TDLU	TDLU	TDLU
Stromal histology	Adipose » connective	Intralobular, interlobular connective » adipose	Intralobular, interlobular connective » adipose	Intralobular, interlobular connective » adipose
No. of ductules/TDLU (Types 1, 2, and 3, respectively)	N/A	11, 47, 81	9, 24, 64	Not defined
No. of galactophores	1	~ 8–15	2	1
Epithelial proliferation	Concentrated in endbuds and alveoli	Concentrated in endbuds or TDLU	Concentrated in endbuds or TDLU	Peripheral zones of TDLU
Response to estrogen	Endbud	Epithelial	Endbuds, TDLU	Epithelial
Response to progesterone	Alveoli	Negative, or No Effect	No Effect	No Effect
Mammary tumors	Spontaneous, viral origin	Spontaneous	Rare, few cases	Rare, few cases
Tumor precursor	AH, ADH, DCIS	ADH, DCIS	Unknown	Unknown
Tumor hormone dependence	Rare	50–60%	Unknown	Unknown

Abbreviations: TDLU: terminal ductal lobular unit, AH: alveolar hyperplasia, ADH: atypical ductal hyperplasia, DCIS: ductal carcinoma *in situ*, TDLU: terminal ductal lobular unit.

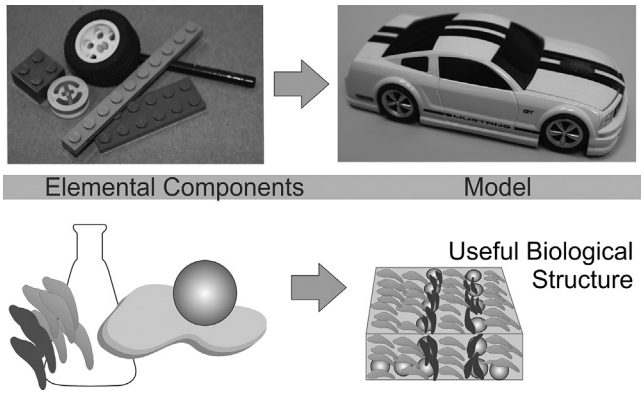

FIGURE 36.2
Top row shows the Lego™ approach where plastic blocks are used to build complex models. The analogy to biofabrication of a tissue test system is shown in the bottom row where the elemental components, cellular units, biomaterials such as beads, fibers, or gels, and biochemical agents are assembled to produce a tissue model.

2) What are the elemental components (i.e., the Lego blocks) and how are the elemental components arranged to make the biological model?
3) How will the elemental components be assembled and the final tissue cultured?

One cannot survive in the world without mentally forming cause and effect relationships — models — that provide useful advice about behaviors of the systems around us. Many natural systems are understood through models which provide useful information, such as predicting the future state of a system for a change in environmental conditions. As an example, the behavior of a resistance may be described by the simple Ohm's law mathematical model of the voltage across the resistance equals the resistance multiplied by the current, $v = Ri$. This model is 'useful' to make calculations such as how many light bulbs could be connected to an electric circuit. The Ohm's law model is easy to use, scales to more complex problems, is consistent and reliable since it works for anything that is a pure resistance, has been verified by experimentation, is cost effective to use, can be extended to account for additional physical phenomena, and increases understanding of the system. Thus, even though the Ohm's law model is not a copy of the resistance it provides useful information. A tissue test system holds the same promise to provide a model that facilitates the creation and sharing of new knowledge of breast tissue processes.

The Ohm's law resistance model has obvious limitations. When the model is tested under unmodeled conditions like very high temperatures, the information from the resistor model becomes less accurate and hence less 'useful'. In this case the resistance model can be updated by changing the constant resistance to a function of temperature, i.e., $R(T)$. Specifically, the resistor model must be designed to include the phenomena that affect the accuracy and resolution needed to provide useful information for a specific question of interest. Thus, a breast tissue test system must be defined to match the expectations of the user to provide useful information. Practically, a tissue test system must be defined to meet the user's needs and likely should not be an exact duplication of the *in vivo* biology; rather, it should capture only the salient physiological, mechanical, biochemical, morphological, and biological elements needed to study a specific phenomenon.

A general schematic of the process of designing, fabricating, and using a tissue test system is shown in Fig. 36.3. Tissue test system development begins with sampling the physical system

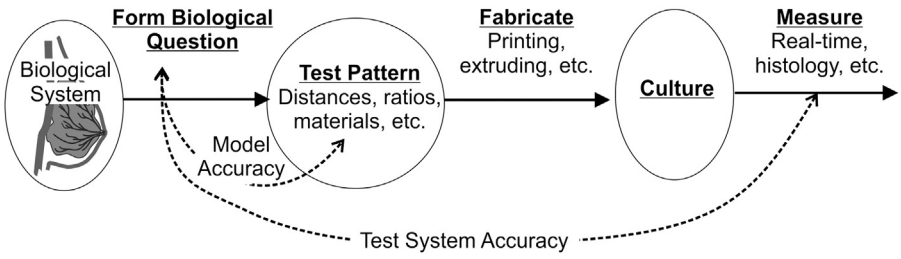

FIGURE 36.3
Process of developing biologically relevant tissue test systems.

to identify the scope and features needed in the test system model. The first challenge in using a tissue test system is defining what behaviors in the native system must be included to address a specific question of interest; that is, what does one intend to learn from the tissue test system and what are the first-order factors that affect the behavior under study. Model accuracy is used here to describe the translation of a biological question into a test pattern or structure for the tissue test system. Note that high model accuracy does not mean that the test pattern must be an exact reproduction of the biological structure, in this case the breast; rather, it must be a representation of the underlying chemical, physical, electrical, etc. phenomena that are useful for answering a specific biological question regarding breast tissue. In fact, a well-designed test system will exclude factors that confound answering the biological question of interest. Hence, different biological questions will require different test system models. As an example, there are currently test systems, such as those addressing adhesion assays, that comprise cells seeded onto a plate. These are relatively straightforward test systems to implement and hence are often the first assays performed. Such a flat, two-dimensional (2D) system can answer important questions; for example, adhesion assays were used to study the expression and function of Laminin-511 during metastatic breast cancer progression [115]. These results can inform decisions regarding incorporation of Laminin-511 in a breast tissue implant, or regarding selection of reconstructive biomaterials that may promote or inhibit production of this extracellular protein. Note, however, that 2D cultures are not amenable to the study of breast ductal structures or questions regarding spatial co-location [114]. Following Fig. 36.3, the test pattern must be fabricated, for example by cell printing, and then cultured. The fabrication system to produce a desired test system and then the culture system, e.g., a bioreactor, to produce the environmental conditions, such as temperature or pH, present significant instrumentation challenges. Test system accuracy describes the overall degree to which the biological question of interest can be studied in the *in vitro* culture.

An established tool in breast cancer studies that is also applicable to breast reconstruction research is the breast tissue organoid, which is formed by digesting harvested tissues into duct-like structures that resemble the original organ in appearance or function. While it is obvious that an organoid model formed by removing tissue and allowing the remaining tissue to grow in a new manner is not a physical copy of the breast tissue, it has been shown that this simplified model can produce useful results. For example, in the work by Cellurale and coworkers [116] the authors were able to isolate the role of cJun NH2-terminal kinase signaling in mammary gland development and tumorigenesis using mammary organoid cultures. In other studies [117], organoids were used as the basic building block of human mammary epithelial cell cultures. The organoid model uses an elemental component that already has significant structure and is directly derived from the biological source. There are more general approaches to building test systems that use biofabrication techniques to assemble smaller elemental pieces. Fig. 36.4 shows additional considerations in biofabricating a test system

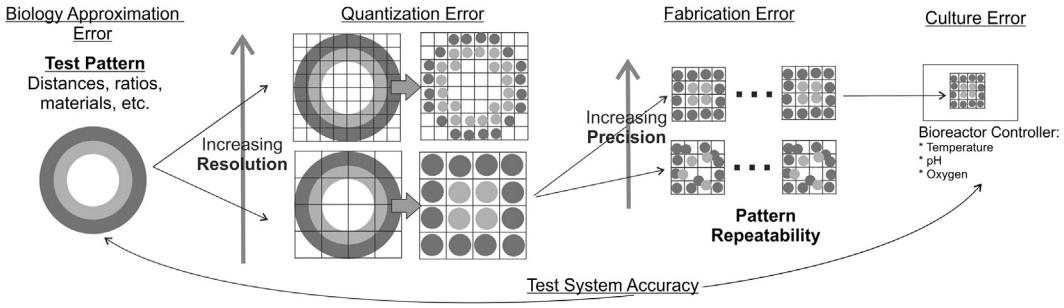

FIGURE 36.4
Sources of error in biofabrication of tissue test systems include the quantization error that arises from resolution of the fabrication instrumentation, the fabrication errors that arise from the precision and repeatability of the fabrication instrumentation, and the culture errors that arise from the ability of the culture controller to reproduce environmental conditions.

model. The first source of potential error is the discretization of a proposed test pattern based on the resolution of the fabrication system. The two-dimensional pixel error is illustrated in the second column of Fig. 36.4 where it can be seen that the biofabrication system with the coarser resolution produces a lower-fidelity replica of the test pattern. The 3D equivalent of pixel error is the voxel error which includes the pixel error as well as a depth component. For example, if a test pattern is defined at the cellular level using micron resolution, such as a scan of a histology slide, then a biofabrication technique that has a smallest fabrication component on the order of a millimeter can only produce an approximate copy of the desired pattern. Thus the approximation of continuous biology with discrete fabrication components leads to quantization error.

The second fabrication error type is in the ability of the biofabrication technique to result in placement of elemental components at a desired location. As illustrated in the third column of Fig. 36.4, high precision means that the system can closely replicate the discretized test pattern. The ability of a process to produce identical replicates of a test pattern is then defined as the pattern repeatability. This is defined as different from the machine precision because occurrences such as cell settling in storage reservoirs can cause evolution of the biofabrication instrumentation precision over time. Note that there is much debate about how much quantization error and fabrication error can be tolerated in building a useful tissue structure. One consideration is that tissue structures will self-assemble from an appropriate starting condition [118]. Specifically, placing cells 'close enough' will allow them to move and assemble based on their natural behaviors. Any approach to tissue fabrication will require that nature take over for the final stage of tissue assembly, the idea of 'close enough' is actively debated. There are two main technologies used to place the cellular components to fabricate a tissue system (see Fig. 36.5a). In the first approach, known as drop-on-demand deposition, a fixed quantity of cells and medium are deposited as a single drop. A representative method of forming these droplets is inkjet printing [119]. Inkjet printing uses either a thermal or mechanical process to eject a fixed droplet size (on the order of 100pL). This same process is used in many home color printers. A biofabrication system based on thermal inkjet printing is shown in Fig. 36.5b. A second technology is the deposition of a small block or pellet of cells through extrusion or separation from a feedstock. Speed, cost, damage to cells, and resolution are the differences that distinguish these technologies.

The ability of the tissue culture system to reproduce environmental conditions can greatly affect overall results, the culture error shown in the last stage of Fig. 36.4. An important part of specifying the tissue test system is to define the environmental conditions, e.g., dissolved oxygen level, that will be used to incubate the tissue. The capability of the culture system to produce these conditions must be considered; limitations occur in the ability of the instrumentation to measure a quantity of interest and the ability of the control system to modulate that quantity. As with all aspects of the tissue test system, the environment is a simplified model of the actual environment and as such must be designed at the start of a project to address a specific question of interest.

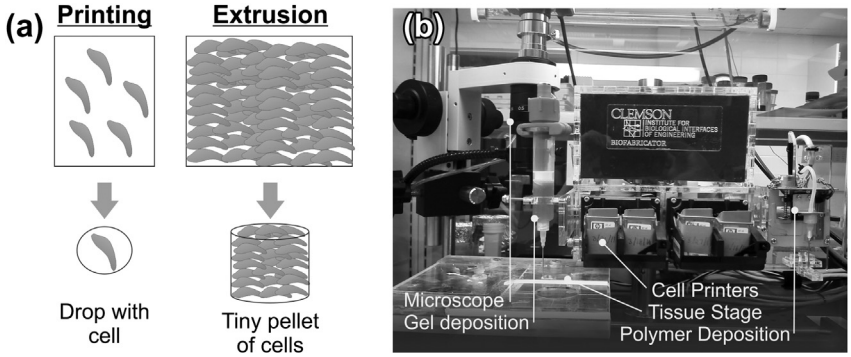

FIGURE 36.5
(a) Two general approaches to cell deposition, printing of cells in medium and extrusion of small cell pellets. (b) Photo of a biofabrication system with multiple deposition stations for cells and biomaterials.

As a final consideration, the state or feature of interest must be measured in the tissue or medium during and/or after culture. A tissue test system can present the same imaging and measurement challenges as *in vivo* experiments – real-time measurements like oxygen levels, discrete measurements like magnetic resonance imaging, and endpoint analysis like classical histology may all be used but have the same fundamental instrumentation limitations. However, there are several important advantages of a tissue test system over an *in vivo* model. First, there is the possibility that sensors can be embedded directly into the tissue design. For example, it is possible to embed a temperature sensor in the center of a tumor mass as it is fabricated. Second, the tissue is potentially more amenable to direct observation than *in vivo*, e.g., a tumor model could be grown on a microscope stage and images automatically taken at regular intervals to observe spreading or growth. There is generally improved access to both inputs and outputs of the culture in a test system, agents can be applied without metabolism or complex pharmokinetics distorting the input. As an example, it is possible to apply an anti-angiogenic agent to a cell culture and know the local concentration without measurement because the pharmokinetics can be easily modeled as a mixing problem in a fixed volume. Similar arguments suggest that observation of outputs, such as metabolic byproducts, are more accessible in a test system. Perhaps the most important advantage that the tissue test system can have over an *in vivo* test is that, given low fabrication errors and culture errors, each copy of the test tissue should be nearly identical. This means that replicates can increase the power of observations and increase the ability of outside groups to repeat experiments and results. In general, a tissue test system should make the biology of interest more accessible for observation and manipulation.

In summary, tissue test systems are an evolving approach to modeling complex biological systems *in vitro*. Ethical, economic and scientific drivers will ensure that this technology continues to evolve. However, the use and expectations of the test system must recognize that the tissue and culture environment is an approximate model that must be designed to address a specific question of interest. As researchers and clinicians begin to appreciate all of the subtleties of designing, fabricating, culturing, and assessing tissue test systems, these approximate models will help reveal the complexities of breast biology and disease processes and therefore will provide the insight to engineering breast tissue. As tissue test systems, the basic units of useful biology, become standardized they can be integrated to produce systems capable of answering ever more complex questions.

In silico breast cancer models

Mathematical modeling of breast cancer continues to advance rapidly, so much so that any summary is nearly obsolete at the time of writing. However, a brief overview of some approaches helps demonstrate the potential and challenges of mathematical and computer models. Generally speaking, there are two starting points for deriving a mathematical model, the first is the application of basic chemical and physical equations (first principles) to create a model, and the second is the observation of physical behavior and use of mathematical tools to capture this behavior. A model may be proposed to work at a specific scale, such as at the molecular level, the cellular level, the organ level, the organism-wide level, or the population level. Initially, models were developed to capture the behavior of an isolated system at a fixed scale. As modeling sophistication has increased, multi-scale models [120] of connected subsystems have evolved. Often the models are referred to as '*in silico*' to indicate that the simulation is performed on a computer and to highlight their similarity in role to *in vivo* or *in vitro* models. As with other modeling approaches, the best model is the one that allows the user to address the question at hand.

As an example of a simple model, tumor angiogenesis is modeled as a set of two interconnected differential equations, one subsystem models tumor growth and one subsystem models the carrying capacity of the vascular network [121]. This model appears to be a gross

summary (as an ordinary differential equation) of the physical diffusion processes and the effects of the biological signals that stimulate or inhibit angiogenesis. However, analysis of the model has provided new insight into the scheduling of anti-angiogenic treatments. The traditional dosing of such a therapy centers on applying a constant dose at regular time intervals; however, the model provides the structure to apply optimization and control techniques that suggest a more efficient use of the treatment agent [121,122]. There will always be questions about the resolution and accuracy of any mathematical model. For example, the angiogenesis model may not be sufficient to model clinical cancer treatment. By connecting a second subsystem model, the movement of bone-marrow-derived endothelial progenitor cells to the tumor site and their effect on tumor growth can be used as a starting point to model vasculogenesis [123]. Such a model can clarify further the possible treatment strategies, including chemotherapy and anti-angiogenic therapies aimed at suppressing vascularization, which may be incorporated in a tissue-engineered implant.

CONCLUDING REMARKS

Tissue engineering may provide a means of both assessing and building reconstructive breast implants for a woman undergoing lumpectomy or mastectomy. The development of vascularization and the long-term retention of tissue volume are keys to viable breast reconstruction options. Benchtop tissue test systems will be critically important in addressing these two major issues, by facilitating markedly improved understanding of the normal and diseased states and, accordingly, assessing and developing advanced reconstructive options.

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PART 9

Cardiovascular System

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CHAPTER 37

Progenitor Cells and Cardiac Homeostasis and Regeneration

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INTRODUCTION

Complementary methodologies introduced in various laboratories have provided clear evidence of the dynamic state of the normal and pathologic heart. This novel view of cardiac biology has introduced the concept of the heart as an organ that is permissive for myocardial regeneration. However, the magnitude of myocyte turnover and the cell of origin of the newly formed parenchyma remain a matter of debate. These two relevant issues are discussed in our chapter. Understanding the biological process that leads to myocyte formation is a challenging task that requires the characterization, at the single cell level, of the phenotypical and functional properties of myocytes and non-myocytes within the heart. By this approach, it is possible to discriminate whether myocyte renewal is accomplished by:

a) The commitment of stem cells that generate all specialized lineages within the cardiac tissue;
b) The replication of pre-existing myocytes to produce more cells of identical lineage; or
c) The dedifferentiation of pre-existing cardiomyocytes that lose their specialized function, acquire progenitor properties, re-enter the cell cycle, and, eventually, regain the original specialized phenotype.

These multiple mechanisms of tissue restoration may act in concert to regenerate organs like the heart with a complex structural and functional identity. This information is of critical relevance for the development of novel cell-based therapies that may prevent the onset of heart failure or delay its evolution.

CARDIAC PROGENITORS IN THE ADULT HEART

Because of the controversy concerning the true existence of stem cells in the heart and their role, if any, in cardiac homeostasis and repair, an overview of the characteristics of the multiple progenitor cell classes identified in the adult heart is needed for a better understanding of the cell of origin of the newly formed cardiomyocytes. Several laboratories have demonstrated that the heart contains a compartment of primitive cells with the characteristics of stem cells; however, the identification of the actual cardiac stem cell (CSC), equivalent to the

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hematopoietic stem cell (HSC) in the bone marrow, has been controversial. After the discovery of c-kit-positive CSCs (see the section 'C-Kit-Positive Cardiac Stem Cells'), different classes of progenitor cells have been characterized in the adult heart, but whether they represent distinct categories of undifferentiated cells with diverse functional properties is currently unknown. A variety of surface antigens, transcription factors and functional assays have been used to define these cells.

The first identification of myocardial progenitors was based on the ability of stem cells to expel toxic compounds and dyes through an ATP-binding cassette transporter [1]. This property, used initially to isolate side population (SP) hematopoietic cells, defines a pool of putative cardiac progenitors which form colonies in semisolid media and differentiate into cardiomyocytes. Although a comprehensive analysis of stem cell properties was not performed in this early study, the work by Rudnicki and collaborators introduced the concept of a myocardial stem cell that participates in the response of the heart to ischemic injury.

The presence of ABC-transporter activity has been tested repeatedly by exposing cardiac cells to the DNA-binding dye Hoechst 33342; functionally competent cells clear the fluorochrome, become Hoechst-low, and occupy a side position in the fluorescence-activated cell sorter (FACS) profile [2]. However, the protocol for the separation of bone marrow SP cells cannot be applied to solid organs such as the heart without caveats. This technical difficulty has led to the isolation of several classes of unrelated cardiac SP cells.

SP cells were found to comprise a surprisingly large pool of cardiac cells in the mouse ($\sim$2%) which were Sca1^{high}, c-kit^{low}, CD34^{low}, and CD45^{low}. The ability to extrude dyes was attributed to the expression of the multidrug resistance protein Abcg2. Despite their high percentage and the presence of the vascular marker Sca1, lineage tracing assays indicated that Abcg2-positive cells do not include endothelial cells (ECs) [3]. A distinct side cell population was reported to include 4%, 2%, and 1.2% of cells in the fetal, neonatal, and adult rat heart, respectively. Bcrp1 was considered the molecular determinant of the SP phenotype [4]; however, most Bcrp1-positive cells expressed CD31 and were located within the intima of the vessel wall. CD31-negative Bcrp1-positive SP cells were also detected in the peri-vascular region and myocardial interstitium; they expressed CD29 and N-cadherin at the interface with myocytes and smooth muscle cells (SMCs). After injury, SP cells generated predominantly vimentin-positive fibroblasts and calponin-positive SMCs; a small fraction of cells acquired the myocyte and EC lineage [4].

An atypical subset of SP cells was considered to represent embryonic fetal remnants of neural crest-derived cells. These cells formed clonal spheroids, expressed markers of neural precursors, including nestin and Musashi-1, and generated *in vitro* neuron-like dendrites and peripheral nerve cells [5]. Following, transplantation into chick embryos, these SP cells formed cardiomyocytes.

The unusual and dissimilar properties of the multiple SP pools identified in the adult heart emphasized the need to introduce substantial modifications to the original protocol employed in the bone marrow and establish a methodology better suited for cell isolation from the adult mouse myocardium [2]. The SP functional assay was combined with labeling of the surface markers Sca1 and CD31 to select for Sca1-positive/CD31-negative cardiac cells, a subset of cardiac SP cells with enriched cardiomyogenic potential [6]. By this approach, functionally homogenous SP cells were obtained and their molecular properties clearly defined. SP cells express in a developmentally regulated manner the P-glycoproteins Abcg2 and Mdr1: Abcg2 is responsible for dye efflux during postnatal development, whereas in adulthood this function is mediated exclusively by Mdr1. Sca1-positive, CD31-negative, cardiac SP cells can acquire the molecular and functional characteristics of adult myocytes [6]. Abcg2 promotes proliferation and survival of SP cells inhibiting differentiation. Deregulation of Abcg2 may alter the fate of

these progenitors, resulting in uncontrolled cell growth or death [7]. Additionally, the canonical Wnt signaling plays a crucial role in the maintenance of the self-renewal ability of this cell class [8]. Bone marrow SP cells do not contribute to the maintenance of the cardiac SP cells in physiological conditions, but can repopulate the resident pool after injury.

Different protocols have resulted in the isolation of distinct Sca1-positive cells, which do not possess the functional properties of stem cells. Sca1 progenitors were found to represent 2% of heart cells and 15% of the myocyte-depleted fraction [9]. In culture, only 3–4% of Sca1-positive cells displayed sarcomeric proteins, and the *in vivo* delivery of these cells led to modest cardiomyogenesis mediated by fusion with resident cells. A distinct immunoselection strategy resulted in the isolation of a pool of murine Sca1-positive cells that accounted for 0.3% of the myocyte compartment [10]. Approximately 10–40% of the cells expressed the pan-leukocyte marker CD45 and the hematopoietic/endothelial epitope CD34. After exposure to oxytocin, Sca1-positive CD45-negative cells showed cardiac transcription factors and contractile proteins organized in sarcomeric structures [10]. In permissive media, Sca1 cells differentiate into osteocytes and adipocytes, strongly suggesting that the Sca1 antigen identifies a heterogeneous cell population composed of hematopoietic, mesenchymal, endothelial, and cardiac progenitors. Monolayered sheets of Sca1-positive cells placed over the necrotic myocardium prevented negative remodeling and improved cardiac function after infarction [11]. This finding raised the possibility that cell transplantation acts through the release of humoral factors, activating endogenous CSCs or recruiting bone marrow cells to the infarcted myocardium. In addition, these cells generate SMCs, ECs, adipocytes, and osteoblasts.

State of the art imaging protocols have been developed to follow *in vivo* the destiny of Sca1-positive cells [12]. Sca1-positive cells were isolated from transgenic mice that constitutively express luciferase and EGFP, enabling *in vivo* tracking by non-invasive imaging and post-mortem identification by immunolabeling [12]. The robust bioluminescence signal at day two decayed with time and the non-viable infarcted portion of the wall was not reduced by cell treatment. Consistently, echocardiographic and MRI measurements did not show functional improvement; an extremely small number of EGFP-positive regenerated myocytes and vessels were found at sacrifice. Poor survival and rapid death of injected cells are common outcomes when neonatal cardiomyocytes, mesenchymal stromal cells, bone marrow mononuclear cells, and human embryonic stem cell-derived cardiomyocytes are adoptively transferred [13]. This phenomenon has prompted the development of novel strategies involving pre-activation with growth factors, application of bioengineering methods, and genetic modifications to achieve long-term homing to the injured myocardium. However, the role of Sca1-positive cells in cardiac homeostasis remains to be defined.

An alternative source of progenitor cells has been identified in the epicardium, which represents an epithelial sheet on the cardiac surface. The epicardium derives from an extracardiac transient structure, the proepicardium, which is located near the venous pole of the developing heart [14]. In avian species, the proepicardium is viewed as the site of origin of hemangioblasts, a pool of immature cells that are considered critical for the development of the coronary circulation. Hemangioblasts migrate from the proepicardium to the avascular heart tube, giving rise first to the epicardial sheet and subsequently to the endothelial and smooth muscle layers of coronary vessels [14].

Proepicardial cells may not contribute directly to cardiomyogenesis in birds but favor the expansion of myogenic precursors through a paracrine mechanism [15]. In mammals, the identification of an equivalent hemangioblast remains controversial [16], although the existence of a murine hemangioblast has been claimed by *in vitro* studies of embryonic stem cell differentiation. Cells expressing brachyury and flk1 generate in culture hematopoietic cells and, at later time-points, myocytes, ECs, and SMCs [17]. Whether brachyury-flk1-positive cells exist *in vivo*, however, is unknown.

The embryonic fetal epicardium hosts several classes of progenitor cells, uncovering previously unexpected functions of this outer cardiac layer. The epicardial markers WT1 and Tbx18 mark distinct pools of progenitors that travel from the proepicardium to the myocardium, where they form the epicardium and give rise to electrically coupled cardiomyocytes [18,19]. A population of c-kit-positive epicardial cells has been identified in the human heart. These cells accumulate in the subepicardial space of hearts affected by ischemic cardiomyopathy [20]. Experimentally, c-kit-positive epicardial cells migrate from the epicardium to the infarct, where they proliferate and differentiate into myocyte precursors and vascular cells [21]. This process is coupled with upregulation of fetal epicardial markers [22]. The recognition of growth factors modulating the behavior of epicardial progenitors may allow their activation *in situ*, possibly influencing the treatment of the human disease.

The expansion of progenitor cells in non-adhesive substrates leads to the formation of floating spheres. This peculiar form of anchorage-independent growth has been used for the expansion of cardiospheres from endomyocardial biopsies [23]. Cardiospheres contain a core of c-kit-positive primitive cells, several layers of differentiating cells expressing myocyte proteins and connexin 43, and an outer sheet composed of mesenchymal stromal cells. C-kit-positive cells within the aggregates do not correspond to a uniform class of progenitors because of the heterogeneity dictated by the uncommitted or early committed state of the cells, their quiescent or cycling condition, or their migratory properties. This may explain the observed differences in the regenerative potential of single-cell-derived clonal c-kit-positive CSCs [24] and c-kit-positive cells sorted from cardiospheres [25].

The expression of connexin 43 at the interface between immature and differentiated cells may play a dual role. Connexin 43 in undifferentiated progenitors favors their proliferation, whereas connexin 43 in cells committed to the myocyte fate promotes electric coupling with the surrounding cells and the acquisition of functional competence. The presence of intercellular gap junctions within the cardiospheres raises the possibility that the differentiated cells may function as supporting cells. If this were the case, then the cardiospheres would reconstitute *in vitro* the complex structure of the cardiac niches identified *in vivo* [26].

The ISL1 transcription factor is associated with the commitment to the myocyte lineage of cardiac cells that have lost their undifferentiated stem cell fate. ISL1 and GATA4 are transcriptional coactivators of the myocyte transcription factor MEF2C. The cardiomyocyte specification dictated by the expression of ISL1 is coupled with a modest ability of these cells to divide and form clones and, most importantly, to the lack of self-renewal ability. ISL1-positive cells are restricted to the embryonic fetal heart and are no longer present at birth [13], making the possibility of their clinical implementation extremely unlikely. By lineage tracing [27], a few myocytes and vascular ECs and SMCs were found to originate from ISL1-positive cardioblasts, but the frequency of this event was very low.

Collectively, these findings indicate that the heart contains populations of immature cells, which, to a different extent, give rise to cardiomyocytes participating in tissue repair. However, the role of these cell categories in cardiac homeostasis and pathology has to be determined, and the clinically relevant question of whether the human heart possesses a class of CSCs to be employed therapeutically remained to be defined.

C-KIT-POSITIVE CARDIAC STEM CELLS

Cardiomyocytes, as the majority of cells in adult organs, are long-lived cells. If cells persist for most of the lifespan of an animal or individual, their functional capacity is expected to decline with time, compromising organ performance. According to the dogma, ventricular myocytes are terminally differentiated cells and their lifespan corresponds to that of the individual. The number of myocytes attains an adult value a few months after birth, and the same myocytes are believed to contract 70 times per minute throughout life. However, several reports have

provided evidence supporting the view that myocytes die and new cells are constantly formed in the normal heart at all ages. Both processes are markedly enhanced in pathologic states and the imbalance between cell growth and cell death may be an important determinant of the onset of ventricular dysfunction and its evolution to terminal failure.

The c-kit receptor tyrosine kinase was detected originally in a class of murine HSCs with long-term reconstituting ability in irradiated recipients. More recently, c-kit has been found in several populations of stem cells in the adult lung, liver, brain, and pancreas [13]. Several lines of evidence have been accumulated in favor of the view that cardiac cells expressing the c-kit receptor are *bona fide* stem cells; they include the capacity to self-renew, form multicellular clones, and give rise to a committed progeny *in vitro* and *in vivo* [24,28–30] (Fig. 37.1). However, differentiation assays of stem cell clones *in vitro* have inherent limitations including the possibility that culture conditions result in the preferential acquisition of a selective lineage phenotype, masking the *in vivo* potential of the founder cell. Similarly, the identification of multiple phenotypes in the progeny of transplanted non-clonal stem cell populations does not provide a direct evidence of the multipotentiality of each administered cell. This problem has been overcome by the delivery of single-cell-derived clonal CSCs to the injured myocardium;

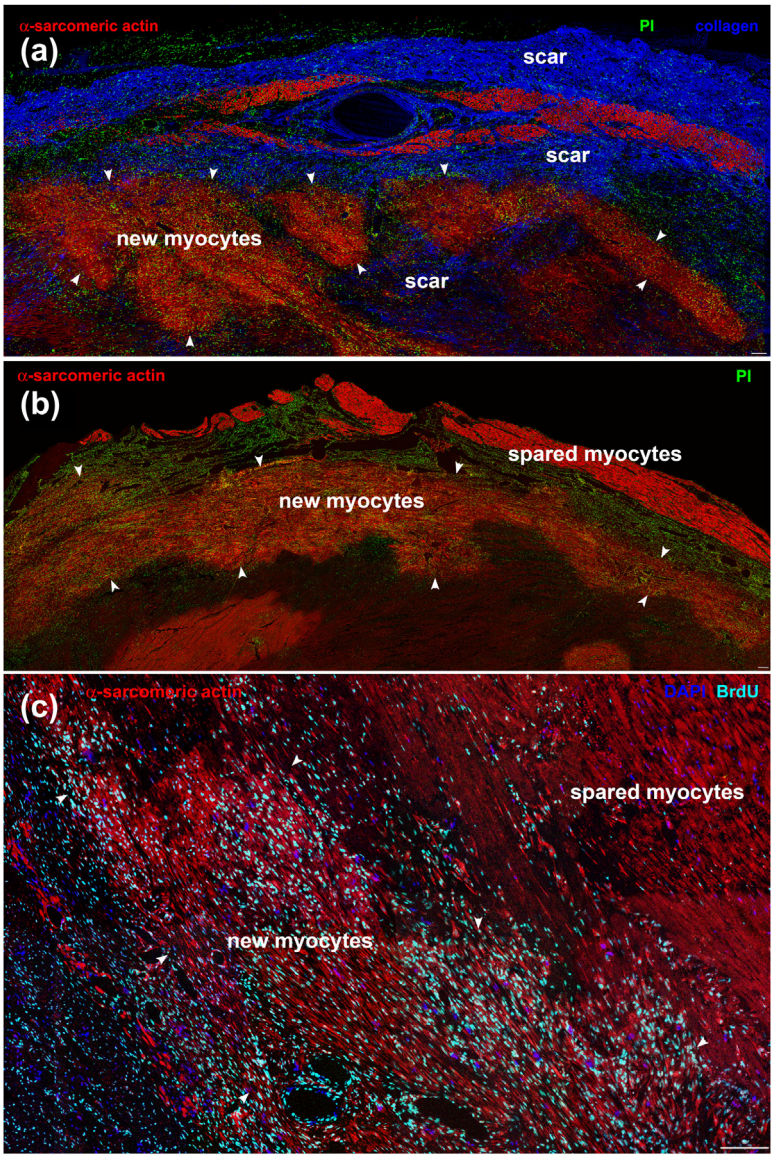

FIGURE 37.1
Myocardial regeneration after infarction. (a) Non-treated infarcted canine heart: the healing process is characterized by the formation of a scar (collagen I-III, blue). (b) Newly-formed myocytes are clustered (α-sarcomeric actin, red, arrowheads) within the area of damage. (c) Bright blue fluorescence in nuclei corresponds to BrdU labeling of accumulated newly formed myocytes.

by necessity, all regenerated structures derive from the individual founder cell that underwent amplification *ex vivo* [24].

Criticisms, however, have been raised concerning the possibility that serial passaging may modify the original properties of CSCs and that tissue injury may affect in an unpredictable manner the fate of CSCs *in vivo*. Novel protocols have been introduced to document unequivocally that cardiomyocytes and coronary vessels originate from CSCs in the non-damaged heart and during physiological aging. Specifically, viral tagging and clonal marking have been implemented to determine whether a resident stem cell pool is present in the myocardium and participate in organ homeosatsis physiologically [30]. Genetic tagging with retroviruses was introduced more than 20 years ago for the characterization of individual HSCs and their progeny [31]. The analysis of the clonality of CSCs and myocyte turnover cannot be performed in humans since it requires genetic tagging of the undifferentiated cells so that the clonal marker of individual mother cells is traced in the specialized progeny *in vivo*. C-kit-positive CSCs located in the niches of the atrio-ventricular groove and apex of the mouse heart were infected with a lentivirus carrying EGFP and the destiny of the labeled cells was determined 1−6 months later [30], providing the opportunity to assess the behavior of tissue-resident primitive cells in the non-injured heart. Although the rate of myocyte turnover in the intact heart is slower than the rapid pace at which cells renew themselves in the presence of damage, the intrinsic properties of CSCs are better characterized when tissue lesions are absent. A common integration site was identified in isolated c-kit-positive CSCs, cardiomyocytes, ECs and fibroblasts, documenting the multipotentiality of CSCs and the clonal origin of the differentiated cells [30]. During a six month period, each EGFP-positive CSC divided around eight times giving rise to 230 cardiomyocytes. These findings, together with data obtained with BrdU pulse-chase assays [26,30,32], indicate that activation and differentiation of CSCs is an ongoing process which results in a significant renewal of cardiomyocytes in the adult mouse heart.

Although viral clonal marking represents the only protocol that can establish the multipotentiality of CSCs *in situ*, limitations involve the low efficiency of CSC infection and the impossibility to collect serial samples of the transduced progeny in small animals. Moreover, whether the insertion site confers a selective advantage or disadvantage to the growth of single cells may be easily assessed in blood cells but cannot be established with certainty in the heart. An additional variable that may influence the assessment of myocyte formation from tagged CSCs involves the insertion of the proviral integrant in repressive regions of the mouse genome [33]. However, silencing of the reporter gene interferes with the recognition of labeled cells by immunohistochemistry but does not affect the analysis of integration sites by PCR.

EGFP is a widely used fluorescent tag for the analysis of the fate of progenitor cells *in vivo* following adoptive transfer, and in lineage tracing and viral clonal marking assays. The immunogenic potential of this foreign protein has raised questions on the appropriateness of its utilization in long-term studies. Processed peptides derived from EGFP may be presented by the major histocompatibility complex on the cell surface, potentially inducing a T cell immune response against the labeled cells. Cells transduced with genes perceived as foreign proteins by the recipient may actively engraft but may be subsequently cleared by the immune system [34]. The magnitude of immunological rejection of cells carrying EGFP remains controversial and most likely context-dependent. Different degrees of bone marrow ablation from sub-lethal irradiation to minimal conditioning have been employed to prevent rejection of EGFP-infected hematopoietic cells [35]. This phenomenon may result in an underestimation of the number of EGFP-positive CSCs and their tagged progeny.

An important aspect of the clonal analysis by viral marking of CSCs *in vivo* consists of the possibility to study the behavior of these primitive cells within their natural habitat, the CSC niches.

CARDIAC STEM CELL NICHES

Stem cells are sheltered in specialized structures called niches, which provide a microenvironment designed to preserve their rarely dividing and lineage negative phenotype [36]. Interstitial structures with the architectural organization of niches have been found in the heart of animals and humans [24,26,37,38–40]. CSCs, progenitors, precursors and early differentiating cells are clustered together in the niche and are coupled with the supporting cells, myocytes and fibroblasts, by gap and adherens junctions [26]. Gap junctions are intercellular channels formed by individual structural units called connexins while adherens junctions are composed of cadherins [41]. Some of the molecular mechanisms that regulate growth, migration, and differentiation of CSCs within the cardiac niches have been identified recently.

In the niches, stem cells divide symmetrically and asymmetrically. When stem cells divide symmetrically, two self-renewing daughter stem cells may be formed. The purpose of this modality of replication, which occurs in active phases of growth during prenatal organ development, is the expansion of the stem cell pool. Stem cells can also divide symmetrically into two committed daughter cells; this type of cytokinesis is triggered by an emergency situation requiring the restoration of the lost tissue upon a pathological insult. With asymmetric division, two differently fated sibling cells are generated: one daughter-stem cell and one daughter-committed cell. The non-stem cell sister is a short-lived committed progenitor cell that divides and simultaneously differentiates, i.e. the amplifying cell [36]. When the amplifying cell has acquired complete maturation, it cannot divide further; it has reached terminal differentiation and growth arrest. The objective of asymmetric division is the maintenance of a steady state in which organ homeostasis is conditioned by a tight balance between stem cell formation and the production of a committed progeny. The developmental choice made by CSCs at any given time has a direct impact on the number of stem cells, progenitors, precursors, amplifying cells and, ultimately, mature cells in a tissue. The size of the amplifying myocyte pool conditions the magnitude of the homeostatic and regenerative response in damaged heart.

The inhomogeneous intracellular segregation of selective proteins in daughter cells at the time of mitosis constitutes the intrinsic determinants of CSC fate in animals and humans. Genes, including numb, α-adaptin and members of the Notch pathway, interact to enable single primitive cells to produce differently destined sibling cells [42]. Numb can segregate to one of the two daughter cells or be equally distributed in the cytoplasm of both daughter cells [43]. Numb is expressed during mitosis, from late prophase to telophase, and in the early stages of life of the new daughter cell. Numb localizes to endocytic vesicles and binds to the endocytic protein α-adaptin, inducing the internalization and inactivation of the Notch receptor.

CSCs that receive Numb become unresponsive to Notch while Numb-negative CSCs retain their responsiveness to Notch and adopt the phenotype associated with Notch activation. Signaling through the Notch receptor can occur only between closely adjacent stem cells and supporting cells [44]. The Notch ligands are transmembrane proteins which upon binding cleave the Notch receptor so that its intracellular domain is translocated to the nucleus where it forms complexes with transcription factors of the recombinant DNA-binding protein (RBP). These effector pathways are operative in the heart and Notch1 activation by the Jagged1 ligand promotes upregulation of the cardiac-specific transcription factor Nkx2.5 and the commitment of CSCs to the myocyte lineage within the cardiac niches in the mouse heart [37,38]. Activation of Notch1 receptor is a critical determinant of the transition of adult CSCs to the compartment of amplifying myocytes, and inhibition of this pathway has dramatic negative consequences on the adaptation of the heart to ischemic myocardial injury (Fig. 37.2).

In addition to Notch1 activation, CSC commitment within the niches may be influenced by the transfer of ions and small molecules from terminally differentiated cardiomyocytes to adjacent stem cells through gap junctions within the niches. Local activation of resident CSCs by growth factors acutely after infarction results in a significant recovery of ventricular muscle

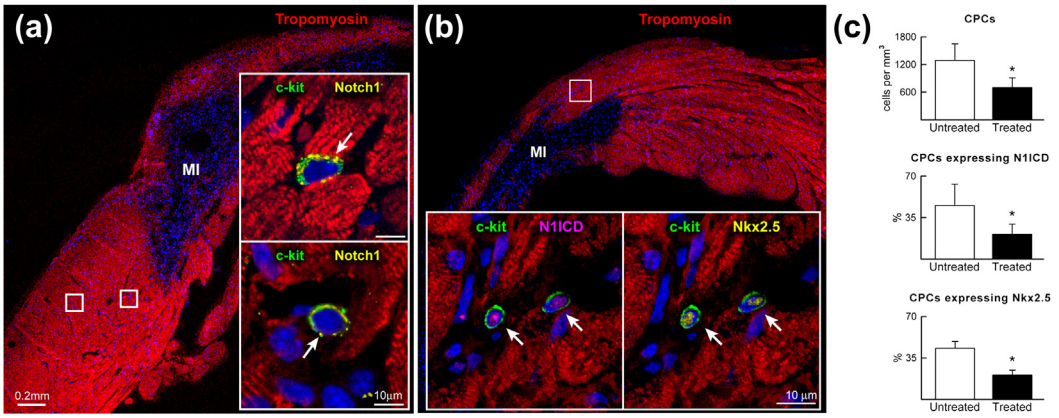

FIGURE 37.2

Notch1 function in the infarcted heart. (a) CSCs are detected in the viable myocardium of the border zone of an infarcted mouse heart. The areas included in rectangles are shown at higher magnification in the insets. CSCs express c-kit (green, arrows) and Notch1 (yellow). Myocytes, red (tropomyosin). MI: myocardial infarction. (b) Following Jagged1 stimulation, the Notch1 receptor is cleaved and its intracellular domain (N1ICD) transfers to the nucleus. N1ICD (magenta) in CSCs is consistently accompanied by the presence of Nkx2.5 (yellow). (c) Notch blockade with *in vivo* administration of γ-secretase inhibitor reduces the number of CSCs and the fraction of CSCs positive for N1ICD and Nkx2.5 in the border zone. $P < 0.05$ vs. untreated mice.

mass. However, only 20% of the regenerated myocytes acquire the adult phenotype over a period of four months while the vast majority of cells display fetal–neonatal characteristics [45]. Similarly, the intramyocardial injection of CSCs induces a substantial restoration of the infarct but the newly formed myocytes are small and resemble fetal–neonatal cells [24,28–30,32,37,40]. In contrast, the occasional migration of CSCs from the border zone to the remote myocardium results in the formation of myocytes which are indistinguishable from the pre-existing adjacent cells. The differences in the phenotype and organization of regenerated myocytes in the infarcted region and in the distant non-infarcted myocardium are apparent not only experimentally but also in the aging and diseased human heart [46–48]. With acute and chronic infarcts, a large number of myocytes is formed but the new cells consist predominantly of clusters of small, proliferating, mononucleated myocytes. The recognition that CSCs can differentiate into two cell populations, i.e., small poorly differentiated and large mature myocytes, is interesting and puzzling at the same time.

In areas of injury, the orderly organization of the niches is destroyed, raising the possibility that alterations in the control of CSC fate prevent the full maturation of cardiomyocytes. Although the mechanism regulating the growth of CSCs into small versus large myocytes is currently unknown, coupling of CSCs with post-mitotic myocytes within intact niches may be critical in determining their fate. MicroRNAs (miRs) are small RNA molecules that may have the ability to traverse gap junctions, and by this means may migrate from cardiomyocytes to CSCs dictating their destiny. MiR-499 was found to traverse gap junction channels and translocate from cardiomyocytes to structurally coupled hCSCs favoring the activation of the differentiation program [39]. These findings emphasize once more that the control of stem cell growth is not intrinsic to stem cells, but depends on communications with the microenvironment. The lack of an appropriate connection between CSCs and the surrounding myocytes may oppose CSC differentiation. The mircrine effect of cardiomyocytes on CSCs is coupled with downregulation of Sox6 and Rod1 in the primitive cells and upregulation of lineage-specific genes. Collectively, these observations are consistent with the view that the heart possesses an intrinsic capacity for regeneration, which may be exploited therapeutically.

ORIGIN OF NEWLY FORMED CARDIOMYOCYTES

Numerous reports indicate that cardiomyocytes in the adult heart derive from activation of resident stem/progenitor cells. Alternatively, it has been proposed that the origin of the

regenerated cells can be found in a non-stem cell source and consists of pre-existing myocytes, which retain the ability to proliferate or undergo dedifferentiation. The analysis of the newly formed progeny provides important information that may retrospectively lead to the recognition of the founder mother cell (Fig. 37.3). Cyclin and cdk activity, BrdU incorporation, and expression of the cell cycle proteins Ki67, MCM5, Cdc6, and phospho-histone H3 have been recognized in myocyte nuclei [13]. The identification of the mitotic spindle and contractile ring during karyokinesis and cytokinesis has further demonstrated the existence of a subpopulation of immature myocytes in the adult heart [49]. In all cases, replicating cardiomyocytes are typically mononucleated, have a 60–80% smaller volume than non-cycling myocytes, and show a disorganized contractile apparatus with modest accumulation of myofibrils [46–49].

This pattern of growth is incompatible with the notion that mature cardiomyocytes correspond to a functionally homogenous pool of cells that, upon need, re-enter the cell cycle and divide to generate new cells. According to basic principles of cell biology, this process would imply that differentiated cardiomyocytes have to double in size prior to cytokinesis and would form two daughter cells of average size. Adult myocytes in animals and humans measure ~20,000–25,000 μm^3; if these cells were to re-enter the cell cycle and divide, they would need to achieve a volume of ~40,000–50,000 μm^3 before daughter cells are formed. This sequence of events does not occur physiologically and the attempts made to reactivate the cell cycle in mature myocytes have generally been unsuccessful. In this regard, forced re-entry of myocytes into the cell cycle results in abortive mitosis with formation of anaphase bridges and apoptotic cell death [50].

As mentioned above, replicating myocytes are small and mononucleated, suggesting that these cells may derive from dedifferentiation of post-mitotic myocytes, proliferation of pre-existing immature myocytes, or activation and commitment of stem cells. To discriminate among these

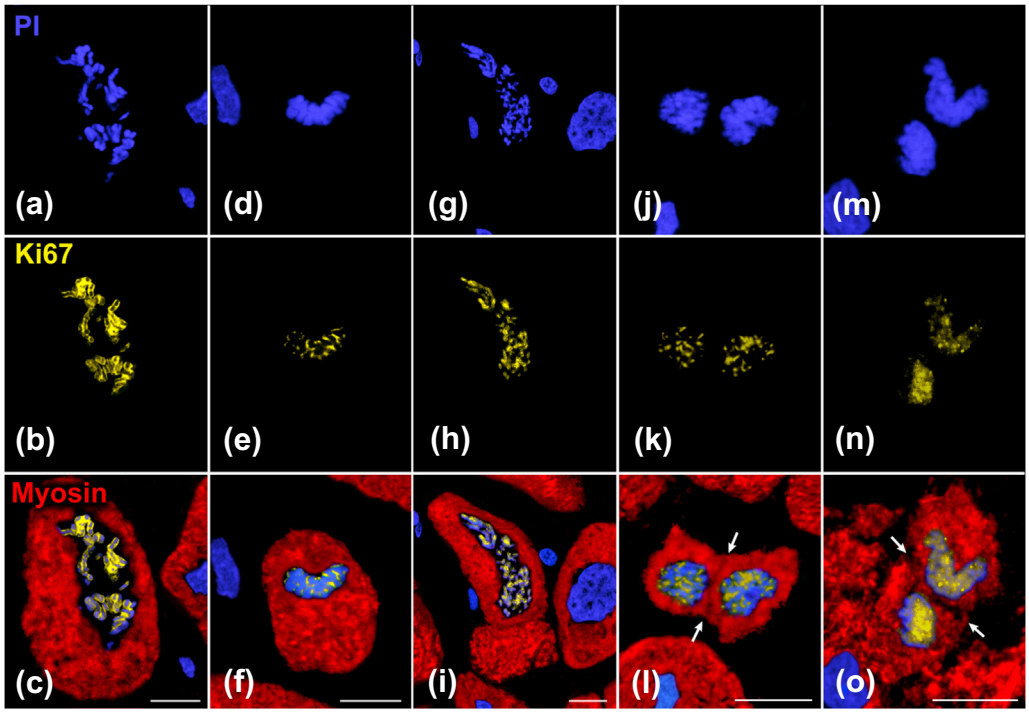

FIGURE 37.3

Mitosis, karyokinesis, and cytokinesis in myocytes. a, d, g, j, and m illustrate chromosomes by the blue staining of propidium iodide (PI). b, e, h, k, and n show Ki67 in chromosomes (yellow). c, f, i, l, and o depict the combination of cardiac myosin (red) in myocytes with PI and Ki67 staining of mitotic nuclei. Arrows point to cytokinesis.

possibilities and to prove or disprove that progenitor cells sustain cardiomyogenesis in the adult heart, studies at the single cell level must be performed. It is surprising that although stem cells are defined as single cellular entities able to self-renew and differentiate, CSCs are rarely studied clonally. Population-based analyses of stem cell behavior often fall short in defining mechanisms that may be unique to these specialized cells. The implementation of clonal assays for the study of individual stem cells is experimentally complex but is crucial for their identification and for the understanding of the mechanisms that govern stem cell growth and fate.

Clonal-based protocols include isolation and separation of single cells which are then tested retrospectively *in vitro* and *in vivo* and genetic tagging *in situ*. These methodologies have been applied infrequently to the heart and are restricted to the study of the properties of c-kit-positive CSCs. Based on clonal analysis of CSCs [24,30], the phenotypic characteristics of newly formed myocytes have been documented to reflect those of transit amplifying cells, a population that is typically present in stem cell-regulated organs [51]. Transit amplifying cells occupy an intermediate position in the hierarchy of cells within an organ and have a unique ability to divide and simultaneously differentiate. By this mechanism, amplifying cells increase the number of irreversibly committed cells generated by stem cell growth. *In vitro* and *in vivo* findings strongly suggest that replicating myocytes are transit amplifying cells derived from the lineage determination of primitive cells, supporting the view that cardiomyogenesis is controlled by a pool of resident CSCs [24,28,30,52].

With pathological cardiac hypertrophy, idiopathic dilated cardiomyopathy, acute myocardial infarction, or in the presence of hibernating myocardium, myocytes with decreased myofibrils and expansion of the undifferentiated cytoplasm have been found [13]. Partial loss in the normal distribution pattern of titin, desmin, and cardiotin has been reported, together with the re-expression of fetal genes, including α-smooth muscle actin, atrial natriuretic peptide, α-skeletal actin, and α-smooth muscle actinin. Cardiomyocytes with these structural characteristics have been interpreted as the product of cell dedifferentiation, resulting in the acquisition of an immature proliferative cell phenotype. Dedifferentiation of adult cardiomyocytes has also been claimed following inhibition of p38-MAPK [53], myocardial injection of the extracellular matrix component periostin [54], or the growth factor neuregulin [55], although the effect of periostin on myocyte replication *in vivo* has been challenged [56].

Myocytes essentially identical to those commonly involved in the prenatal and early postnatal development of the myocardium reappear in the diseased human heart, and this process may be mediated by the release from macrophages of a member of the IL-6 inflammatory cytokines, oncostatin M [57]. However, enhanced delivery of oncostatin M by macrophages in an *in vivo* model of dilated cardiomyopathy or myocardial infarction fails to promote myocyte regeneration, raising questions about the claimed beneficial effects of myocyte dedifferentiation on ventricular remodeling and cardiac hemodynamics. Similarly, the dedifferentiated cardiomyocytes identified in the human dilated and infracted heart never reached karyokinesis or cytokinesis. The expression of the fetal gene program with hemodynamic overload may simply reflect regenerated myocytes derived from commitment of resident CSCs, rather than dedifferentiation of terminally differentiated post-mitotic myocytes, which acquire an unwanted, mechanically inefficient cell phenotype [13].

Oncostatin M has also been argued to reverse *in vitro* the structural organization of post-mitotic myocytes resulting in the upregulation of several stemness genes, including c-kit [57]. Although the c-kit protein was never identified and the potential contamination of culture preparations from resident c-kit-positive progenitor cells was not excluded, a reinterpretation of the data on c-kit-positive CSCs was offered [58]. Despite the absence of *in vivo* findings, the validity of multiple demonstrations of resident c-kit positive CSCs was challenged, but the rationale for this scientific view is unclear.

Recently, the possibility of dedifferentiation was tested in fetal myocytes, obtained from mice at E16-E18 expressing EGFP under the control of the c-kit promoter; these cells should be more prone to reprogramming with respect to adult myocytes [59]. Mitotic and non-dividing myocytes expressed different levels of sarcomeric proteins but were all EGFP/c-kit-negative, excluding the occurrence of cell dedifferentiation and the reacquisition of a progenitor-like phenotype. Importantly, the generation of myocytes by dedifferentiation of mature cells, if it occurs, would have a minor role in physiological cell renewal, being restricted to cardiomyocytes. Similarly, dedifferentiated myocytes would be severely limited in their ability to sustain or improve ventricular performance following injury. Myocytes in the absence of adequate vascular supply and tissue oxygenation would be mechanically inefficient. Conversely, CSCs are multipotent and form cardiomyocytes and coronary vessels, fundamental components of myocardial repair, in a coordinated manner.

Following disassembly of the sarcomeric contractile apparatus, which physically impedes cytokinesis, differentiated cardiomyocytes have been claimed to replicate, reconstituting up to 20% of the zebra fish ventricle after resection. By cre/lox cell lineage analysis, the regenerating myocardium has been shown to derive primarily from a subpopulation of GATA4-positive cardiomyocytes rather than non-myocyte sources such as stem cells. The poorly organized sarcomeric structure of replicating GATA4-positive myocytes has led to the hypothesis that dedifferentiation constitutes the primary regenerative step [60]. However, the failure in the induction of the early myocyte commitment genes Nkx2.5 and Hand2 in the replicating myocytes indicate that the cells of origin underwent limited dedifferentiation or, most likely, that replicating GATA4-positive cardiomyocytes correspond to cells at the late stage of commitment of CSC differentiation. In the mouse heart, Nkx2.5 expression precedes GATA4 in transit amplifying cardiomyocytes originated by the lineage specification of CSCs [37].

A comparable regenerative response has been observed after surgical resection of the apex of the left ventricle in the neonatal mouse heart [61]. Again, cardiomyocyte proliferation was suggested to be the crucial cellular adaptation supporting the repair process. Despite the need of single cell analysis for the understanding of stem cell biology, population-based genetic fate mapping strategies have been employed to distinguish the contribution of pre-existing myocytes and progenitor cells to the regenerative process in the zebrafish and mouse heart. Unfortunately, this approach is inadequate to answer this question since CSCs engineered with fluorescent constructs placed under the control of promoters encoding myocyte-specific transcription factors and sarcomeric proteins show transgene protein expression [62]. Although the GATA4 and α-myosin heavy chain promoter promoters are generally considered to be active only in mature cardiomyocytes, the expression of the reporter gene in c-kit-positive cells reflects very early stage of cardiogenic lineage commitment of CSCs [37,62]. These observations support the view that myocyte regeneration in the zebrafish and neonatal mouse heart may be mediated by CSCs destined to a myocardial fate.

The intrinsic limitations of lineage tracing approach imply that the evidence of dedifferentiation is based exclusively on cellular morphology. Cells characterized by an intermediate phenotypical phase between a primitive and a fully mature phenotype have arbitrarily been considered the product of dedifferentiation. However, the lack of structural markers typical of a given cell type is not by itself indicative of the developmental stage of the cell; it is impossible to define *in vivo* whether the cell of interest is undergoing differentiation or whether it is in the process of reverting to an earlier differentiation state.

MYOCYTE TURNOVER AND CARDIAC AGING

The controversy on the growth reserve of the adult heart in animals and humans has not been resolved, and the extent of myocyte renewal claimed by different groups varies significantly. On the basis of retrospective carbon 14 (^{14}C) birth dating of cells, it has been reported that

~1% and ~0.45% replacement of myocytes occurs annually in the human heart at 25 and 75 years of age, respectively [63]. These findings indicate that only 50% of myocytes are renewed during the entire life of the human heart, from birth to death, whereas an equal number lives as long as the organ and organism, up to 100 years of age and longer. Although the possibility of myocyte regeneration was confirmed, the actual magnitude of the process is in contrast with the level of myocyte apoptosis found in the adult human heart [64] and the progressive increase in myocyte loss that occurs with aging in humans.

In a recent study, myocyte turnover was determined in normal female and male human hearts, collected from a large cohort of patients, 19 to 104 years of age, who died from causes other than cardiovascular diseases [48]. Myocyte regeneration was found to increase as a function of age, demonstrating that the age of cardiomyocytes does not coincide with the age of the organ. At all times in life, the heart contains myocytes which are younger than the organism. This discrepancy becomes more apparent in the senescent myocardium, in which a large proportion of myocytes is ~5 years old, or younger, in both women and men. The older the human heart, the younger is its myocyte compartment. From 19 to 104 years of age, essentially none of the myocytes present at birth are preserved in the young adult, middle-aged or senescent heart. In the female heart, myocyte replacement occurs at a rate of 10%, 14%, and 40% per year at 20, 60, and 100 years of age, respectively. Corresponding values in the male heart are 7%, 12%, and 32% per year, documenting that myocyte turnover involves a large and progressively increasing number of parenchymal cells with aging [48].

The striking differences between these results and the data derived from the integration of ^{14}C into the DNA of myocyte nuclei may be dictated by several variables that have been analyzed in original articles, reviews, and commentaries [13,65,66] and will not be discussed extensively here. Briefly, an inherent limitation of ^{14}C birth dating is related to the need to introduce mathematical models with assumptions that affect the computed cell turnover values [63]. Additionally, the use of troponin I expression as a marker for the isolation of a representative pool of myocyte nuclei restricts the selection to a population of $p16^{INK4a}$-positive senescent cells that exhibit marked alterations in the permeability of nuclear pore complexes [65].

It is important to emphasize that the study of cardiomyogenesis with age has to follow a stringent approach to minimize variables that confound the accurate measurement of cardiomyocyte turnover and later in life the etiology of the aging myopathy and its evolution. Therefore, specific selection criteria have to be introduced clinically, anatomically and histologically. The latter is particularly relevant, because multiple focal areas of myocardial damage and tissue fibrosis, indicative of extensive myocyte loss, are not detectable grossly and are frequently associated with an apparently normal cardiac weight, resulting from reactive hypertrophy of the remaining viable myocytes. Additionally, the concept of aging has to be carefully defined.

'Chronological age' corresponds to a time-dependent process. Old age is associated with increased morbidity and mortality and chronological age does not take into account the health of the individual. Conversely, 'biological age' is the objective assessment of a human being's health and therefore reflects the presence or absence of disease. The expression 'functional aging' has been introduced to emphasize the limitations inherent in the definition of health of an individual with respect to chronological age. This principle is based on what human beings can do in relation to others in society but it may be extended to characterize the level of functional competence retained by tissues and organs in the elderly. Finally, the concept of 'successful aging' suggests that this process is the product of a balance between gains and losses [67]. Compression of morbidity and enhanced quality of life is the foundation of successful aging.

The structural and functional alterations of the heart that accompany advancing age in otherwise healthy individuals have been interpreted as adaptive to the arterial changes that occur with aging [68]. Aging results in arterial stiffening and increased arterial systolic pressure that,

together with a mild increase in peripheral vascular resistance, leads to aortic dilatation and vessel wall thickening. In turn, these modifications promote an increase in thickness of the left ventricular wall, myocyte death and deposition of collagen [68]. Collectively, these adaptations are accountable for the decrease in reserve of the old heart. The changes in cardiovascular reserve are insufficient to produce clinical heart failure but they affect the threshold for its symptoms and signs, severity and prognosis [68]. Although cardiac aging may represent a successful adaptation of the organ to the burden of time, aging is the major independent risk factor for chronic heart failure (CHF) in the Western world. Morbidity and mortality for CHF continue to increase and parallel the extension in median lifespan of the population. At present, there are 5.7 million patients with CHF in the United States alone with an incidence of 670,000 new cases per year [69]. And the overwhelming majority of individuals with CHF are 65 years of age or older.

Studies in humans have been conducted by comparing individuals of different ages and only chronological age was considered in the interpretation of the results. However, as discussed above, chronological age and biological age do not necessarily coincide, and organism and organ aging do not necessarily proceed at the same pace. Moreover, the chronological age of individual cells in an organ is highly heterogeneous, being conditioned by the birth date of the cells; additionally, biological age differs according to the extent of damage that cells have suffered. If these variables are not considered, the possibility that an aging myopathy and the manifestations of clinical heart failure in the elderly occur independently of coronary artery disease and hypertension cannot be tested.

CARDIAC STEM CELL SENESCENCE

The demonstration that the heart harbors stem cells capable of creating functional myocardium accounts for the partial repair that occurs acutely after infarction or in the senescent heart in humans. However, it also raises the question why this regenerative response is incomplete and resident CSCs cannot reconstitute the muscle mass lost after an ischemic insult or as a result of the chronic process of aging. In the bone marrow, self-renewal of old HSCs is limited by telomere attrition [70]. Longitudinal studies in primates have documented that the expansion phase of the most primitive HSC pool coincides with the presence of long telomeres. The loss of 2–3 kbp of telomere sequences promotes a change to a functional mode characterized by a decrease in the rate of blood cell turnover. Under these circumstances, the preservation of cell number can be achieved only by increasing the lifespan of the progeny which inevitably results in the accumulation of old cells [71]. A similar condition is encountered in the aging heart.

During the course of life, CSCs undergo progressive telomere erosion which is dictated by the loss of DNA sequences that occurs at each cell cycle and as a consequence of oxidative damage [72,73]. Shortening of telomeres in CSCs may favor their symmetric division to daughter-committed cells limiting self-renewal and enhancing myocyte formation. However, this adaptive response of CSCs is inefficient because cell death exceeds cell production and newly generated cells may acquire rapidly the senescent phenotype. When differentiating CSCs traverse the transient amplifying compartment, the numerous and repeated rounds of division impose attrition on the telomeric DNA of forming myocytes [24,32,48]. A subset of CSCs with critically short telomeres may remain confined to the niche area where they cannot proliferate and differentiate. Epidermal stem cells with critically short telomeres do not translocate from the bulge of the hair follicle to the basal layer of the skin and the papilla of the hair follicle and this impairs hair growth and cutaneous cell turnover [74]. These possibilities are consistent with the characteristics of the senescent heart phenotype: impaired CSC function and accumulation of senescent myocytes.

Loss of DNA does not affect all telomeres homogeneously. Shortening preferentially occurs in a fraction of telomeres [75]. CSCs may have comparable mean telomeric lengths but exhibit

significant differences in the length of individual telomeres. Telomeric shortening in specific chromosomes can influence the destiny of a CSC and its decision to divide symmetrically or asymmetrically. CSCs with short telomeres in specific chromosomes may be programmed to generate only transit amplifying myocytes through symmetric division and permanent loss of stemness. The non-uniform shortening of telomeres has been documented in old CSCs in an animal model of cardiac aging. Thus, the shortest telomere may be critical for cell viability and chromosome integrity [76].

Telomeric shortening occurs in aging CSCs in spite of the presence of telomerase activity [72,73]. This finding is consistent with results obtained in HSCs. HSCs express low to moderate levels of telomerase, but telomeres shorten considerably with age [77]. Two possibilities have been proposed in an attempt to explain telomeric shortening in the presence of detectable telomerase activity: a suppressor of telomerase function may be upregulated during the cell cycle or a competition may occur between the reassembly of telomeric chromatin and extension of telomeres by telomerase [77]. In HSCs, telomere shortening occurs at a rate of 50–100 base pairs per population doubling; a similar rate of telomere attrition has been found in human CSCs *in vitro* [24] and may characterize the aging process of CSCs *in vivo*.

Throughout life, adult stem cells undergo numerous rounds of divisions but, at the same time, experience long periods of quiescence. Thus, primitive cells are subjected to replicative senescence, with telomeric shortening and accumulation of replication errors, as well as stress-induced senescence, with a considerable load of damaged macromolecules, DNA double strand breaks, and oxidative DNA injury. CSC aging mediated by telomeric shortening triggers the expression of several gene products, which interfere with the cell cycle but mostly with G1-S transition. Surprisingly, the number of CSCs increases with age in the myocardium [32]. However, during aging CSCs become unable to divide and the number of functionally competent cells decreases with time.

Five biomarkers of hCSC senescence which are strictly linked to chronological age have been identified: telomeric shortening, attenuated telomerase activity, increased number of telomere-induced dysfunction foci (TIFs), and enhanced expression of $p16^{INK4a}$ and $p21^{Cip1}$ [78]. Severe heart failure has similar effects on the hCSC compartment. Senescent hCSCs are characterized by the expression of phosphorylated histone H2AX and p53 binding protein. Both γ-H2AX and 53BP1 are markers of DNA double strand breakage and their presence in old hCSCs from donor and explanted hearts identifies DNA lesions at the sites of telomere hybridization spots. TIF-positive hCSCs reflect old cells in which the DNA damage response is activated. This process may precede irreversible growth arrest dictated by the expression of $p16^{INK4a}$ [79].

CONCLUDING REMARKS

The human heart is a highly dynamic organ regulated by a pool of resident hCSCs that modulate cardiac homeostasis and condition organ aging. Hopefully, recent findings will resolve the long debate that has divided the scientific community into strong opponents and passionate supporters of the regenerative potential of the human heart, offering a more biologically valid understanding of cardiac growth and repair. A common ground can now be found to translate this different perspective of cardiac biology into the development of novel strategies for the management of the human disease.

Importantly, hCSCs with preserved growth reserve have been detected in old and explanted hearts at the end of their physiological lifespan. This category of hCSCs is expected to generate a young myocyte progeny within the senescent heart. The compartment of hCSCs in the old heart is composed of a subset of quiescent cells with preserved telomere integrity and a pool of highly proliferating cells that regulate organ homeostasis but lose telomeric DNA during each round of division. From a clinical perspective, the recognition that a subset of telomerase-competent hCSCs with long telomeres persists at all ages and with chronic cardiac diseases has

raised the possibility that autologous cell-based therapy may be feasible in patients with severe heart failure. Two clinical trials in which autologous cardiac-derived cells are administered to patients with sub-acute and chronic ischemic cardiomyopathy are ongoing [80,81]. The initial results are very encouraging warranting the need for large multicenter studies. Recently, a methodology has been developed to isolate from endomyocardial biopsies this compartment of functionally competent hCSCs to be expanded *in vitro*, prior to their intracoronary or intramyocardial delivery in very sick patients [82]. Although this approach has not yet been employed clinically, it generates therapeutically relevant quantity of cells, in the absence of invasive surgical techniques. This strategy is in the process of being implemented in forthcoming clinical trials.

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CHAPTER 38

Cardiac Tissue Engineering

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INTRODUCTION

In the developed world, cardiovascular disease is the leading cause of death. Due to the minimal intrinsic ability of adult heart to regenerate itself following injury [1], myocardial infarction (MI) results in the rapid death of hundreds of millions of cardiomyocytes (CMs), and a vigorous inflammatory response. Over subsequent weeks to months, fibroblasts (FBs) and endothelial cells (ECs) form granulation tissue and a dense collagenous scar, which reduces the contractile function of the heart and leads to a pathological remodeling and, in many cases, heart failure. Adult CMs are terminally differentiated, thus it is not possible to expand them to sufficient numbers starting from small cardiac biopsies. Current clinical trials focus on cell replacement through the application of bone marrow mesenchymal stem cells, peripheral blood mononuclear cells, or resident cardiac cells. Most of these cell types have no intrinsic ability to give rise to a large number of CMs; instead they improve function through paracrine effects. Additionally, cells can be applied alone or in combination with different types and forms of biomaterials (e.g., hydrogels, scaffolds). An appropriate combination of a biomaterial, cell type, delivery method and tissue culture parameters can develop unique tissue constructs for multiple types of heart disease. Here we provide design criteria for generation of functional cardiac patches and discuss different biomaterials and cell types used during the tissue-engineering process.

CLINICAL PROBLEM

Cardiovascular disease is the leading cause of death worldwide. In the United States cardiovascular diseases account for approximately 30% of all deaths, with the cardiovascular disease population constantly increasing [1,2]. In 2008 alone, 86.2 million Americans were living with some form of cardiac disease, and this number is projected to grow to 40.5% of the American population by the year 2030 [3]. Concomitantly, the financial burden of disease is also projected to rise, from present estimates of $300 billion to $800 billion [3]. On a global scale, cardiovascular disease is responsible for 60% of deaths, and will become increasingly important as global obesity continue to rise. Importantly, cardiovascular disease is responsible for 80% of non-communicable diseases in low and middle income countries [4], with grave social and economic consequences.

One of the key limitations for treating cardiovascular disease is the lack of regeneration after myocardial injury. The majority of existing therapies aim to mitigate the progression of heart failure, intervening in the cyclic progression of the neurohormonal cascade, but the options for

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improvement or regeneration of diseased tissue are limited. The most common pathogenesis is ischemic heart disease, which occurs when a portion of the heart does not receive a supply of oxygen from the blood. Coronary artery disease, or a narrowing of the lumen in the coronary arteries, most often by atherosclerotic change, limits perfusion of certain sections of the heart. Limiting oxygen delivery below a certain threshold leads to angina, characterized by reversible discomfort or heaviness to the chest. Complete occlusion of an artery, or MI, is associated with a typical pathological progression. Depletion of adenosine triphosphate (ATP) occurs within seconds, leading to irreversible cell damage by 20–40 minutes [5]. Coagulative necrosis begins ~30 minutes after coronary occlusion, followed by a robust inflammatory response that begins with the release of reactive oxygen species and neutrophil invasion ~24 hours post-infarction, and continues for the next 2–3 days, in parallel to the continued necrosis. Macrophages ultimately dominate the infarcted zone by 5–7 days post-infarction, and are responsible for removing dead cells and creating granulation tissue. Weeks to months after infarction, collagen deposition dominates, and a fibrous scar is formed [5].

Heart failure, which is the inability of the heart to adequately pump blood, ensues most commonly after ischemic injury, but may also have other etiologies, including valvular disease, hypertension, or genetic cardiomyopathies [6]. An increase in cardiac work requirements leads to one of two patterns of dysfunction in the heart; one where pressure overload dominates, leading to concentric hypertrophy of cardiomyocytes and a thickening of the myocardial wall, or a second one where volume overload dominates, leading to eccentric hypertrophy of cardiomyocytes and a thinning of the myocardial wall [5]. In either case, increased fibrosis, abnormal gene expression, and insufficient vascular function lead to pump dysfunction and an activation of the neurohormonal system, in which the sympathetic nervous tone, renin secretion, and arginine vasopressin secretion all increase. Together, these cues lead to myocardial remodeling at the cellular level that contributes to the progression of heart failure, by mechanisms that are not well understood.

Presently, heart transplant and Ventricular Assist Devices (VAD) can improve cardiac function, but the numbers of available donor hearts are limited, and the VAD is only a temporary solution. Therefore, biological treatment strategies that can enhance cardiac function are especially attractive for countering the pathophysiological progression of heart failure. With the advent of induced pluripotent stem cells (iPS), there is newfound promise for cardiac regeneration using patient-specific cells, since cardiomyocytes were previously unattainable by any other means. Present approaches involve direct cell injection or the creation of a cardiac patch. Cell injections are attractive due to relative simplicity, though poor cell retention is a recurring obstacle [7,8]. A cardiac patch approach would be aimed at replacing or repairing the specific lesion created by a MI, using a lab-grown piece of contractile cardiac tissue. Continuing challenges are the vascularization and electromechanical integration of such a construct, and these are among the key areas of active research.

ENGINEERING CARDIAC TISSUE: DESIGN PRINCIPLES AND KEY COMPONENTS

The heart functions as a highly organized physiological pump. The cardiomyocytes, comprising 80–90% of the heart volume, are not only elongated and hypertrophied, but are also aligned and electrically coupled to surrounding cardiomyocytes. They are constantly active, stimulated to beat, and therefore have a high metabolic demand for oxygen. Supporting cell types – endothelial cells and smooth muscle cells – organize themselves into a dense vascular network supplying nutrients to the cardiomyocytes. Fibroblasts support the cardiomyocytes and generate a collagen-dense matrix. On an organ level, pacemaker cells spontaneously generate action potentials that propagate the volume of the heart, generating a synchronous contraction. The flow of blood through the heart necessitates mechanical stress on it, as a preload that stretches the myocardium and an afterload to push against.

Fabrication of a functional cardiac patch depends on a multitude of parameters that collectively recapitulate some aspects of the complexity and function of the heart. Since cardiomyocytes are terminally differentiated, current studies are focusing on deriving a renewable source of them from embryonic and induced pluripotent stem cells. Other studies have aimed at recapitulating one or more physiologic aspects of cardiac tissue (e.g., the incorporation of multiple cell types, aligning cardiomyocytes, or electrical stimulation of cardiac tissue). Table 38.1 contains a collection of studies that aim to mimic aspects of the native myocardium. The three classical tenets of the tissue-engineering paradigm have been used for cardiac constructs: cell source, scaffold materials, and biophysical stimulation (Fig. 38.1).

Cell source

The limited ability of human cardiomyocytes to divide and expand has restricted the scope and therapeutic potential of cardiac tissue engineering. The first evidence that the application of cells may be a viable therapeutic approach for MI came from animal studies which used injection of fetal or neonatal CMs, and found that CM injection improved left ventricular function and thickness, thus attenuating pathological remodeling upon MI [1a–4a]. Injected CMs integrated through gap junctions and intercalated disks with the host CMs [5a]. However, these findings have limited clinical relevance, and human fetal and neonatal CMs cannot be readily obtained for transplantation due to obvious ethical issues.

The search for a clinically relevant cell source has led to the transplantation of skeletal myoblasts [6a], embryonic stem cell derived cardiomyocytes (ESC-CMs) [7a–9a], bone marrow derived mesenchymal stem cells (MSCs) [10a–11a] and hematopoietic stem (HS) cells [12a–14a] into animal models of MI (reviewed in references [15a] and [16a]). Among these cell sources, skeletal myoblasts and MSCs were pursued into clinical trials. A meta-analysis of recent clinical trials with injection of bone marrow and peripheral blood mononuclear cells demonstrated a significant, albeit low (3%), increase in left ventricular ejection fraction (LVEF) as well as a significant reduction in infarct size (−5.6%) and end systolic volume (−7.4mL) in patients treated by intracoronary cell injection after acute MI [17a]. A dose-response effect of the injected cell volume on LVEF change was also reported [17a]. During the last year, resident cardiac stem cells, either c-kit+ (SCIPIO) or those derived from cardiospheres (CADUCEUS) demonstrated promising functional improvements in Phase I clinical studies and restoration of viable tissue per MRI imaging, presumably due to the new CMs in addition to vascular cells [18a,19a].

Although these studies are encouraging, modest long-term improvements in function upon cell injection have motivated the investigation of new cell sources and methods that increase survival and retention of injected cells. Pluripotent stem cells such as hESCs or iPSCs can both give rise to bona fide CMs and be expanded to sufficient numbers (millions/patient) using existing technologies. The discovery of human iPSCs [20a] and the ability to generate CMs from them [21a] could provide effectively unlimited numbers of autologous CMs for cell therapy without the ethical concerns raised by the use of hESCs has enormous implications. Studies from a number of groups have shown that it is possible to generate CMs from mouse [58] and human ESCs [53] and iPSCs [21a]. The most efficient protocols to date are those that replicate the signaling pathways regulating lineage commitment in the early embryo [53]. These protocols are now being adapted to defined culture conditions, with the use of small molecules such as glycogen synthase kinase 3 inhibitors and chemical inhibitors of wingless-int (WNT) signaling [22a].

While in many ways iPSC-derived cardiomyocytes are ideal, the exact approach for their utilization is not entirely clear. Though the differentiation process has become more specific than previous work relying on stochastic cell differentiation, further refinements are possible and necessary. In particular, there are multiple phenotypes of cardiomyocytes, each with a different set of functions [10]. Certain cardiomyocytes are pacemakers or chamber-specific myocytes, and fine control over the generation of these phenotypes remains elusive. Laflamme

TABLE 38.1 Design parameters for cardiac tissue engineering

Native cardiac attribute	Engineering method	Results	References
Cell Source	Sequence of Activin and BMP-4 cytokines induces cardiac cell differentiation in human embryonic and iPS cells.	Flow cytometry at multiple timepoints revealed high sensitivity of multiple ES and iPS cell lines to concentrations of induction factors. However, optimization of the times and concentrations lead to improved differentiation yields.	Kattman et al., Cell Stem Cell 2011 [51]
	Lentiviral iPSC technology using epigenetically specific cell sources, ventricular cells vs. fibroblasts.	Ventricular cells as material for iPSCs improved cardiac differentiation potential when compared to using fibroblast cells, suggesting epigenetic memory for iPSC cell lines.	Xu et al. Cell Research 2012 [52]
Patient cell source	Induced pluripotent stem cells were derived from fibroblasts of patients with Long QT syndrome type I using infection with retrovirus encoded with Oct3/4, Sox2, KLF4, and c-MYC. They were subsequently differentiated into cardiomyocytes.	Generation of induced pluripotent stem cells was confirmed with Nanog staining, and generated cardiomyocytes showed striations when stained with cardiac troponin T. LQT1 disease phenotype of lengthened ADP 50 and ADP 90 was confirmed by single cell patch clamp. Myocytes generated from LQT1 patients showed KCNQ1 channel localization to the endoplasmic reticulum, suggesting a possible mechanism for the disease.	Moretti et al. NEJM 2010 [11]
	Induced pluripotent stem cells derived from patients with familial dilated cardiomyopathy were generated using Oct4, Sox2, Klf4, and c-MYC. They were subsequently differentiated into cardiomyocytes using the protocol developed by Yang et al. [53]	Immunofluorescence images of Oct4, Nanog, TRA-1−81 and SSEA-4, as well as hypomethylation as seen by quantitative bisulfite sequencing confirmed the generation of iPS cells. Multielectrode array analysis of dilated cardiomyopathy derived cardiomyocytes displayed similar beat frequency, interspike intervals, and field potential durations as the control group. Immunohistochemistry and transmission electron microscopy (TEM) displayed similar cell size, but dilated cardiomyopathy (DCM) derived cells had a higher relative percent of disorganized cells based on α-actinin staining. Overexpression of Serca2a resulted in restoration of contraction force as measured by atomic force microscopy (AFM). Treatment of cells with β-blockers decreased the percentage of disorganized cells.	Sun et al. Science Translational Medicine 2012 [14]
Multiple Cell Types	Porous PGS scaffolds were pre-seeded with cardiac fibroblasts encapsulated in Matrigel for five days and were subsequently seeded with cardiomyocytes for another five days. This was compared to constructs of cardiac fibroblasts co-seeded with cardiomyocytes.	Immunofluorescent analysis of constructs pre-seeded with fibroblasts showed a greater percentage of actin positive cells compared with vimentin-positive cells, and compared with actin positive cells in the co-seeded group. The pre-seeded group further showed lower excitation threshold, higher fractional area change, and increased fluorometrically measured DNA and protein content.	Radisic et al. J Biomed Mater Res Pt A 2007 [21]
	Endothelial cells were co-cultured in cell sheets with cardiomyocytes at different ratios.	Higher seeding ratios of endothelial cells resulted in higher density of vascular networks as appreciated by fluorescent images. Enzyme-linked immunosorbent assay (ELISA) for	Sekine et al. Circulation 2008 [22]

		secreted factors showed higher levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) in endothelial co-culture groups compared to cardiomyocytes alone. Fluorescent views of implanted co-cultured cell sheets showed neovascularization into the myocardium.	
	External endothelial cell addition to cardiac tissue constructs.	Increased cardiomyocyte DNA content demonstrated a 35% increase in cardiomyocyte proliferation due to addition of endothelial cells, validating co-culture effects of endothelial cells. Additionally vascular engraftment was shown when implanted with patent blood vessels.	Tulloch et al. Circ Res 2011 [23]
High Cell Density	Cell sheet method, where confluent layers of cells are detached from a temperature-dependent poly(N-isopropylacrylamide) substrate and stacked on top of other cell sheets using a cell sheet manipulator. Cell sheets were compared to cell injection in an infarct model.	There was a greater *in vivo* bioluminescence of implanted GFP-expressing cell sheets compared to injected GFP-cells. Macroscopic fluorescent views of cell sheets versus injected cells showed dense localization 4 weeks post transplantation. Immunohistochemistry showed a greater density of cells. TUNEL staining showed a significantly lower level of TUNEL positive nuclei compared to cell injection.	Sekine et al. Tissue Eng Pt A. 2011 [8] Haraguchi et al. Nat Protoc. 2012 [42]
Ultrastructure	Microcontact printing of fibronectin into rectangles of various aspect ratios and seeded with cardiomyocyte pairs.	Connexin-43 immunosignal and conductance as measured with dual-voltage clamp was greater in the 5.2 length:width ratio as compared to 3.5.	McCain et al. Am J Physiol-Heart C 2012 [54]
Alignment/ Anisotropy	Soft lithography of poly(glycerol) sebacate into accordion-like honeycombs of overlapping diamonds.	Differential long and short axis elastic moduli and excitation threshold that mimic the anisotropy of the heart. Immunofluorescent actin images demonstrating elongation in the preferred direction	Engelmayr et al. Nature Mater 2008 [31]
	PDMS microcontact printing of fibronectin into shapes of various aspect ratios.	Image analysis of immunofluorescence actin images demonstrated greater anisotropy in shapes with $>$ 2:1 aspect ratio as compared to the 1:1 group	Bray et al Cell Motil Cytoskel 2008 [34]
Extracellular matrix	Neonatal hearts were decellularized with antegrade coronary perfusion of SDS and were recellularized with neonatal cardiomyocytes, fibrocytes, endothelial cells, and smooth muscle cells.	Decellularization and recellularization were confirmed by histological analysis and staining. The perfused recellularized heart showed synchronous contraction as measured by electrocardiogram (ECG) and left ventricular pressure (LVP) after electrically stimulated depolarization. Ejection fraction totaled 25% of an equivalently aged healthy fetal heart.	Ott et al. Nat Med 2008 [25]
	Decellularized sheets of human myocardium were used as a scaffold for human mesenchymal progenitor cells suspended in fibrin and implanted into infarcted rat hearts.	Histological staining of extracellular matrix (ECM) proteins and tensile testing showed that decellularized tissues were similar to native tissue. Echocardiographs performed on constructs transplanted on rat hearts preserved left ventricular systolic area and fractional area change.	Godier-Furnemont et al. Proc Natl Acad Sci 2011 [26]
	Poly(glycerol sebacate) scaffolds were prepared at different stiffnesses by altering the curing time and were subsequently seeded with neonatal rat cardiomyocytes.	Low stiffness groups were found to have the greatest functional change (contraction amplitude) and also the greatest compressive stiffness.	Marsano et al. Biotech Prog 2010 [55]

Continued

TABLE 38.1 Design parameters for cardiac tissue engineering—*Continued*

Native cardiac attribute	Engineering method	Results	References
Cardiomyocyte Hypertrophy	Molded rings of neonatal rat cardiomyocytes, Matrigel, and Collagen I, and cultured in different hypertrophic stimuli (angiotensin II and phenylephrine versus hypertrophic inducing serum) on a cyclic stretch device for 12 days.	Immunofluorescent analysis of single cells in the angiotensin II and phenylephrine group showed no change in length but displayed increased width and total volume suggesting concentric hypertrophy. Similar analysis on the 'hypertrophic inducing serum' group displayed significant elongation without a widened morphology suggesting eccentric hypertrophy. Further evidence for hypertrophy in both groups is supported by high levels of gene expression of ANP and low levels of gene expression of alpha/beta myosin heavy chain (α/β-MHC).	Tiburcy et al. Circ Res 2011 [29]
Electrical stimulation	Carbon electrodes field stimulation.	Ultrastructurally improved contractile apparatus and gap junctions. Histology and immunostaining showed increased linear organization. Contracting force increased and improved electrical maturity.	Radisic et al. Proc Natl Acad Sci 2004 [48]
	Gold nanowire impregnated alginate scaffolds.	Electrical conductance through gold impregnated scaffolds was increased. Connexin 43 expression was doubled compared to non-impregnated controls.	Dvir et al Nat Nanotech 2011 [56]
	hES cardiomyocytes transplanted in ablated large animal heart model.	ECG mapping co-localized injected cardiomyocytes to ectopic ventricular pacing, demonstrating pacing potential of injected hES pacemakers.	Kehat et al. Nat Biotech 2004 [57]
Vascular Perfusion	Perfusion bioreactor through channeled scaffolds.	Perfused channeled scffolds showed nearly 50% increase in viable cells compared to non-perfused controls. Finite element modeling provides a rational approach for vascular perfusion design through engineered cardiac tissue.	Radisic et al. Nat Protoc 2008 [39]
	Omental pre-vascularization of cardiac patch.	Prevascularization improved engraftment on the infarcted heart, and mitigated decline in cardiac function based on echocardiography.	Dvir et al. Proc Natl Acad Sci 2009 [41]
Force generation	Engineered heart tissues, ring-shaped cardiomyocyte aggregates in a mixture of Matrigel and collagen, were placed onto load-adjusted coils to apply a passive, auxotonic, load on the tissues.	Engineered heart tissues on auxotonic load displayed a greater twitch tension by isometric force contraction analysis. Four weeks after implantation onto an infarcted rat heart, echocardiographs, MRI, and catheterization together demonstrated decreased left ventricular volumes, lower left ventricular end diastolic pressures, and shorter relaxation times when compared to sham-operated rats.	Zimmerman et al. Nat Med 2006 [30]
	Cardiomyocytes in porous chitosan-collagen scaffolds were stretched by moving four pins on the scaffold. Nominal strain of approximately 20% were applied at a frequency of 1 Hz for 6 days.	Regions of high local stress were determined using a mathematical model. Immunohistochemical analysis showed high levels of connexin-43 staining at the regions of high stress. Histological analysis and scanning electron microscopy (SEM) also demonstrated elongated morphologies in areas of high stress in comparison to areas of lower stress	Zhang et al. J Tissue Eng Regen M 2011 [33]

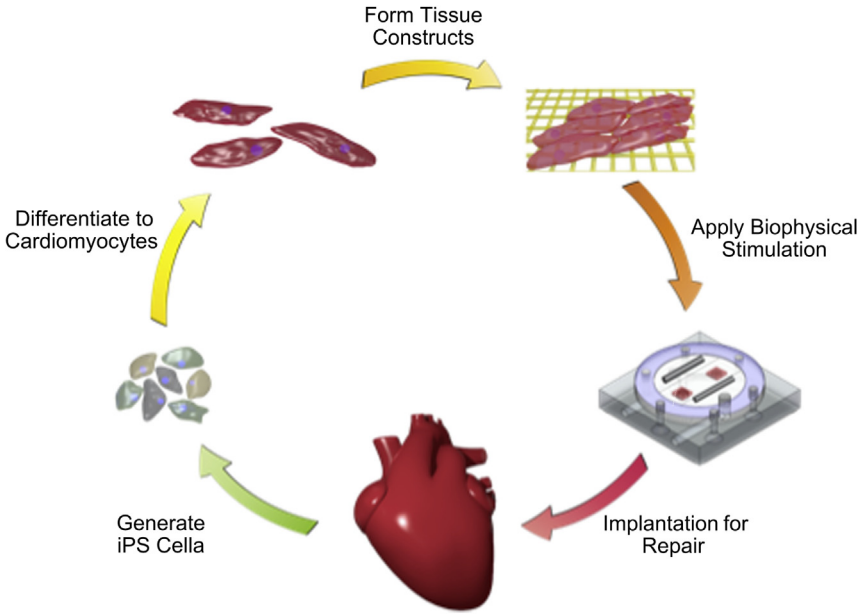

FIGURE 38.1
Cardiac tissue engineering approach.

and colleagues recently demonstrated conclusively that hESC-CMs can electrically couple and suppress arrhythmias in hearts upon MI induced by cryoinjury [63]. As cell death is a major problem for cell injection studies – with up to 90% of injected cells dying or being washed away from the injection site – the authors used a pro-survival cocktail consisting of Matrigel and various growth factors to enhance cell persistence upon injection [38].

Another issue is the cell maturity [18–20]. Being recently differentiated, iPSC-derived cardiomyocytes are relatively young, and further phenotypic maturation may be needed to grow them into adult-like myocytes with their inherent contractile properties. Furthermore, even after differentiation and maturation, it is unclear whether a pure population of cardiomyocytes would be most apt for building cardiac tissue, since co-culture effects improve function, mimicking the heterogeneity of cells found natively [21–24]. The cell-related parameters of interest for building a cardiac patch include cell identity that accounts for specific cardiomyocyte type, relative ratios of different cells, cell density, and cell maturity.

Scaffold

Scaffolds provide a three-dimensional environment in which the cells are cultured. The constituents and organization of the scaffold dictate the organization, maturation, and function of the forming tissue constructs. The scaffold material itself ranges from the native heart matrix itself [25–27] to natural hydrogels such as collagen and Matrigel [7,28–30] to synthetic polymers, such as poly(glycerol) sebacate (PGS) or polyacrylamide [31–33]. The scaffolds differ in the way they are processed (e.g., decellularization for native heart matrix versus temperature related gelation for Matrigel), their mechanical properties, their ultrastructure, and their biodegradability. With the wide library of materials to choose from, scaffolds can be made into virtually any size or shape, depending on the application. Micropatterning has been employed as a way to control cardiomyocyte alignment and cell-cell interactions on a single cell level [34–36], while macro-sized constructs offer amenability to force generation and animal implantation studies [24,37,38].

Due to the large metabolic demand of cardiomyocytes, scaffold design must take oxygen and nutrient delivery into account. The cardiac tissue engineering scaffolds are in general porous and perfusable to enable oxygen supply *in vitro* [19,39,40] and designed to promote angiogenesis to enable oxygen supply *in vivo* [37,41] Some methods of cardiac tissue engineering do not utilize scaffolding material; for example, the cell sheet method [8,42–44] relies on stacking confluent sheets of cardiomyocytes and the extracellular matrix these cells alone produce.

Biophysical stimulation

Further phenotypic maturation of cardiac constructs can be achieved using biophysical stimulation. Perfusion of engineered constructs helps alleviate the diffusional limitations [37,39,40], which is of particular importance for the highly metabolically active cardiac tissue. The flow of medium across or through the engineered construct mimics the vasculature found in native tissue in providing fresh media and nutrients while removing toxic metabolic products from the cells. These approaches are critically important for generating large, thick, clinically sized cardiac constructs with homogenous distributions of cells.

Other stimulation modalities rely on the excitation-contraction coupling property that is inherent to heart tissue [45]. Stimulation systems can excite cells using either electrical depolarization or mechanical strain. Mechanical stimulation systems use active or passive tension enhancing cellular organization, morphology and contractile force generation. Auxotonic systems maintain passive tension on engineered cardiac constructs, providing a tonic resisting force for the cells to pull against [30,46]. Phasic systems provide active, cyclic strain to improve cardiac function, though some studies have shown improved twitch forces created using the auxotonic method [23,30,33]. Electrical field stimulation uses electrodes to provide a depolarizing stimulus [20,40,47–50]. The electrical field which excites the cells of the construct creates a voltage difference across the two electrodes. This cyclic depolarization improves electrical synchronization of cardiac constructs, while also improving contractile function and cellular organization. Both of these modalities of biophysical stimulation lead to functional enhancements, such as improved cardiomyocyte ultrastructure, improved sarcomeric linearization and organization, and increased functional gap junctions.

DIRECTED CARDIAC DIFFERENTIATION OF HUMAN STEM CELLS

Cardiac tissue engineering requires a reliable source of cardiomyocytes. Human adult cardiomyocytes are unsuitable as they do not have the ability to proliferate. Recent advances in hESC and iPSC technologies have allowed for the generation of human cardiomyocytes from healthy progenitors as well as from diseased individuals. We describe here the differentiation of cardiomyocytes from an embryonic stem-cell-derived progenitor. We further discuss the purification and characterization of cardiomyocytes, as well as current efforts to generate patient-specific cardiomyocytes.

Derivation of cardiomyocytes from embryonic stem cells

One of the first accounts of generating human cardiomyocytes from an embryonic stem cell source comes from Yang et al. in 2008 [53]. This study focuses on the discovery of a common cardiovascular progenitor capable of generating the different cell types dominant in the adult heart, that is, cardiomyocytes, endothelial cells, and smooth muscle cells. This method uses a combination of several factors to recapitulate cardiac development (Fig. 38.2a,b), resulting in a large percentage of troponin positive cells (Fig. 38.2c). The embryonic stem cell cultures were first induced with activin A and BMP4, generating a *T*+ population, or one which overexpressed brachyury, a transcription factor important for defining the primitive streak and mesoderm. The cultures were next induced with Dickkopf-related protein 1 (DKK1), a canonical WNT inhibitor, and vascular endothelial growth factor (VEGF). DKK1 was necessary for generating cardiomyocytes, as demonstrated by a subsequent increase in ISL1, a marker for the secondary heart field, NKX2.5, a marker for cardiac differentiation, and increases in cardiac-specific proteins MLC2A and cardiac troponin T.

Interestingly, flow cytometric data of embryoid bodies at various stages of cultivation demonstrated three distinct populations at day 6 of induction (Fig. 38.2d). Stage I corresponds to a KDR^{low}/C-KIT^{neg} population, stage II corresponds to a KDR^{neg}/C-KIT^{pos} population, and stage III corresponds to a KDR^{high}/C-KIT^{pos} population. Kinase insert domain receptor (KDR)

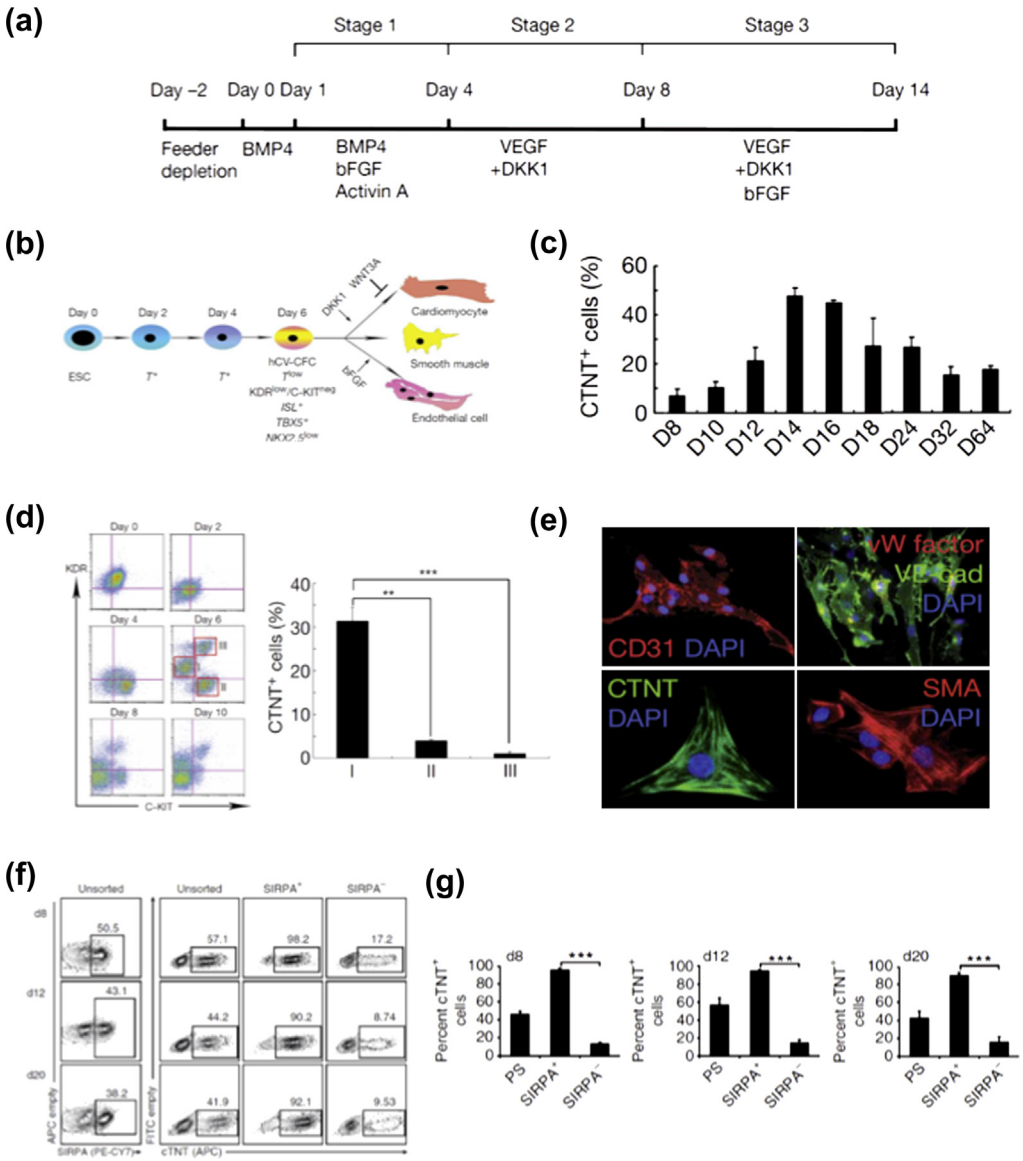

FIGURE 38.2

Generation of cardiomyocytes from embryonic stem cells. (a) Cardiomyocyte derivation protocol and (b) corresponding schematic of development of cardiovascular lineages. (c) Percentage of troponin positive cells over time using the protocol in (a). (d) Flow cytometry reveals three populations on day 6 of differentiation KDR^{low}/C-KIT^{neg} (I), KDR^{neg}/C-KIT^{pos} (II), and KDR^{high}/C-KIT^{pos}, where differentiation of the KDR^{low}/C-KIT^{neg} population yielded the highest percentage of troponin positive cells. (e) Immunostaining for endothelial cell markers CD31 and vWF, cardiomyocyte marker cTNT, and smooth muscle marker smooth muscle actin (SMA) on cells differentiated from the KDR^{low}/C-KIT^{neg} lineage. (f) Flow cytometric analysis of human embryonic stem cell (hESC) derived cardiomyocytes sorted for signal regulatory protein alpha (SIRPA) on various days (8, 12, 20) of differentiation. (g) SIRPA positivity selects for a cardiomyocyte progenitor, resulting in up to 98% troponin positive cells.

and C-KIT were chosen for further investigation based on their significance in mouse stem cell studies. In particular, in mice KDR was shown to give rise to cardiac progenitors, and C-KIT was shown to derive hematopoietic and vascular lineages [58]. Low KDR expression and C-KIT positivity defined a cardiac progenitor, developing into cells with a greater number of cardiac troponin positive cells (>50%) compared with the other subpopulations (Fig. 38.2d). As lineages of cardiovascular cells are induced, significant populations of cardiomyocytes, endothelial cells, and smooth muscle cells form in culture (Fig. 38.2e). Transplantation of these progenitors into murine hearts has led to differentiation into the same three cell types — cardiomyocytes, endothelial cells, and smooth muscle cells, and resulted in an increase in

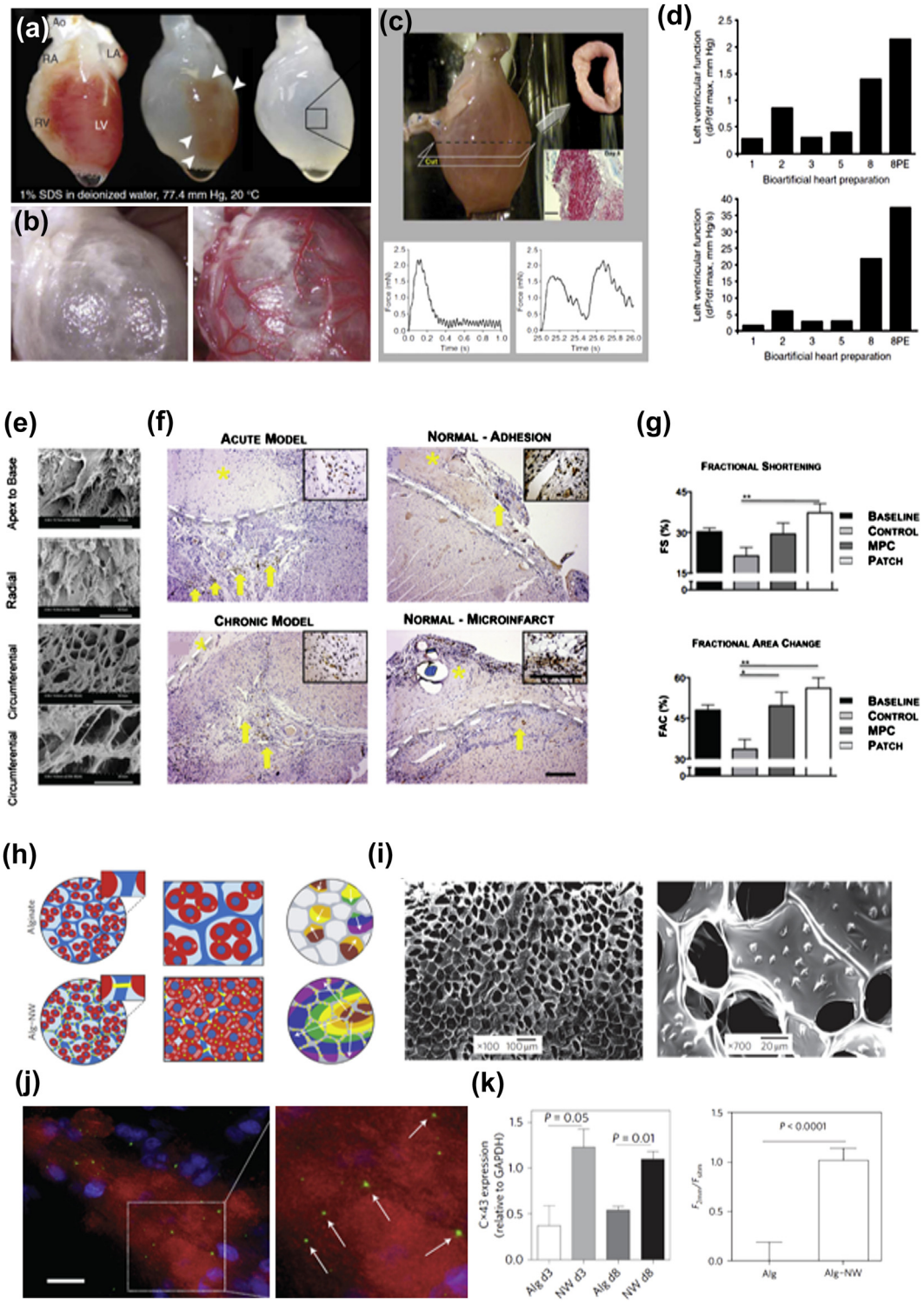

FIGURE 38.3

Scaffolds for cardiac tissue engineering. (a) Decellularization of whole neonatal heart with 1% SDS over the course of 12 hours. (b) Perfusion of the decellularized heart with the host vasculature through the aorta demonstrates maintenance of the decellularized blood vessels. (c) Hearts recellularized with cardiomyocytes beat spontaneously after four days in culture and can generate force when paced at 1 Hz or 2 Hz. (d) Maximum pressure and dP/dt after 8 days in culture and after stimulation with phenylephrine (PE). (e) Scanning electron microscope images of various slices of heart yield different pore sizes. (f) Migration of mesenchymal progenitor cells (MPCs) from MPC/Scaffold/transforming growth factor beta (TGFβ) constructs to acute infarcts, chronic infarcts, or normal myocardium four weeks after implantation. (g) Fractional shortening and fractional area change for 3d post-infarction rats (baseline) which were subsequently stratified into three groups: a control which received no additional intervention, an MPC group which received an injection of MPCs, and a patch group, which

ejection fraction (56% versus 39%). Whole cell patch clamp and microelectrode arrays of the cardiomyocytes demonstrated results consistent with cardiac phenotype. Together, these results suggest the identification of a cardiovascular progenitor which gives rise to cardiomyocytes, endothelial cells, and smooth muscle cells.

Purification and use of stem cell derived cardiomyocytes

While this original study and subsequent studies have identified ways of generating cardiovascular lineages [59], one major challenge was to separate the cardiomyocytes from the non-myocyte cell types. In a screen for a marker of human embryonic stem-cell-derived cardiomyocytes, signal regulatory protein alpha (SIRPA) appeared to specifically select for cardiomyocytes above the other cell types (including endothelial cells and smooth muscle cells) [60]. Indeed, cell sorting with this marker and depletion of endothelial cells and smooth muscle cells resulted in a 98% troponin positive population of cells (Fig. 38.2f,g). The generation of a pure cell source is of particular importance since implantation of undifferentiated cells may lead to teratoma formation. Further, a purified cell source allows for greater control over the cell densities and types present in tissue-engineered constructs.

Given the precision required to generate specific cell types from stem cells, protocols must be optimized. For example, the procedure originally proposed by Yang and coworkers has been further modified and applied to multiple cell lines, including iPSCs [51]. Use of microbioreactor arrays allows control over the three-dimensional cellular microenvironment in a multiplexed fashion, for experimental optimization of cell derivation procedures on a small scale [61]. Additionally, these devices allow to control more than simply the concentration or type of cytokines. Various electromechanical cues can be added in order to further recapitulate the cardiogenic niche, allowing true optimization of stem cell derivation procedures [62].

SCAFFOLDS

Cells alone do not compose functional tissues; other critical components include the substrate and extracellular matrix that surrounds and instructs the cardiac cells. Natively, the extracellular matrix provides microenvironmental cues, mechanical support and architectural guidance, acting as the scaffold upon which cardiac cells grow and function. Previous tissue-engineering scaffolds have used polymeric materials, lyophilized collagen sponges, and micropatterned anisotropic materials. These artificial materials have design advantages in that they can be microfabricated, functionalized and are highly reproducible, but other approaches have used decellularization of native matrix as a different starting point, providing a scaffold with improved biological activity at the cost of some of the versatility of the synthetic approaches.

Decellularization approach

Decellularization of native heart material is a powerful approach to easily recapitulate the *in vivo* architecture and extracellular composition of the heart. In 2008, Ott et al. decellularized whole rat hearts using a perfusion system of 1% sodium dodecyl sulfate (SDS) in deionized water, as shown in Fig. 38.3a [25]. The subsequent washes with detergent removed all of the cellular components from the native heart, leaving behind a 'ghost' heart, translucent in appearance, composed of thin walls of native heart matrix retaining the macroscopic and microscopic architecture.

received composite scaffolds containing MPCs in the decellularized scaffold. (h) Schematic of cardiomyocytes (red) in an alginate matrix or in the nanowired composite. (i) Transmission electron microscope images of alginate-nanowire composite scaffolds, where nanowires of approximately 5 μm in length were incorporated into the scaffold material. (j) Connexin-43 (green), troponin (red), and 4',6-diamidino-2-phenylindole (DAPI) (blue) immunostained cardiomyocytes organized in the nanowired scaffold. (k) Connexin-43 and actinin expression by western blot relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in nanowired group versus the alginate only group 3 or 8 days after seeding with cardiomyocytes.

When reperfused with a native blood supply, the decellularized hearts clearly showed maintenance of vascular channels, demonstrating the preservation of overall native morphology (Fig. 38.3b). When reseeded with neonatal rat cells, the heart regained a cellularized appearance, and sections cut from the reseeded heart were capable of beating and matched the pacing rate applied through an external electrical field, either at 1 or 2 Hz (Fig. 38.3c). Over the eight days of culture, there was an increasing trend of contractile function and pressure generation. The whole heart preparations were also exposed to phenylephrine, which increased contractile pressures, suggesting pharmacological responsiveness (Fig. 38.3d). Overall this study provided a basis for whole heart decellularization with subsequent repopulation, providing a cardiac pump function at 2% of the adult rat heart output.

Since cardiac MIs are localized within the whole heart, sometimes only a specific section, importantly the left ventricle may need to be the target of therapy [26]. Decellularized scaffold patches can be made from native human heart sections using sequential detergent washes. However, the direction of sectioning is important, as different planes of section result in different scaffold architectures and different pore sizes (Fig. 38.3e).

When vascular progenitor human MSCs are seeded onto these native scaffolds, they act as depots of vasculogenic factors, improving the recovery of left ventricular function damaged by infarction. As shown in Fig. 38.3f, the presence of infarct improved the migration of the vasculogenic MSCs into the damaged tissue, in both acute and chronic models, and suggested that the stimulus provided by injury improved the responsiveness of cardiac tissue to the therapeutic cells. Notably, cells did not stay at the surface epicardium of the heart; instead they penetrated into the myocardium even when only microinfarcts were created using sutures alone to damage the myocardium. These native cardiac patches acted as a vehicle for a vasculogenic cell-based therapy and showed improved heart function, using echocardiographic metrics such as fractional shortening and fractional area change of the regions infarcted (Fig. 38.3g). The use of the native matrix scaffold improved recovery above cell injection alone, proving additional benefit as a delivery vehicle for the vasculogenic cells.

Artificial scaffolds

Despite advances in using native matrices as scaffolding agents for the delivery of cells to the injured heart, the rational design of artificial scaffolds is still an active area of research. One primary limitation of fabricated scaffolds was their lack of electrical conductivity, a property that might enhance cell-cell communication and synchronization of the heart. Dvir et al. used gold nano-wires impregnated in a conventional scaffold material (alginate) to bestow conductive properties to the material [56]. Fig. 38.3g outlines how the conductive scaffold allows electrical signal propagation. Since alginate was chosen as the base scaffolding material, it maintained many of its familiar features. Even with the addition of gold nano-wires (Fig. 38.3h), the viscosity and material properties of alginate were not altered enough to change the porosity of the final scaffolds. The presence of the nano-wires improved the expression and organization of connexin 43, the primary gap protein associated with electrical-mechanical coupling and communication between cells, as assessed using immunofluorescent staining and western blot quantification (Fig. 39.3i). When calcium transients were investigated, the nano-wire-impregnated scaffolds showed fluorescent signals consistent with electrical propagation through the scaffold material, providing electrical stimulation to the cells. Engineered materials can thus add unique properties to cardiac scaffolds and tailor their functionality to that found in native heart muscle.

Freed and colleagues created an accordion-like scaffold using laser boring of 250 μm thick poly(glycerol sebacate) layers [31]. The accordion-like honeycomb was made by overlapping two 200 by 200 μm squares at an angle of 45°. The pore walls and struts were ~50 μm thick. The scaffolds were pretreated with cardiac fibroblasts followed by seeding of enriched cardiomyocytes. At the end of cultivation, the authors obtained contractile cardiac grafts with

mechanical properties closely resembling those of the native rat right ventricle. In addition, the cells in the pores were aligned along the preferred direction.

In another study, Feinberg et al. seeded a layer of neonatal rat ventricular cardiomyocytes on a polydimethylsiloxane membrane that could be detached from a thermo-sensitive layer of poly(isopropylacrylamide) at room temperature. Called Muscular Thin Films, these cell-covered sheets could be designed to perform tasks such as gripping, pumping, walking and swimming by careful tailoring of the tissue architecture, thin-film shape and electrical-pacing protocol [23a].

BIOPHYSICAL CUES

Biophysical stimulation has been used to improve the phenotypic maturity of cardiac tissue-engineered constructs. These stimuli are designed to be biomimetic, and simulate the native heart environment to facilitate proper cardiac growth and maturation.

Electrical stimulation

One of the primary features of the heart is its electrical connectivity and synchronization. Previous studies have shown improved conductive and functional characteristics of heart tissue grown in the presence of a stimulating electrical field. These results validate the use of electrical stimulation as a functional cue to improve phenotypic maturation of cardiac tissue, however, the precise techniques to apply electrical stimulation to engineered tissues is a topic of further exploration.

To establish baseline parameters, Tandon et al. compared multiple electrode materials and stimulation regimes to optimize electrical field conditioning of engineered heart tissue [50]. Petri dishes were outfitted with 1/8 inch rod shaped electrodes, spaced 1 cm apart, and 4 cm in length made from the following materials: carbon, steel, titanium nitride, and titanium. When comparing injected charge and recovery, the electrodes made of carbon recovered the most charge (95%), meaning had the least amount of lost charge due reaction products in the solution (Fig. 38.4a,b). The carbon electrodes were also best able to maintain a current through the bioreactor over the 2 ms time range for the depolarizing stimulus. With respect to effect on cell function, the results comparing various electrode materials was more modest, but carbon electrodes still had a favorable trend in their ability to properly capture the pacing of cardiac constructs as well as a lowered excitation threshold (Fig. 38.4c,d). This suggests that the carbon best communicates with the tissue-engineered constructs with minimal side reactions.

As seen with these and other studies, the effect of field stimulation improves cardiac tissue function, shown by increased Troponin and Connexin 43 markers, key proteins in both cell-cell connectivity and contractile apparatus (Fig. 38.4e).

Mechanical stimulation

Mechanical stimulation approaches the electrical-mechanical coupling of heart tissue from the stretching and mechanical contraction perspective. It has been shown that passive or active tension can increase the cardiac functionality and orientation of cardiac cells. In one study, chitosan, a polysaccharide-based scaffolding material, was processed to have defined channel pores for perfusion and high porosity for cell seeding (Fig. 38.4f–h). Due to its attractive mechanical properties (Fig. 38.4i), it was used with a radial stretch device to characterize contraction using active tension. Strain maps of the scaffolds could be generated modeling the surface mechanics of the chitosan, showing stereotyped stress fields around the large pore areas (Fig. 38.4j). Interestingly, increased cell density and organization followed the predicted stress map suggesting that the mechanical stress provides a biophysical cue for cellular organization and communication (Fig. 38.4k).

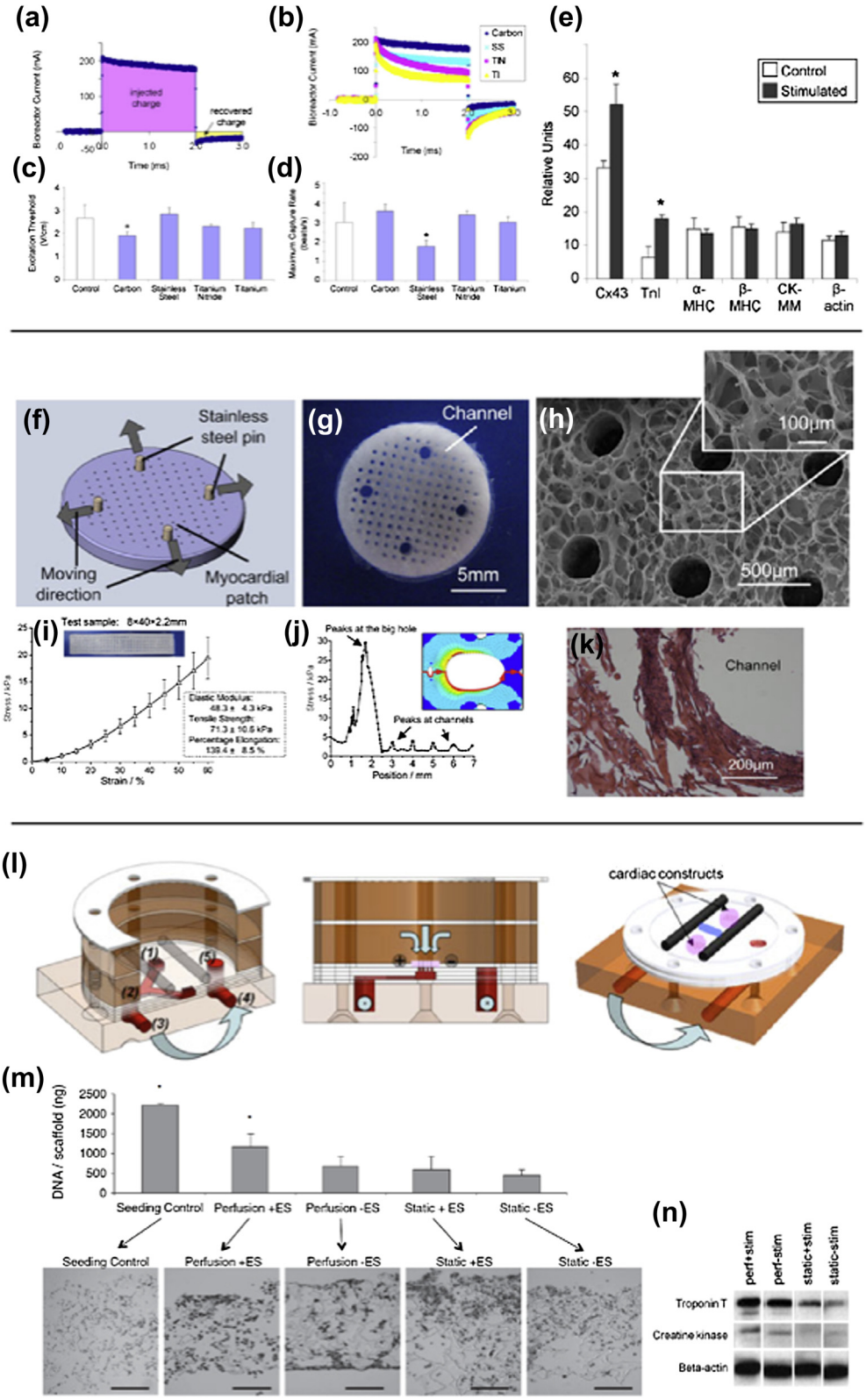

FIGURE 38.4

Biophysical cues for cardiac tissue engineering. (a) Bioreactor current over time depicting injected and recovered charge. (b) Current-time curves for different materials (carbon, stainless steel, titanium nitride, and titanium). (c) Excitation threshold and (d) maximum capture rate for cardiomyocytes stimulated with different materials or not stimulated at all (control). (e) Relative protein amounts of cardiac-related proteins in stimulated groups versus control. (f) Schematic scaffold design with pins for mechanical stimulation and pores for perfusion. (g) Macroscopic image and (h) scanning electron microscope image of

Perfusion

Another critical biophysical stimulus — perfusion — is required for proper metabolic function. The transport of fresh oxygen and nutrients along with removal of metabolic products is particularly essential for cardiac tissue. Native tissue combines active perfusion and electrical-mechanical coupling, and in Maidhof et al, these two critical stimuli were combined in a novel bioreactor to enhance cell density and contractile protein expression of troponin [40]. A perfusion system, forcing fluid through a channeled scaffold was built around the standard paradigm of carbon electrodes. (Fig. 38.4l). This resulted in a more uniform cell distribution due to removal of diffusion limitations encountered when growing thicker pieces of cardiac tissue. When electrical stimulation was also applied, the cells were both more numerous while maintain an even density distribution throughout the tissue. (Fig. 38.4m) The two stimuli, perfusion and electrical stimulation, had an additive effect on troponin expression (Fig. 38.4n). The three biophysical stimuli — electrical stimulation, mechanical stretch and perfusion — all can contribute to the functional maturity of engineered cardiac tissues.

IN VIVO APPLICATIONS OF CARDIAC TISSUE ENGINEERING

The ultimate goal of cardiac tissue engineering is the implementation of cells or tissue constructs in an injured heart and subsequently improving cardiac function. An 'ideal' cardiac patch would not only beat spontaneously, but would also connect with the host vasculature, couple electrically with the surrounding myocardium, and generate force to improve the function of a failing heart. We describe in this section two methods of fabricating a cardiac patch, the first of which focuses on using mechanical load to create force-generating constructs, and the second of which focuses on introducing multiple cell types to enhance vascularization of the cardiac patch. Open questions for *in vivo* systems include the functional differences between cell injection and scaffold implantation, and the type of arrhythmogenic or immunogenic response the foreign cells will have when near the host tissue.

Engineered heart tissue

Zimmermann and colleagues (2006) outlined an approach to generate contracting rings of cardiac tissue which can be implanted into infarcted rat hearts, resulting in an improvement in cardiac function [30]. Engineered heart tissues were created by casting neonatal rat cardiomyocytes, collagen I, and Matrigel in a circular mold, followed by seven days of culture in 40% oxygen, under static, 'auxotonic' load, and in media containing insulin. Five of these loops were then stacked to create a large (15 mm diameter x 1—4 mm thick), fused, synchronously beating tissue assmbly amenable to implantation (Fig. 38.5a).

Structural and electrical integration with the host myocardium as well as whole heart function were examined in rats with infarcted hearts implanted with engineered heart tissues. Immunostaining of the engineered heart tissue four weeks after implantation showed an elongated, sarcomeric pattern suggestive of highly differentiated cardiomyocytes (Fig. 38.5b). Additionally, there was neovascularization within the tissues from the donor cells that connected to the host vasculature (Fig. 38.5c). Electrical coupling was improved in the engineered heart tissues, as demonstrated by lower total activation times and higher QRS amplitudes. *In vivo* studies allow for the functional examination of whole hearts. Rats with engineered heart tissues demonstrated shorter left ventricular end diastolic diameter and a lower maximum left ventricular volume when compared to sham-operated rats, suggesting no additional dilation

fabricated chitosan-collagen scaffold. (i) Stress-strain curve of scaffold material with corresponding elastic modulus, tensile strength, and percent elongation. (j) Stress distribution along the length of the scaffold. (k) Hematoxylin and eosin (H&E) staining of cardiomyocyte laden scaffold in a region near a channel. (l) Schematic of perfusion-electrical stimulation bioreactor. (m) DNA content per scaffold and H&E stained sections of cardiac constructs that underwent different stimulation procedures. (n) Western blot of troponin T, creatine kinase, and beta-actin for constructs that were perfused and/or electrically stimulated.

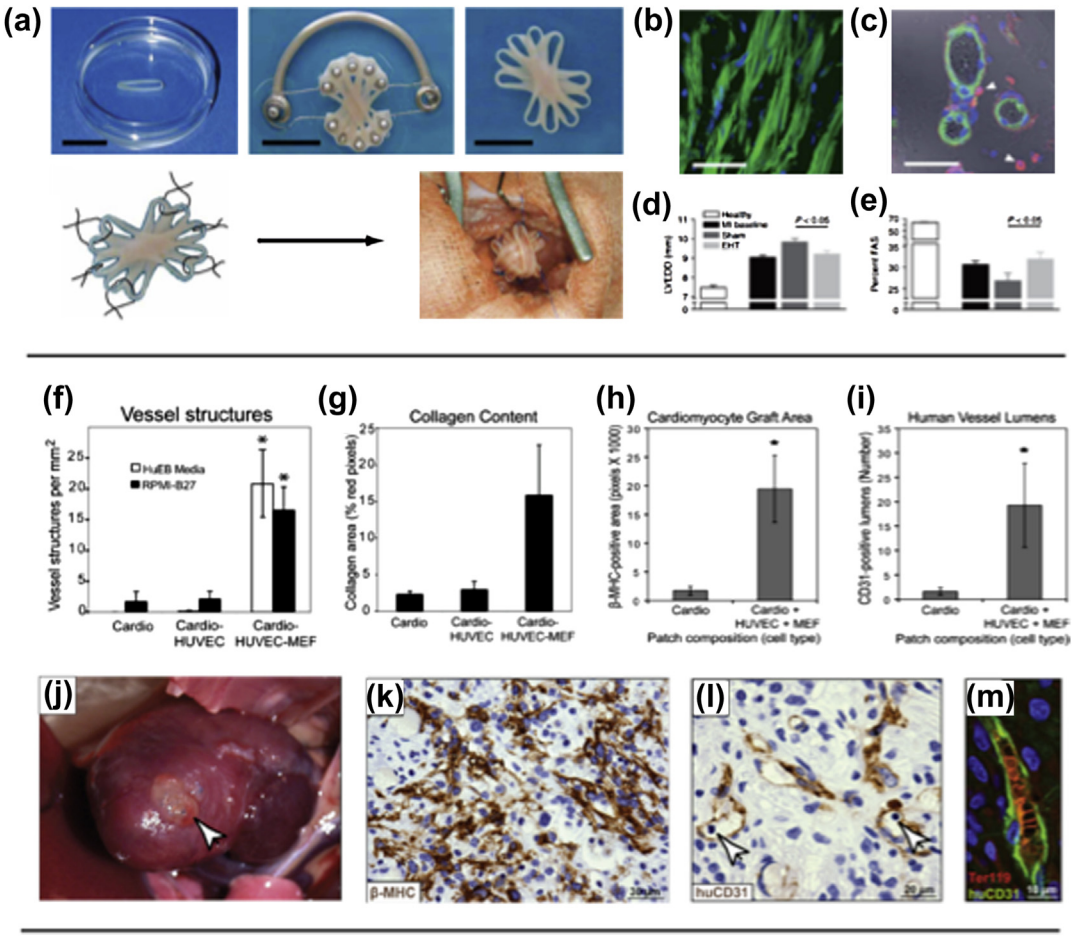

FIGURE 38.5

***In vivo* applications of cardiac tissue engineering.** (a) Single ring constructs are stacked in an auxotonic loading system and sewn on a rat heart using six sutures. (b) Troponin (green) and DAPI (blue) staining of engineered heart tissue four weeks after implantation showing cardiomyocyte elongation and sarcomeric alignment. (c) Vessel structures where asterisks represent the vessel lumens and arrows represent associated macrophages. (d) Left ventricular end diastolic diameter and (e) Percent fractional area shortening or healthy rat hearts compared to myocardial infarction (MI) baseline, sham surgery, and engineered heart tissue (EHT) implanted groups. (f) Number of vessel structures present in the cardiomyocyte only group, cardiomyocyte-HUVEC group, and tri-cultured group for two different media compositions after eight days of culture. (g) Collagen content of constructs after 8 days of culture. (h) β-MHC positive area and (i) CD-31 positive lumens based on histological immunostains in constructs implanted in skeletal muscle for one week. (j) Tri-cultured patches on the rodent heart one week after implantation. (k) β-MHC and (l) CD-31 immunostain of the constructs. (m) Vessel lumen in engineered tissue construct filled with Ter119-positive red blood cells from the host vasculature.

of the infarcted rat hearts as might be expected (Fig. 38.5d). Further, there was no decrease in fractional area shortening of the heart after the operation (Fig. 38.5e).

Vascularized cardiac patches

Vascular integration of the cardiac patch with the host myocardium is important for the prolonged survival of thick tissue patches, especially given the high oxygen demand of cardiac tissue. Stevens et al. demonstrated the utility of prevascularizing cardiac tissue prior to transplantation into the infarcted area [24]. Cardiac patches were created from human embryonic stem-cell-derived cardiomyocytes and human umbilical vein endothelial cells, on mouse embryonic feeders. Several experimental groups were investigated: a cardiomyocyte only group, a group consisting of cardiomyocytes and endothelial cells, and a group consisting of all three cell types. Histology showed that the group consisting of all three cell types resulted in the largest number of vessel-like structures (Fig. 38.5f), and the stiffest construct with higher collagen contents, that were most similar to native myocardium (Fig. 38.5g). Upon

implantation into a skeletal muscle environment, the tri-culture group demonstrated great area staining for β-myosin heavy chain, the greatest number of vessel lumens (Fig. 38.5h,i), and neovascularization and anastomosis with the host vasculature (Figs. 38.5j–m).

Electrical coupling of cardiomyocytes in the heart

One question remains whether or not cardiomyocytes, when applied to the heart, will form adequate connections with the host tissue, or if the engineered tissue constructs are proarrhythmic, thus potentially negating any therapeutic effect. In one recent study, Shiba et al. showed that these grafts may electrically couple with the host and suppress arrhythmias [63]. Human embryonic stem-cell-derived cardiomyocytes were transplanted onto a guinea pig cryoinjured heart and were subsequently analyzed for electrical coupling with the host tissue. They showed not only 1:1 coupling with the host myocardium, but also that the heart had reduced susceptibility to premature ventricular contractions, and sustained ventricular tachycardia. As a result, this study represents one of the first accounts that cardiomyocytes are non-arrhythmogenic when implanted. This supports the continued study of the interactions between the native host myocardium and the tissue-engineered construct.

MODELING OF DISEASE

In vitro models of disease represent an important avenue for studying disease and for identifying potential therapeutic options. Here we describe the use of patient specific cells to generate diseased cardiomyocytes. We further describe the utility of tissue engineering in providing a faithful representation of diseased myocardium, and how this may be used in high throughput screens and drug studies.

Generation of patient-specific cardiomyocytes

Induced pluripotent stem cells have allowed for the generation of patient-specific cardiomyocytes. One of the first accounts came from Moretti et al., who generated iPS cells from patients with a particular genetic mutation leading to Long QT syndrome and subsequently differentiated them into cardiomyocytes (Fig. 38.6a,b) [11]. Localization of the mutated channel (KCNQ1) in the endoplasmic reticulum confirmed this channel as the likely cause of disease (Fig. 38.6c,d). These cardiomyocytes displayed the electrophysiological phenotype of Long QT syndrome, including the classic lengthened action potential duration (Fig. 39.6e,f). The use of induced pluripotent cells to recapitulate the disease phenotype is particularly amenable to patients with genetic mutations. Since the study on Long QT syndrome, cardiomyocytes from patients with other diseases, including arrhythmogenic right ventricular cardiomyopathy and familial dilated cardiomyopathy, have been generated [13,14]. The iPS cells from patients harboring genetic cardiac mutations have been differentiated into CMs. These include cells from Timothy [12], Long QT [11], and LEOPARD [24a] syndromes and dilated cardiomyopathy patients [14].

Engineered heart tissue model for diabetes

While induced pluripotent cells are useful in generating cells from patients with specific genetic mutations, many cardiac diseases are multifactorial and require a biomimetic environment to faithfully reproduce the disease phenotype. Engineered heart tissue has been used by Song and colleagues to study the cardiac effect of diabetes and the effect of drug therapy on diabetic engineered heart tissues [64]. Briefly, the diabetic rat heart and high glucose cultivation conditions exhibited diminishing electrophysiological properties and increased ratio of myosin heavy chain isoform β to α, indicative of diseased states.

Tissue engineering as a platform for pharmacologic studies

Given the active nature of cardiac tissue, new methods have been developed to study the function of engineered cardiac tissues in multiplexed *in vitro* systems. [65] Particularly, Schaaf

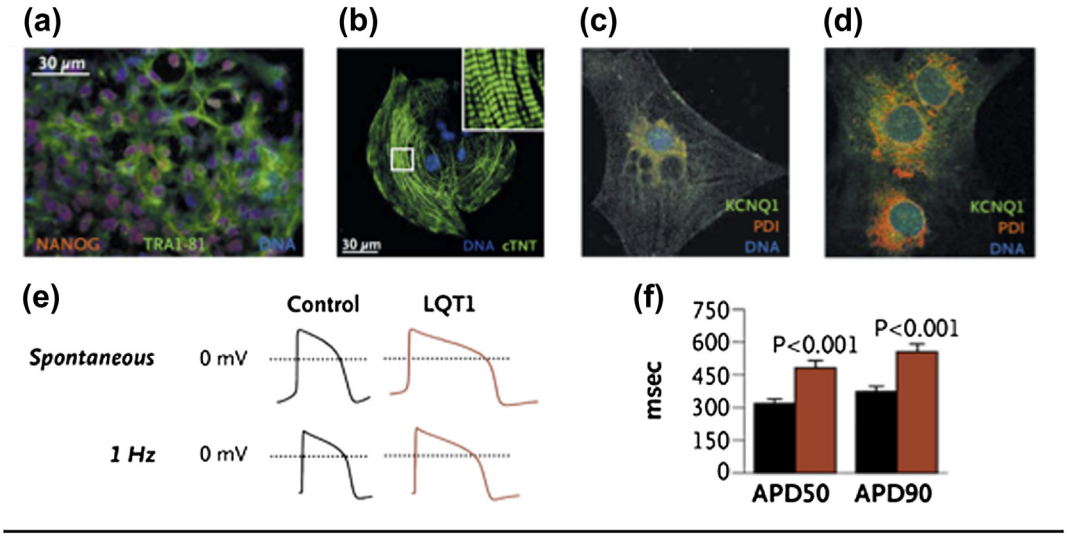

FIGURE 38.6

***In vitro* models of disease.** (a) Immunostain of induced pluripotent cells from LQT1 patients for Nanog and Tra1—81. (b) Troponin stain showing sarcomeric organization in induced pluripotent stem cell (iPS) derived cardiomyocytes. Potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) (green) and protein disulfide isomerase (PDI) (red) staining for control cardiomyocytes (c) and patient cardiomyocytes (d). (e) Single cell action potentials of control and patient derived stem cells spontaneously beating or stimulated at 1 Hz. (f) APD50 and APD90 for control (black) or patient derived (red) cardiomyocytes.

et al. cast human embryonic stem cell derived cardiomyocytes in fibrin across a 24-well format which allowed the real-time measurement of force generation. [66] This system was then used to examine the effect of various proarrhythmic drugs (e.g., E-4031, quinidine, procainamide) on the beating dynamics of the heart tissue. As expected from the known electrophysiological effects of the drugs, the engineered tissues displayed irregular beating at low relaxation velocities.

In a separate study, sheets of engineered cardiac tissues were fabricated in a similarly multiplexed format which allowed the measurement of stress exerted by the cells [67]. Myocytes were seeded on a micropatterned surface to facilitate alignment of cardiomyocytes. The system was imaging compatible, allowing for the quantification of images or videos to determine contractility, action potential propagation, and cytoskeletal architecture.

SUMMARY AND CHALLENGES

The increasing population of patients with heart disease, and the limited availability of transplant organs, motivates the field of cardiac tissue-engineering, which aims to generate robust tissue for implantation and subsequent improvement in cardiac function. The stringent requirements of cardiac tissue, including: the ability to generate contractile force, substantial metabolic requirements, and the need to electrical integrate with host tissue have resulted in a multitude of techniques to develop *in vitro* cardiac tissue. Different scaffolds, including decellularized native heart and various artificial materials have been used to recapitulate the native architecture of the heart while providing conduits for vascularization. The addition of biophysical stimuli, including mechanical stretch and electrical stimulation, are aimed to phenotypically mature the cells. When considering these various strategies implemented to design and fabricate improved cardiac tissue, one overall obstacle as the field advances is standardization of experimental and measurement techniques.

This is even more critical with the recent advent of high yield differentiation strategies to generate iPS and ES derived cardiomyocytes, enabling for the first time, a human cell source with the potential to generate patient-specific cardiac tissues. The field of pluripotent stem cell mediated therapies is still in early development, and many fundamental obstacles still remain

before their full promise is realized. However, we are primed to leverage autologous, nonimmunogenic, patient-specific cardiomyocytes with advanced techniques for the cultivation of engineered heart tissue. The creative combination of these cells with three-dimensional vascularized matrices and biophysical stimulation will allow the generation of functional tissues and provide an opportunity to intervene on the global epidemic of heart disease [25a–29a].

Acknowledgments

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CHAPTER 39

Blood Vessels

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INTRODUCTION

In the early 1900s, Alexis Carrel first described the utility and shortfalls of autogenous and synthetic grafts, including the unique challenges of small diameter synthetic grafts. Voorhees first utilized a synthetic vascular graft for larger artery replacement in the 1950s [1]. In 2012, small diameter arterial grafting continues to be associated with worsened long-term patency rates compared to autogenous vessels.

Since living with cardiovascular disease has become more prevalent in the last 100 years and people's lifespan across most of the world has been extended substantially in this time, there has been a growing need for vascular interventions. There are between 600,000 and 1.4 million bypass operations annually in the United States, and this number has remained stable in recent years. There has also been a significant growth in endovascular procedures for the lower extremity, and this has correlated with a decrease in major amputations [2]. Despite initial benefits of decreased early mortality and morbidity, these procedures are usually of inferior durability or outcome, and sometimes both. Thus there is a critical need to create small vessel vascular conduits and apply bioengineering approaches to endovascular therapies to improve the capacity and quality of patient care in the treatment of cardiovascular disease and injury.

This chapter discusses the experimental and clinically available bypass grafts, the modifications of these grafts to facilitate healing, current approaches to promoting arteriogenesis and therapeutic angiogenesis, to creating tissue-engineered vascular grafts, and concludes with tissue-engineering approaches in endovascular therapeutics.

CURRENT STATUS OF VASCULAR CONDUITS

Conduit patency and failure

Large diameter grafts, such as those used for aortic reconstruction, have a five year patency rate approaching 95%, but when synthetic grafts are used in the infrapopliteal region, the one year patency rates are less than 50% [3]. The best autogenous conduit is the left internal mammary artery for coronary artery bypass grafting; it has a greater than 90% 5-year patency. Vein, particularly good quality greater saphenous, is the conduit of choice for multi-vessel coronary artery bypass grafting and extremity vascular reconstruction. However its primary and secondary patency rates at one year in a patient population with critical limb ischemia were

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recently reported to be 61% and 80%, respectively [4]; the results are not better in the coronary vasculature. Thus there are clinical needs that drive novel conduit creation and existing conduit remodeling.

A variety of mechanisms can lead to bypass failure, and they occur in a defined temporal sequence. Immediate graft failure is usually the result of technical error from the operation or the patient having a hypercoagulable condition; failure in the first month following graft placement is likely the result of thrombosis secondary to distal flow resistance. Small diameter grafts are prone to early thrombosis because of their lower flow rates and higher resistance. Thus graft thrombogenicity is of primary concern early after graft placement.

Anastomotic myointimal hyperplasia (IH) is the most common reason for graft failure from six months to three years after graft insertion. Small diameter grafts are particularly susceptible to IH.

Later graft failure is frequently due to the progression of distal atherosclerotic disease and beyond the direct influence of the conduit. However stiffness in the conduit or the target artery itself may contribute to the worsened prognosis after intervention in diseased compared to healthy arteries via flow disturbances, and the predilection towards inward remodeling. We have recently demonstrated that circumferential microstructural collagen alignment in the arterial media is associated with increased stiffness in distinct primate arteries (Fig. 39.1). One can envision endovascular approaches being individuated according to the stiffness of the target artery in order to promote favorable remodeling post-procedurally.

Pathophysiologically, denuded intima or exposed luminal area of a graft may lead to thrombosis via platelet deposition and activation of the coagulation cascade, and over time it promotes pathologic smooth muscle cell (SMC) migration, proliferation, and extracellular matrix (ECM) deposition, leading to IH. IH in turn narrows the vessel lumen (re-stenosis) disturbing blood flow to the point that it may occlude, thrombose, or cause symptomatic

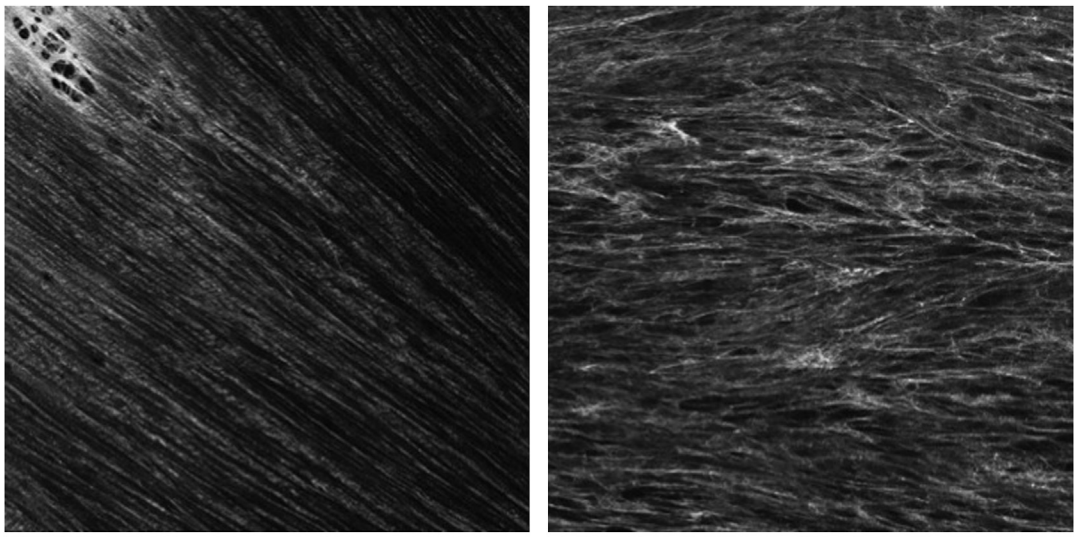

FIGURE 39.1
Differences in circumferential collagen alignment in the arterial media. Representative multiphoton image of differences in microstructural collagen alignment in the arterial media of the primate femoral (left) and carotid (right) artery.

ischemia in the relevant distal end organs like the brain (cardiovascular accidents), heart (myocardial infarctions), and extremities (critical limb ischemia). Since thrombogenicity and intimal hyperplasia represent the most common causes of graft failure and are both mediated at the luminal interface of the vessel or graft, the inner lining of grafts has been the subject of much investigation.

Cellular and molecular mediators of graft outcome

ENDOTHELIUM

In the absence of disease or injury, native blood vessels possess an endothelial lining that constantly secretes bioactive substances inhibiting thrombosis, promoting fibrinolysis, and inhibiting SMCs from switching from a contractile to a synthetic phenotype. The intima cues the vessel media to maintain blood flow and perfusion to meet distal tissue demands. Thus re-establishing this type of intimal lining quickly and completely in vascular grafts is vital to the patency of small diameter vascular grafts. Unfortunately, and unlike most animal models in use that spontaneously endothelialize synthetic grafts, humans manifest only limited endothelial cell ingrowth not extending beyond 1–2 cm of both anastomoses. However, endothelial islands have been described in the midportions of grafts at significant distances from the anastomosis, suggesting that other EC sources for graft endothelialization may exist. Interstitial tissue ingrowth accompanied by microvessels from the perigraft tissue is one potential source. There is also evidence that circulating endothelial cells, endothelial progenitor cells (EPCs), or stem cells can be directed to these areas. Such homing can be promoted through affixation of EC attractant antibodies to the grafts in a similar fashion to that utilized in coronary artery stents [5].

However, ECs growing on prosthetic graft surfaces are not necessarily the same as their normal quiescent counterparts in uninjured vessels. These ECs are often 'activated', secreting bioactive substances (e.g., PDGF) that actually promote thrombogenesis and changes in SMC phenotype. This has been seen in the perianastomotic region, which is the most frequent site of interventional failure after operation. SMCs found within the myointima of prosthetic grafts are also functionally altered. They produce significantly higher amounts of PDGF, as well as various ECM proteins, compared to those of the adjacent vessel, which along with the body's inflammatory reaction to prosthetic material, contribute to the development of intimal hyperplasia [6].

INFLAMMATION

The inflammatory response to vascular interventions is complex because these patients have systemic inflammation which may influence the degree and direction of local inflammation. Potent chemoattractants like complement 5a (C5a) and leukotriene B_4 recruit neutrophils to the graft surface where they localize in the fibrin coagulum of the graft's inner and outer capsule via β2 integrins. Also, IgG binds to the neutrophils' Fcγ receptors activating neutrophils' pro-inflammatory response while inhibiting normal clearance of bacteria. Neutrophils also interact with various other deposited proteins including C3bi and Factor X, and they adhere to the endothelial cells in the perianastomotic region through selectin- and integrin-mediated mechanisms. L-selectin is thought to modulate neutrophil/endothelial cell interactions by presenting neutrophil ligands to both E and P-selectin on the vascular endothelium. In addition, selectin-carbohydrate bonds are important for the initial cellular contact while the integrin-peptide bonds are responsible for strengthening this adhesion, as well as the transmigration of neutrophils. Both intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the EC surface bind these integrins as well, and ECs upregulate ICAM-1 and express VCAM-1 when stimulated by inflammatory agonists like IL-1, TNF, lipopolysaccharide, and thrombin. Further, activated neutrophils

release oxygen free radicals and various proteases, which result in matrix degradation and may inhibit both endothelialization and tissue incorporation of the vascular graft.

Circulating monocytes/macrophages are also attracted to areas of injured or regenerating endothelium, especially in response to IL-1 and TNF-α. There are many plasma monocyte recruitment and activating factors, including LTB_4, platelet factor 4, and PDGF. This process is propagated in the presence of these plasma activating factors, driving monocytes to differentiate into macrophages that direct the host's chronic inflammatory response via the release of proteases and oxygen free radicals.

A variety of cytokines are released from the inflammatory cells activated by vascular grafts. Lactide/glycolide grafts are composed of bioresorbable materials that are phagocytosed by macrophages; in culture these materials stimulate macrophages to release mitogens that stimulate vascular cells. This mitogenic activity appears to be related to the secretion of FGF-2 since pretreatment of the culture media with a neutralizing anti-FGF-2 antibody significantly diminishes the stimulatory effect on SMC growth in culture [7]. Cultured monocytes and macrophages incubated with Dacron and expanded polytetrafluoroethylene (ePTFE) have been demonstrated to produce different amounts of IL-1β, IL-6, and TNF-α that are biomaterial specific [8]. TNF-α is one of the factors that may contribute to the enhanced proliferation of SMCs caused by leukocyte-biomaterial interactions, while IL-1 may be partly responsible for the increased SMC proliferation caused by leukocyte-EC reaction. IL-1 also induces up-regulation of IGF-1 expression in ECs, and co-culture of neutrophils with IL-1β-treated ECs dramatically increases PDGF release.

Since the inflammatory reaction elicits a cascade of growth processes, it has been proposed that approaches attenuating the initial inflammatory reaction may improve long-term graft patency; alternatively, directing the inflammatory response to promote favorable cellular and protein responses may promote intimal generation and tissue incorporation.

PHYSICAL OR CHEMICAL MODIFICATION OF CURRENT GRAFTS TO IMPROVE DURABILITY

The long-term patency of vascular grafts depends upon the intrinsic properties of the graft itself and the hemodynamic environment in which the graft is placed, as well as patient variables (e.g., diabetes, renal failure, etc.), and may or may not be improved by prior or concomitant interventions such as proximal or distal angioplasty. Since it is now clear that tissue incorporation is important for long-term graft function, grafts that have this ability are now desirable for medium and small caliber vessel replacement. Polyethylene terephthalate (PET, Dacron) and ePTFE are the predominant materials currently used in prosthetic vascular grafts, but both of these react with blood components and perigraft tissues in concomitantly beneficial and detrimental fashions. All grafts, regardless of their composition and structure, evoke complex but predictable host responses that begin immediately upon restoration of perfusion, and this improved understanding of the cellular and molecular components of biomaterial/tissue interactions has led to more intelligent designs of grafts that maximize beneficial ingrowth while minimizing the chronic inflammatory changes that lead to graft dilation or occlusion. Thus, the chemical composition, construction parameters and biomechanical characteristics of a vascular graft are the predominant mediators of host interaction, and largely determine graft fate.

Surface characteristics

The thrombotic interaction at the grafts' luminal interface is dependent on both the chemical and physical properties of the graft (e.g., surface charge, surface energy, and roughness). A negative surface charge attenuates platelet adhesion and a positive charge promotes it, while a heterogeneous charge density distribution is also thought to be thrombogenic. A myriad of

approaches have been designed to limit the thrombotic reaction, including modification of surface properties, incorporation of antiplatelet or anticoagulant substances onto the graft surface, and endothelialization of the luminal surface. In addition to thrombogenic reactions to the luminal surfaces, the rate and extent of endothelialization will vary depending on the characteristics of the surface [9]. Therefore properties must be optimized for both reduced thrombogenic reaction and maximized endothelialization.

Surface modifications

The simplest modification of a graft surface is to coat it with a relatively inert polymer. Since the 1960s, it has been known that carbon coating decreases surface thrombogenicity through its negative charge and hydrophobic nature. Experimentally, the carbon-impregnated prosthetic graft was found experimentally to reduce platelet deposition, but the advantage of these grafts was not confirmed in a prospective multicenter clinical study of 81 carbon-impregnated ePTFE and 79 standard ePTFE grafts for below-knee popliteal and tibial/peroneal artery bypasses. Here the investigators failed to show a significant difference in patency rate between the two groups at up to 12 months after implantation [10].

Silicone polymer coating is another approach to alter the luminal surfaces of grafts; this process produces a smooth surface that is devoid of the usual ePTFE graft permeability and texture, and when followed by plasma glow discharge polymerization, it effectively abolishes pannus tissue ingrowth as well as graft surface neointimal hyperplasia in a baboon arterial interposition graft model. Further Nojiri et al. [11] have developed a three layered graft consisting of PET for the outer layer (to promote perigraft tissue incorporation), non-porous polyurethane in the middle layer (to obtain a smooth surface), and a 2-hydroxyethyl methacrylate and styrene (HEMA-st) copolymer coating for the inner layer (to establish a non-thrombogenic blood interface). These grafts with an ID of 3 mm were implanted in canine carotid arteries and remained patent for over one year. Only a monolayer of adsorbed proteins was described on the luminal surface of the grafts with no pannus ingrowth from the adjacent artery, no thrombus, and no endothelial lining or neointimal formation.

Protein adsorption

Another approach is to cover vascular grafts' lumens with proteins. Protein coating has been used as an alternative to preclotting with blood to decrease the initial porosity of Dacron grafts in order to limit transmural blood loss. Knitted Dacron prostheses coated with albumin, gelatin, and collagen have all been available for clinical use. As the impregnated proteins are degraded, the graft undergoes tissue ingrowth.

The most abundant serum proteins are albumin, fibrinogen, and IgG. They adsorb to grafts almost instantaneously following exposure to the systemic circulation. Subsequently there is a redistribution of proteins, known as the Vroman Effect, according to each protein's relative biochemical and electrical affinity for the graft surface and their relative abundance [12]. Since platelets and blood cells interact predominantly with the bound proteins and not with the prosthetic material itself, the constitution and concentration of bound protein has profound influence over the type and degree of cellular interaction with the graft. Fibrinogen, laminin, fibronectin, and vitronectin all have an arginine-glycine-aspartate (RGD) sequence that is recognized by platelets' glycoprotein (GPIIb/IIIa) receptor and initiate platelet activation. RGD sequences are also recognized by β_2 intergrin, which directs leukocyte adhesion to the graft. Additional plasma proteins, including complement components, can also be differentially activated directly by different synthetic surfaces. For example, the generation of the monocyte chemoattractant, C5a, is greater following implantation of Dacron compared to ePTFE grafts in an animal model [13]. In addition, the rapid accumulation of coagulant proteins such as thrombin and Factor Xa on the luminal surface after implantation contributes to the thrombogenicity of vascular grafts.

Porosity

The prevalence of open spaces or pores determines the porosity of a scaffold or synthetic graft, while the permeability of a graft is defined by its ability to permit passage of a substance through itself. Since ePTFE is composed of a number of solid nodes inter-connected by a matrix of thin fibrils with no uninterrupted transmural spaces, it is best categorized by the distance between these nodes, which is defined as the internodal distance (IND). This spacing, when above approximately 5–6 μm, as is commonly used in clinically available prostheses, allows for cellular ingrowth, but transinterstitial ingrowth is not strictly a function of porosity. We have shown that the extent of ingrowth varies greatly among different biomaterials (e.g., polyglycolic acid (PGA) and Dacron) despite these biomaterials having similar porosity [14].

Still, the rate of tissue ingrowth can be improved by optimizing graft porosity or permeability. Clowes et al. have demonstrated enhanced tissue ingrowth and complete re-endothelialization of 60 μm or 90 μm internodal distance ePTFE grafts in a baboon model [15]. However, transinterstitial capillary ingrowth was not seen with the more commonly used 30 μm internodal distance ePTFE. Human trials using ePTFE with these expanded internodal distances failed to show any advantage in platelet deposition compared to the standard 30 μm internodal distance ePTFE grafts [16].

Compliance

The compliance mismatch between arteries and grafts causes flow disruption *in vivo* which may contribute to anastomotic pseudointimal hyperplasia [17]. It is for this reason that various surgeons have suggested interposing a segment of vein between the synthetic graft and artery, creating a composite graft at the distal anastomosis. This has led some investigators to design more compliant grafts using more flexible materials and/or changing the parameters of graft construction to improve graft compliance. Although animal experiments have suggested concept validity, the clinical benefit of this approach remains controversial. Many factors may contribute to this confusion, including longitudinal variability in the diameter and compliance of the arterial tree and the effect of activated endothelium on intimal hyperplasia. Further there is a robust fibrotic response after implantation that leads compliant grafts to become incompliant after implantation; thus even if a compliance match were attained initially, it would not likely persist. In the para-anastomotic region there are dynamic changes in compliance that vary over time. First a para-anastomotic hypercompliant zone exhibits a 50% gain in compliance, then later its compliance is lessened 60% from baseline [18]. It is likely that this bimodal effect limits the practical value of this approach.

Thromboresistance

Early platelet deposition on vascular grafts is mediated by von Willebrand Factor (vWF) and platelet membrane glycoproteins. After adherence to a graft, platelets degranulate, releasing many bioactive substances, including serotonin, epinephrine, ADP, and thromboxane A2. These substances in turn activate additional platelets and promote a prothrombogenic reaction. Activated platelets also release growth factors, such as PDGF, EGF, TGF-β, which promote SMC migration and proliferation as well as ECM degradation and ECM protein synthesis. In addition, platelets release monocyte chemoattractants such as platelet factor 4 and β-thromboglobulin, which mediate the recruitment of macrophages to the graft. Platelet deposition and activation continues chronically after graft implantation as evidenced by increased thromboxane levels and decreased systemic platelet counts one year after Dacron graft implantation in a canine model [19], and human studies have confirmed platelet adhesion to grafts up to one year after implantation.

Since the deposition and activation of platelets elicits various pathologic cascades, the thrombogenic nature of the synthetic graft surface can lead to both early and late graft failure. A myriad of approaches have been studied to attenuate platelet deposition, aggregation, and

degranulation. Antiplatelet agents directly targeting platelet/graft-binding molecules such as platelet surface GPIIb/IIIa and different functional domains of thrombin have been shown to at least transiently decrease the accumulation of platelets on Dacron grafts [20]. Also the surface thrombogenicity of grafts can be altered experimentally as described earlier.

Similar approaches can be utilized to decrease thrombogenicity by disrupting the activation of the blood system's coagulation cascade on thrombogenic surfaces, such as cardiovascular stents and synthetic grafts. These are now commercially available in various formulations. Genetic approaches to increase thromboresistance have been employed by multiple groups through the over-expression of thrombotic inhibitors, but since ECs themselves are antithrombotic, there may be limited benefit of this approach when compared to a functioning endothelium.

Resistance to infection

Vascular graft infection is rare, but it is catastrophic when it occurs; as demonstrated by an amputation rate of approximately 50% and a reported mortality rate that ranges from 25–75%. In an attempt to limit this dreaded complication, penicillin and cephalosporins have been successfully bound to Dacron and ePTFE grafts and found to limit *Staphylococcus aureus* infection in animal models. Rifampin-bonded gelatin-sealed Dacron grafts have also been shown *in vitro* to lessen bacterial colonization [21]. Intuitively, tissue ingrowth itself may also provide resistance to infection.

Biological modification through exogenous sources

The delivery of potent angiogens or genes that promote EC-specific mitogenesis or chemotaxis upon prosthetic surfaces may be used to regenerate a rapid and complete endothelium after vascular intervention. Such prosthetics could store these genes or proteins and provide a controlled expression or release of these genes or proteins locally to circulating or surrounding ECs in a cell-demanded fashion. Ideally, this kind of prosthetic would be available as an off-the-shelf alternative to autogenous vein.

PROTEIN THERAPY

Although tissue incorporation is a desirable process for implanted prostheses, excessive vascular cell proliferation as well as ECM deposition can lead to intimal hyperplasia and ultimately graft failure. The ideal healing process in vascular grafts would be rapid endothelialization of blood contacting surfaces concomitant with a spatially and temporally limited subendothelial SMC growth, and followed by phenotypic and functional differentiation of cellular components and the subsequent remodeling of a mature ECM. The recent expansion of knowledge concerning the mechanisms responsible for the migration and proliferation of ECs and SMCs, angiogenesis, ECM deposition and remodeling, and physiologic parameters provides optimism for the possibility of manipulating the healing process through the directed manipulation of the microenvironment within the graft and perigraft tissue.

Since ECs have only limited capacity for regeneration, re-endothelialization of the relatively large surface areas encountered clinically exceeds the normal mitogenic and chemotactic capacity of surrounding ECs. Thus endothelialization of large surfaces requires the recruitment of ECs from sites beyond the anastomotic border via the circulation or through transinterstitial migration from the surrounding tissue and/or the vasa vasorum. This is possible under the direction of localized angiogenic stimuli, and to a limited degree this is what occurs *in vivo* as protease-driven ECM changes and local availability of growth factors, stimulate ECs, SMCs and fibroblasts to enter the cell cycle.

Platelet deposition and the perigraft inflammatory response lead to the release of many cytokines and proteolytic enzymes, which in turn incite secretion of a cascade of growth factors. Some of these growth factors are potent angiogens (e.g., vascular endothelial growth

factor (VEGF) and fibroblast growth factor (FGF)-family members) while others are angiogenic inhibitors (e.g., platelet factor 4, thrombospondin); still others (e.g., tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β)) exhibit both activities.

The local delivery of growth factors or selected ECM may be utilized to promote desirable cellular events, such as endothelial ingrowth of synthetic grafts. For example, local delivery of exogenous angiogenic factors through a biologic delivery system (e.g., fibrin) may induce transmural capillary ingrowth *in vivo*, which can be the source of cells for an endothelial lining within synthetic grafts. Bioresorbable grafts may also facilitate local angiogenic responses by stimulating macrophage release of angiogenic proteins. Fibroblast growth factors, notably FGF-1 (acidic FGF) and FGF-2 (basic FGF) have potent mitogenic, chemotactic, and angiogenic activity on vascular cells, but in order to deliver them locally to direct intimal regeneration, they require a delivery system that provides predictable local release with bioactivities preserved over a specific interval of time. Their ability to promote endothelial ingrowth has been tested experimentally after application to grafts. Our lab has evaluated the affixation of FGF-1 to synthetic surfaces. In early attempts, FGF-1 was applied to various synthetic grafts via a fibrin glue delivery system that, due to its structural orientation and state of polymerization, had been found not to be thrombogenic [22]. After delivering FGF-1 from fibrin glue to 60 μm IND ePTFE in both canine aorto-iliac and thoracoabdominal aortic models, there was a significant increase in luminal EC proliferation as assayed by *en face* autoradiography, and a more rapid development of a confluent Factor VIII positive endothelial blood contacting surface [23–24] (Fig. 39.2). There was also extensive transinterstitial capillary ingrowth observed throughout the graft wall. Cross-sectional autoradiography did find a significant increase in subendothelial myofibroblast proliferation in these treatment grafts at one month, but this returned to baseline at later time points. Still, treated grafts developed a significantly thicker pseudointima (139 μm ± 178 μm versus 93 μm ± 89 μm and 67 μm ± 151 μm) at 140 days. In order to limit this IH response, we have developed site-directed mutants based on the FGF-1 angiogen and bioactive

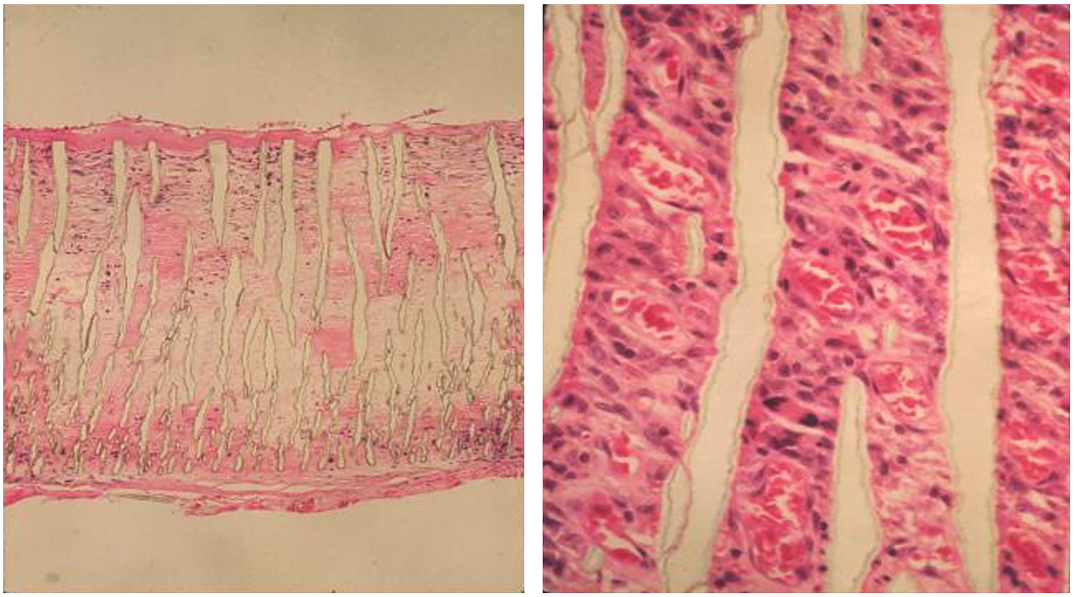

FIGURE 39.2

Transmural induction of endothelialization by FGF-1 on prosthetic graft. Fibrin glue delivery of exogenous FGF-1 promotes ePTFE endothelialization. The untreated control is pictured on the left side at 117x, and the FGF-1 treated graft is seen on the right at 486x. The treated graft demonstrates robust capillary ingrowth and cellular coverage not seen in the control graft. *Reprinted from the Journal of Surgical Research, Vol. 57; JL Gray, SS Kang, GC Zenni, DU Kim, PI Kim, WH Burgess, W Drohan, JA Winkles, CC Haudenschild, HP Greisler; FGF-1 affixation stimulates ePTFE endothelialization without intimal hyperplasia, pp. 600, 602; 1994, with permission from Elsevier.*

chimeric proteins that promote favorable characteristics including prolonged bioactivity, EC specificity, or increased potency, while removing unfavorable characteristics such as heparin-dependent activity and susceptibility to thrombin-induced proteolysis [25–26].

Co-immobilization of FGF-2 and heparin in a microporous polyurethane graft by crosslinked gelatin has also been demonstrated to accelerate tissue regeneration on synthetic grafts, associated with a greater extent of endothelialization via perianastomotic and transmural capillaries ingrowth, in a rat aortic grafting model [27]. A consistent 'neointima', approximately 40 m thick with intermittent endothelialization as well as SMCs and fibroblasts underneath the luminal surface were observed in the middle portion of treatment grafts, whereas the control grafts were only covered with a fibrin layer. However because of the cross-talk that exists in the vessel wall between endothelial cell (EC), smooth muscle cell (SMC), and fibroblasts, multimodal therapies that promote EC coverage while limiting activation of vascular smooth muscle cells (VSMCs) or the delayed delivery of cell cycle inhibitors may be required to optimize graft healing.

GENE THERAPY

Gene therapy shares the same promise as proteins, but it may also allow sustained or controlled protein expression in a desired location that is not possible with protein formulations. These qualities could obviate some of the current limitations encountered with the direct application of growth factor proteins to tissue beds for the regeneration of the endothelium.

This approach shows much promise as a delivery system, but single gene therapy trials have not yielded straightforward results. For example, although VEGF does improve endothelialization consistently, it does not reliably limit IH (and sometimes even promotes IH) in the literature. Gene therapy still requires cellular transduction or infection, and this has a variable effect on the cellular behavior. Also controversial results have been reported in the literature related to the proliferation, adhesion, and retention of genetically modified ECs on the surface of synthetic grafts. A major concern is that genetically modified ECs display poor retention on graft surfaces *in vivo*. This was demonstrated at six weeks in canine thoracoabdominal aortic ePTFE grafts seeded with lacZ-infected ECs compared to non-infected control ECs [28]. Further, Dunn [29] reported only 6% retention of ECs that had been retrovirally infected with thromboplastin on Dacron grafts after two hours of exposure to flow *in vivo*. For these reasons, little success has been documented to date concerning the long-term benefit of genetically modified EC seeded grafts *in vivo*.

CELLS

In addition to the emphasis on the endothelialization of the flow surface, the function of other cell types, particularly the SMCs, in the vascular wall have become better appreciated. It has been suggested that ECs by themselves cannot produce a stable intima without SMCs or fibroblasts underneath. In support of this contention, tissue fragments containing multiple cell types, including venous tissue, adipose tissue and bone marrow, have been seeded onto grafts and found to accelerate graft-healing processes. Interestingly, bone marrow cell seeding was also reported to induce an abundant capillary ingrowth in the graft wall and a rapid, complete endothelialization of the inner surface without intimal hyperplasia. Since the bone marrow stem cells have the ability to differentiate in response to their microenvironment and to proliferate as well as secrete cytokines critical to their survival, they may provide a useful cell source for blood vessel tissue engineering.

THERAPEUTIC ANGIOGENESIS AND ARTERIOGENESIS

Therapeutic angiogenesis

In addition to stimulating graft re-endothelialization via transmural angiogenesis, a number of cardiovascular pathologies and surgical interventions could also benefit from the ability to

therapeutically stimulate new blood vessel formation (neovascularization). Peripheral vascular disease, myocardial ischemia, wound healing, and tissue engineering are just a few of the many fields that may benefit from this approach.

GROWTH FACTOR THERAPY FOR ANGIOGENESIS

The process of neovascularization is controlled by a complex spatial and temporal expression of proteins, and to date most strategies have attempted to stimulate angiogenesis by injecting growth factors (as proteins) systemically or directly into target tissues. VEGF, FGF-1, and FGF-2 have been utilized frequently, reaching the point of clinical trials for the treatment of myocardial and/or peripheral limb ischemia. Many other growth factors also play a role in neovascularization and are under investigation in animal and *in vitro* models. The angiopoietins (Ang-1 and Ang-2), placental growth factor (PlGF), FGF-4, hepatocyte growth factor (HGF), ephrin-B2, and platelet derived growth factor BB (PDGF-BB) are a few of the many other proteins with the potential to be used for therapeutic neovascularization.

Due to short protein half-lives *in vivo* and their rapid diffusion out of target tissues, growth factor therapies require high initial concentrations, and repeat injections are required in order to achieve a noticeable response. Yet sustained protein levels are most likely needed in order to form a stable microvasculature. Since it is the local microenvironment concentration that determines the structure of the resultant microvasculature [30], these initially high concentrations can lead to demonstrable side effects, such as hyperpermeable or malformed vessels. Even sustained local concentrations may not elicit a sufficient angiogenic response because ischemic tissue may have an altered ability to respond to growth factors [31].

GENE THERAPY FOR ANGIOGENESIS

The genes of these same proteins have also been investigated as neovascularization therapies. As with gene delivery to vascular grafts, the goal of these approaches is typically local overexpression of the protein. Delivery of a single gene or protein has shown promise in animal models, but clinical results have fallen short of expectations. Overexpression of multiple proteins may have a synergistic effect on neovascularization. PlGF combined with VEGF has been shown to be significantly more potent than each factor alone in an animal model that was refractory to a single protein [32]. Combined delivery of adenovirus-mediated VEGF and Ang-1 has also been shown to promote greater perfusion and vessel stability in muscle flaps than VEGF alone [33]. Combined therapies may also be more effective in treatment of diseased or elderly patients who are known to have an impaired response to a single protein.

CELLULAR THERAPY FOR ANGIOGENESIS

Cellular therapies can also be used to enhance neovascularization. ECs injected into ischemic or engineered tissues are thought to be capable of mimicking vasculogenesis and to assemble into capillary structures, but may also act as sources of pro-angiogenic factors. Studies have shown that these cells can fuse with invading host vessels, recruit perivascular cells, and establish flow [34], and that support cells can prolong the existence of transplanted vessels [35]. Here ECs seeded alone into fibronectin-collagen type I gels and implanted into mice showed little perfusion and regressed from the gels after 60 days, while ECs combined with mesenchymal precursor cells resulted in the formation of vessels that established flow through connections with the mouse circulatory system. EPCs [36–37] may also be used to increase neovascularization. These cells may have increased proliferative capacity relative to mature ECs, and may be less sensitive to the short-term hypoxic conditions in tissues prior to establishing a blood flow. Injected EPCs selectively localize in ischemic tissues and may increase vascular density [36]. However, the mechanism for this increase is not clear. EPCs may develop

into new vascular structures or may increase neovascularization indirectly by recruiting monocytes/macrophages that then secrete angiogenic factors [37].

EPCs can also be genetically modified to enhance their therapeutic function. EPCs transfected to express VEGF stimulate a greater improvement in blood flow and angiogenesis in animal models of ischemia than EPCs alone [38]. While they have a longer life than fully differentiated cells, EPCs isolated from adults have reduced telomerase activity and regenerative capacity relative to embryonic stem cells. EPCs isolated from bone marrow and transfected to express telomerase reverse transcriptase (TERT) are more resistant to apoptosis and drastically increased neovascularization in an animal model of limb ischemia [39].

Modest but statistically significant improvements in the delivery of mesenchymal stromal cells to ischemic lower extremity and heart tissue have been demonstrated recently [40–41].

Arteriogenesis

Facilitating arterialization of capillary beds and expansion in size of smaller collateral arterial pathways (arteriogenesis) rather than formation of new capillary beds (angiogenesis) may be critical to facilitating adequate perfusion needed to overcome tissue ischemia. Recent work has shown that non-viral monocyte chemoattractant protein-1 (MCP-1) gene delivery increases arteriogenesis in a rabbit ischemic limb [42], and results from clinical trials suggest that VEGF gene therapy may also stimulate arteriogenesis. Less specifically, cell delivery can also enhance arteriogenesis.

Monocytes injected intravenously into rabbits following femoral artery ligation have been shown to home to sites of ischemia and stimulate arteriogenesis. By genetically modifying these monocytes with granulocyte-monocyte colony stimulating factor, collateralization was increased significantly [43]. Although promising, monocytes are also active participants in atherosclerosis, and it may be difficult to direct arteriogenesis without atherogenesis in these patients.

TISSUE-ENGINEERED VASCULAR GRAFTS

Driven by the desire to develop an ideal vascular substitute, the construction of tissue-engineered arterial grafts has been attempted with variable success. The potential benefits of tissue-engineered vascular grafts (TEVG) include the creation of a responsive and self-renewing tissue graft with functional intimal, medial, and adventitial layers (including both cellular and ECM components) that can be remodeled by the body according to its needs. Such grafts may improve graft durability and reduce the potential for graft infection by lessening the foreign body reaction and facilitating a more complete integration of the graft into the surrounding tissue.

In vitro TEVG

Weinberg and Bell were the first to develop a TEVG *in vitro* [44]. Using collagen and cultured bovine vascular cells, they demonstrated the feasibility of creating a TEVG, but their graft had prohibitively low burst pressures, hence requiring external Dacron for support. In the following decade L'Heureux et al. constructed a human blood vessel with an acceptable burst strength and a thromboresistant endothelium *in vitro* using cultured umbilical cord-derived human cells [45], but because of the immunogenic effects of the heterogeneic ECs *in vivo*, this graft devoid of ECs had only a 50% patency rate at eight weeks in a canine model. Since neonatal cells have a greater regenerative capacity, the above TEVG was not considered applicable to the aged population; that which would benefit most from a TEVG. Since these early efforts, considerable progress has been made.

ENDOTHELIAL CELL SEEDING

The prototype TEVG was created by seeding ECs onto ePTFE grafts *in vitro*, and then implanting them clinically. A confluent EC monolayer can prevent the development of myointimal hyperplasia by:

1) Preventing the deposition of platelets which release bioactive factors responsible for SMC migration, proliferation and production of ECM,
2) Maintaining a mechanical barrier to VSMC invasion via intimal basement membrane and the internal elastic lamina, and
3) Assuming a quiescent EC phenotype that does not stimulate SMC activity.

In 1978, Herring [46] first reported that endothelial cell seeding onto a graft surface enhanced graft survival in animal models. One of the early difficulties encountered was due to the relatively low cell density initially applied to the graft. Even though the cell density of the endothelial cell monolayer on a normal vein is approximately 10^3 ECs/mm^2, an initial density of at least 5 x 10^3 ECs/mm^2 is required for immediate confluent EC coverage of a small caliber vascular graft after exposure to the circulation. Studies on the kinetics of EC loss following seeding showed that between 20–70% of initially adherent cells are lost during the first hour and as few as 5% were retained after 24 hours [47]. Retained cells at least partially compensate for the cell loss by migration and proliferation. Preconditioning the seeded EC monolayer with graded shear stress promotes reorganization of the EC cytoskeleton and production of ECM, which in turn enhances the EC retention at flow exposure [48]. Additionally, Dacron and polyurethane have better cell attachment rates than ePTFE.

To maximize immediate cell inoculation density, a two stage seeding procedure is often performed in which endothelial cells are harvested, allowed to proliferate *in vitro*, and then seeded and grown to confluence on the vascular graft prior to implantation. The disadvantages of this technique include the increased potential for infection, the alterations of EC phenotype and function, the requirement of a 3–4 weeks waiting period for expansion of the cell population, and the necessity for two operative procedures. Zilla et al. demonstrated increased patency and decreased platelet deposition in clinically implanted endothelial cell-seeded (two stage approach) ePTFE femoropopliteal bypass grafts over three years as compared to unseeded grafts, and this group more recently reported an overall 7-year primary patency rate of 62.8% for 153 endothelialized femoro-popliteal ePTFE grafts [49]; this is comparable to the patency rate of saphenous vein grafts in this region. However, the seeded grafts have not been reproducibly shown to significantly reduce anastomotic pseudointimal hyperplasia.

There are also concerns about the ultimate function of those endothelial cells on the graft surface, the cells having been injured by the process of manipulation and/or exposure to a non-physiologic environment. Unlike their uninjured counterparts, injured endothelial cells produce a variety of procoagulants like von Willebrand Factor, plasminogen activator inhibitor, thrombospondin and collagen. Higher levels of PDGF and bFGF have also been measured in EC-seeded grafts; this is particularly concerning given their potential role in stimulating the migration and proliferation of SMCs, which can lead to IH.

ARTERIES ENGINEERED *IN VITRO*

The two main components of arterial generation *de novo* are the cells and their scaffolds. These components are not separable in practice, but they are separated in this chapter to emphasize their respective contributions.

There is much discussion about the proper cells to use for cell seeding *ex vivo* or cell homing *in vivo*. The EC is the most fastidious vascular cell to grow, and heterogenous ECs are highly immunogenic. Therefore in the absence of immunosuppression or genetic modification, autogenous ECs are a requirement for TEVG. TEVG media and adventitia can be created by using vascular SMCs or fibroblasts with or without exogenous matrix scaffolding. These cells

can be harvested from the patient in need, but since these patients are typically older with significant co-morbidities, their cells (particularly VSMC and ECs) may not retain sufficient doubling capacity to generate these TEVGs.

This may be due to the loss of telomere length that occurs with aging. Niklason and colleagues have demonstrated an increase in the population doublings of adult VSMCs through retroviral infection with the telomerase reverse transcriptase subunit (hTERT) without evidence of inducing cellular transformation [50]. However these arteries have a low bursting strength, which may be due to intrinsic and hTERT independent limitations of ECM protein production. More recently the Niklason laboratory has utilized multipotent cells to strengthen the matrix in these vessels and then decellularized them. As elastin formation in these vessels is incomplete, the decellularization process is much easier than that of healthy human arteries [51]. However elastin and elastic function of the artery is desired. Thus they are now evaluating ways of increasing elastin levels in TEVG [52].

Using a clinically relevant aged fibroblast-derived media, L'Heureux demonstrated a TEVG that has suitable bursting strengths at insertion, has a functional endothelium, and demonstrates mechanical stability in a variety of animal models out to eight months [53]. More recently this group has shown reasonable effective of this graft as a dialysis conduit [54–55].

Clinical success has been seen when using TEVG for vessel replacement in the venous system, which is potentially less challenging biomechanically. These TEVGs have been implanted in 42 pediatric patients with congenital heart defects. To date these grafts have been resistant to aneurysmal dilatation and had superb patency [56].

Various cellular and acellular approaches to TEVG scaffolds have also been pursued, and the challenge of the scaffold begins with providing satisfactory retention of cells *in vivo* or *ex vivo* in clinically relevant bioreactors and promoting optimal phenotypic characteristics of those cells. In addition, the scaffold or resulting vessel must have sufficient bursting strength to withstand the physiologic stresses of the circulation. After implantation this scaffold will undergo remodeling, and this can lead to aneurysmal dilatation and subsequent graft rupture. Niklason [57] reported encouraging results with a graft produced by seeding SMCs onto PGA scaffolds which were sodium hydroxide modified to promote cell attachment. This graft was cultured in an *in vitro* pulsatile radial stress environment for eight weeks prior to implantation. These grafts showed contractile responses to serotonin, endothelin-1, and prostaglandin $F_{2\alpha}$, and they expressed the SMC differentiation marker myosin heavy chain. Further, the grafts cultured under pulsatile conditions produced more collagen than those grown without pulsatile stress and exhibited a mechanical strength comparable to native human saphenous veins; this graft ruptured at 2150 ± 909 mmHg versus 1680 ± 307 mmHg for saphenous vein. Autologous ECs were seeded onto the luminal surface and were cultured for three more days before implantation. Four of the grafts were then implanted into swine saphenous arteries, of which two were generated under pulsatile stress and two under static conditions. Two pulsed grafts remained patent up to the fourth week without dilation or rupture while two non-pulsed grafts thrombosed after three weeks; the polymer remnants were no longer visible at four weeks.

Decellularized tissue scaffolds are appealing because they are already composed of native vascular ECM proteins that exhibit reasonable structural characteristics as well as providing instructive cues for cellular ingrowth. Using bone marrow derived cells incubated on decellularized canine carotid arteries, Byung-Soo Kim and coworkers demonstrated cellular incorporation into the scaffold and subsequent differentiation of these cells into endothelial and vascular SMCs and subsequently into three distinct vessel layers [58]. Others have used a more focused approach and induced or applied EPCs into similar scaffolds with promising results. The benefit of EPCs include a robust replication potential ideal for TEVG, and these cells acquire mature endothelial cell markers and function upon seeding into TEVGs [59]; these attributes may be further

augmented by gene therapy. Similarly adipose-derived stem cells have been used successfully as a cell source for TEVG by a number of investigators [60–61].

Using biologic gels, such as those composed of type I collagen or fibrin, one can promote tissue ingrowth and direct remodeling in a bioreactor, thereby promoting favorable characteristics such as improved mechanical strength or vessel reactivity over time [62]; such approaches are easily modified by the addition of growth factors with refined delivery systems in order to enhance and sustain cellular ingrowth [63]. Further refinement of these scaffolds can mimic the differential mechanical properties of the intimal and medial arterial layers. Syedian has recently reported development of TEVG from neonatal fibroblasts in a fibrin gel [64].

In vivo TEVG

Current clinically available synthetic vascular grafts, composed of ePTFE, Dacron, or polyurethane are permanent prostheses within the host after implantation. Theoretically, it is possible with bioresorbable materials to stimulate a rapid and controlled ingrowth of tissue to assume the load bearing sufficient for resistance to dilation, and to incorporate cellular and extracellular components with desirable physiologic characteristics to form a new artery *in vivo*, whereby the synthetic material itself would cease to be necessary following tissue ingrowth. Still, the limited regenerative capacity of aged or diseased cells discussed above may also compromise cellular ingrowth *in vivo*.

BIORESORBABLE GRAFTS

Aneurysmal dilatation of bioresorbable grafts can be expected to occur after sufficient degradation of the graft material and prior to adequate cell growth. The first published report of a fully bioresorbable graft was by Bowald in 1979 [65] and described the use of a rolled sheet of Vicryl (a copolymer of polyglycolide and polylactide). However, these early grafts were prone to aneurysmal dilation and rupture. We have reported that 10 % of woven PGA grafts have aneurysmal dilation within the first three months after implantation, and that this does not increase over the next nine months, suggesting that the critical time for the development of aneurysms is during prosthetic resorption prior to the ingrowth of tissue with a sufficient strength to resist hemodynamic pressures. These studies also demonstrated the ability of bioresorbable grafts to support sufficient cellular ingrowth. Here, four weeks after implantation, these 24 mm by 4 mm grafts contained an inner capsule with a confluent layer of endothelial cells and myofibroblasts amidst dense collagen fibers [66]. Similarly constructed and implanted Dacron grafts demonstrated an inner capsule composed solely of fibrin coagulum with minimal cellularity. Macrophage infiltration and phagocytosis paralleled the resorption of PGA, which was totally resorbed at three months.

In order to limit this catastrophic outcome, several approaches have been developed. One is to combine the bioresorbable material with a non-resorbable material in order to retain a mechanical strut. Another solution involves the combination of two or more bioresorbable materials with different resorption rates so that the more rapidly degraded material evokes a rapid tissue ingrowth while the second material provides temporary structural integrity to the graft. Thirdly, growth factors, chemoattractants, and/or cells can be applied to the graft to enhance tissue ingrowth, structure, and organization.

Using a more slowly resorbed compound, polydioxanone (PDS), we demonstrated 1/28 PDS grafts exhibited aneurysmal dilation with explant times as late as one year. The explanted specimens of these PDS grafts also demonstrated biomechanical characteristics similar to native arteries, being able to withstand static bursting pressures of 6000 mmHg and 2000 mmHg mean pulsatile pressure without fatigue [67].

Using the differentially resorbed approach with composite grafts woven from yarns of 74% PG910 and 26% PDS, we reported a one year patency rate of 100% with no aneurysms in the

rabbit aorta model. The PG910 was totally resorbed by two months and the PDS by six months. The regenerated arteries withstood 800 mmHg of pulsatile systolic pressure *ex vivo* without bursting, and a confluent, functional, von Willebrand Factor-positive endothelial cell layer over circumferentially oriented smooth muscle-like myofibroblasts formed in the inner capsule of both these grafts. Tissue ingrowth into all tested lactide/glycolide copolymeric grafts was observed to parallel the kinetics of macrophage phagocytosis and prosthetic resorption. *In vivo*, the rate of cell proliferation and collagen deposition in inner capsule also paralleled the kinetics of macrophage-mediated prosthetic resorption [68].

Partially resorbable grafts have also been investigated. Since Dacron was found to inhibit the macrophage-mediated arterial regeneration stimulated by the resorbable component PG910, polypropylene was evaluated as a non-resorbable component because of its high tensile strength, low fatigability, low degradation *in vivo*, and minimal inhibitory effect on cellular regeneration of grafts [69]. Composite grafts constructed from yarns containing 69% PG 910 and 31% polypropylene implanted into rabbit and dog arteries again demonstrated superb results without aneurysmal dilation. Galletti [70] used Vicryl (polyglactin 910) prostheses coated with retardant polyesters to temporarily protect the Vicryl from hydrolytic and cellular degradation. When implanted into the canine aorta, prosthetic resorption was noticed at four weeks and was complete by 24 weeks. In separate experiments, another group evaluated grafts prepared from a mixture of 95% polyurethane and 5% poly-lactide [71]. They found that only relatively compliant grafts that induced circumferential smooth muscle development contained elastin and remained mechanically stable without dilating. They concluded that modifications of the graft preparation including SMC seeding help to enhance the optimal orientation of the SMCs and prevent aneurysm formation.

THE LIVING BIOREACTOR

Campbell et al. have developed a modification of the Sparks' mandrel to create a TEVG. Here they utilize the abdominal peritoneum's reaction to foreign bodies as a living bioreactor [72]. After the graft has matured, it is removed from the mandrel and inverted; this creates a TEVG with a mesothelial inner lining. This graft's resistance to aneurysmal dilatation and rupture has not yet been proven.

CELLULAR RECRUITMENT

Since autogenous cells cannot populate a scaffold by migration or proliferation alone, they must be recruited internally via circulating ECs or EPCs or externally from the surrounding tissue or

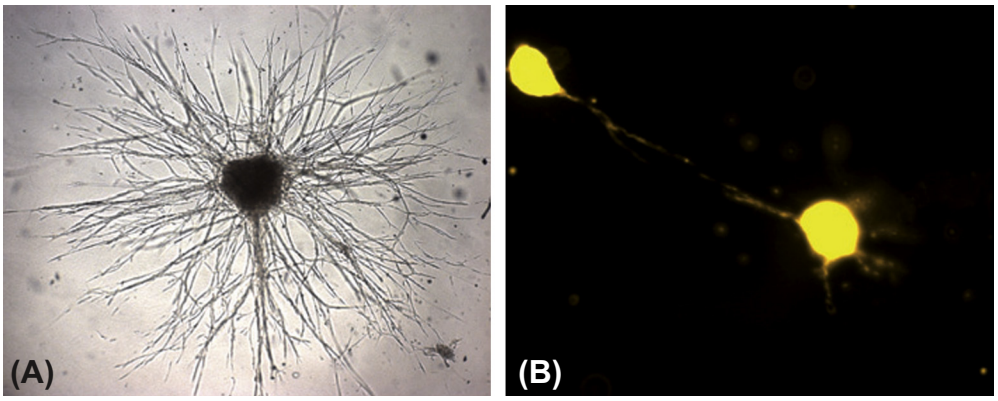

FIGURE 39.3
Three-dimensional *in vitro* induction of a capillary network. Robust capillary formation can be induced in ECs stimulated with angiogenic growth factors in a three-dimensional fibrin matrix. (A) Spherical induction of a radially oriented capillary network from an EC aggregate induced by sustained low-dose FGF-1 (1ng/mL). (B) When two EC aggregates are in proximity to each other, the EC sprouts are preferentially directed towards each other.

exogenous source through angiogenic ingrowth during tissue incorporation of the graft. In addition to the benefit provided by the localization of cells, transmural ingrowth also provides for cellular perfusion that extends beyond the distance supplied by simple diffusion (100 μm). We are currently designing three-dimensional capillary constructs (Fig. 39.3) that could provide grafts with the cellular and metabolic infrastructure requisite for the creation of a living TEVG through the induction of a *vaso vasorum* which can be incorporated into pre-existing capillary networks (inosculation). Proof of concept of this approach has been demonstrated in cardiac sheet grafts [73]. This induction can obviously be supplemented by the delivery of angiogenic proteins or genes to these constructs. With further research, a small diameter, totally resorbable vascular graft may be able to improve the current dismal long-term patency rates of small caliber grafts.

ENDOVASCULAR STENTS AND STENT GRAFTS

Endovascular stents were conceptualized prior to the introduction of angioplasty in 1969, and they have enjoyed increasing popularity in recent years. They have improved the durability of endovascular treatments and led a paradigm shift away from traditional operative approach to vascular disease. Angioplasty, stents and stent grafts are all endovascular interventions; they minimize the size of incisions, decrease lengths of hospital stays, and may confer some short-term survival benefit acutely after intervention. With over 1.5 million percutaneous coronary interventions occurring each year and a prevalence of in-stent stenosis ranging from 15—60% of patients, there is little wonder that much interest has been paid to the drug eluting stent (DES). But DES release broadly suppressive drugs to the surrounding tissue in order to limit neointimal stenosis, and they likely retard myointimal thickening rather than facilitate healing. This may be sufficient for short-term benefit, but may compromise the long-term durability of these vascular interventions. Long-term durability is likely to become more critical in the coming years because of the improved mortality rates in patients with cardiovascular disease. Thus it is critical to avoid complacency in clinical thinking, as what comprises a good result today is dependent on mortality rates that may not be applicable to today's patients. We firmly believe that promoting healing and not limiting the adverse effects of healing will provide for durable results.

Tissue-engineered cardiovascular stents

There are three basic types of stents: balloon expandable stents, which need balloon inflation to expand the stent into the arterial wall; self-expanding stents, allowing delivery in a collapsed form with the stent expanding to its predetermined size after release from the delivery device; and thermally expanding stents, made by shape memory metal alloys which exist in an easily manipulated form and which regain their memorized shape at a certain transition temperature. All these stents have been successfully used in iliac arteries with a two-year patency of approximately 84%. Improved endothelialization has also been reported using VEGF gene application to a modified metal stent [74], and although stenting itself has quickly become an important member of the endovascular armamentarium, the development of biodegradable stents has progressed slowly, mostly due to difficulties in replicating the properties of stainless steel stents [75]. Still the promise of cell-demanded sustained drug delivery in biodegradable stents has led to a renewed interest in this approach. Currently preliminary evidence supports the short-term stability of these stents and the ability of these stents to deliver bioactive agents to the vessel lumen, providing proof of concept for this approach.

Tissue-engineered stent grafts

Stent grafts are a collapsible hybrid product composed of either PET or ePTFE with stents providing for radial support; they are delivered intravascularly to patients' large vessels, in the same way as are cardiovascular stents. These stent grafts maintain flow through their lumen and are commonly used to exclude flow into aneurysmal portions of arteries. Theoretically, the

graft creates a barrier to exclude diseased arterial wall and provides a smooth flow conduit, while the stent support affixes the graft and may enhance luminal patency by resisting external compression. Stent grafts are delivered endovascularly, and since endovascular intervention reduces early operative morbidity and mortality, they have considerable consumer interest. However, the benefits of this approach over traditional operations is not clear, and it appears that outcomes are similar to that of the more traditional operative approach two years after intervention or operation.

The rapid improvements in stent grafts will likely lead to improved durability and decreased frequency of re-intervention. Therefore they will likely have a defined role in the treatment of patients with cardiovascular disease. For this reason it is important to define the known differences in healing between stent grafts and traditional bypass grafts.

Compliance changes between stent/unsupported graft/artery interfaces yield a remarkable hemodynamic disturbance. In addition, delivery procedures such as balloon dilatation may alter both graft intrinsic characteristics such as porosity, wall thickness, etc., as well as creating mechanical injury to the surrounding artery. In addition, unlike a conventional bypass, the endovascular graft is placed within the lumen of arteries with perigraft exposure to diseased arterial intima or to thrombus. All these factors change the healing characteristics of stent grafts compared to synthetic grafts. An inflammatory reaction and progressive thickening of neointima have been observed in both ePTFE and Dacron based stent grafts. When comparing endovascular grafts to conventional bypass grafting using a canine iliac artery model, endovascular stent grafts composed of ePTFE grafts and balloon expandable stents resulted in both a greater rate of endothelialization as well as an approximately five times thicker neointima in the mid-portion of the graft and a higher percentage of stenosis at the distal anastomosis when compared to conventional ePTFE grafts [76]. Pathological remodeling has also been reported after anchoring to the arterial wall, and it has been reported that stent placement may cause a variety of flow disturbances. The stent components themselves also stimulate a non-specific inflammatory reaction and induce neointimal formation, and tissue-engineered approaches including cell seeding with genetically modified cells may limit these complications [77–78]. The accumulation of experience, optimization of devices, and a better understanding of resultant pathological processes will likely allow the easy transfer of the recent advances in tissue-engineered grafts and vessels to stent grafts. This transfer is probably critical to enhancing their durability over time.

CONCLUSION

Much progress has been seen in recent years, and yet old things also become new again. Tissue engineering approaches to promoting graft healing continues to provide scientific successes and clinical limitations. As more patients live with cardiovascular disease than die from it, there is little doubt that promotion of graft healing will be more effective than inhibition of pathologic processes like IH. This creates a need for research investment, clinical trialists' audacity to apply promising ideas, and novel investigations to be tested to refine and improve these ideas.

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CHAPTER 40

Tissue-Engineering Heart Valves

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INTRODUCTION

Heart valve function and structure

The physiological function of a heart valve is to maintain unidirectional non-obstructed blood flow without damaging blood elements, causing thromboembolism, or placing excessive mechanical stress on the leaflets and cusps. The native heart valve is remarkably well adapted to performing these functions. This capability arises from a near perfect correlation of structure to function, enabling the valve to avoid excess stress on the cusps while simultaneously withstanding the wear and tear of 40 million repetitive deformations per year, equivalent to some 3 billion over a 75 year lifetime [1]. There are four valves in the human heart: two semilunar and two atrioventricular. The semilunar heart valves include the structurally similar aortic and pulmonary valves while the atrioventricular heart valves include the tricuspid and mitral valves. The following text describes the relation of structure to function in the semilunar valves from a macroscopic (tissue) to microscopic (cellular) level.

Grossly, the semilunar heart valves are composed of three thin cusps that open easily when exposed to the forward blood flow of ventricular systole, and then rapidly close under the minimal reverse flow of diastole [1]. The three cusps of the aortic valve are the left, right and non-coronary cusps. Each cusp is attached to the aortic wall by a thick base known as a commissure. Despite the force applied to the leaflets during diastole, prolapse is prevented by substantial coaptation of the cusps in a crescent shaped region of the cusp termed the lunula. In addition, the structural elements within the aortic valve cusps are anisotropically oriented in the tissue plane, resulting in disproportionate mechanical properties of the valve cusps with greater compliance in the radial rather than the circumferential direction. This compliance allows the cusp thickness of the aortic valve to vary from 300 to 700 μm throughout the course of the cardiac cycle [2]. Further structural specializations that occur are lengthwise folding of collagen fibers, and orientation of collagen bundles in the fibrous layer toward the commissures. This orientation conserves maximal coaptation, and thereby prevents regurgitation. Thus, both the macroscopic valve geometry and the fibrous network within the cusps work to transfer stresses caused by the diastolic force to the aortic wall and annulus.

Microscopically, the semilunar heart valve is composed of three layers: the ventricularis, spongiosa, and fibrosa (Fig. 40.1). It is the unique extracellular structural characteristics within these layers that create a specialized biomechanical profile necessary for proper function. The fibrosa, which is exposed to the aortic lumen, is composed of primarily collagen fibers, which

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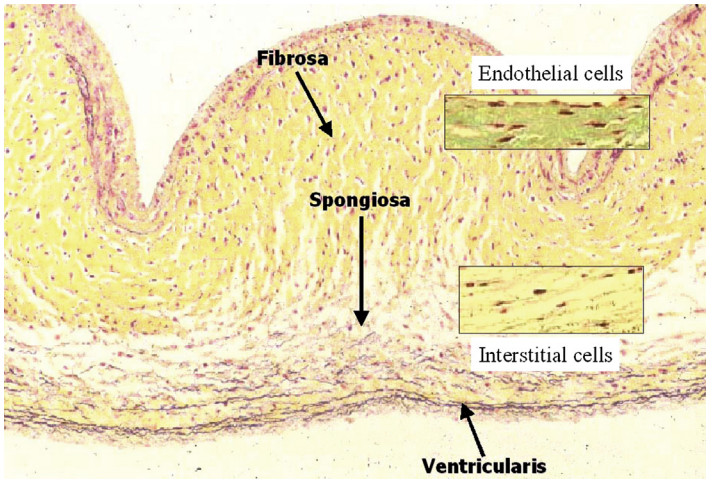

FIGURE 40.1
Histological cross section of a heart valve demonstrating the ventricularis, spongiosa and fibrosa. Also shown are interstitial cells and endothelial cells.

are densely packed and arranged parallel to the cuspal free edge. It is these collagen fibers, mostly type I and III, which provide most of the mechanical strength of the valve [3]. The ventricularis layer faces the ventricle and is composed of collagen and radially aligned elastin fibers. Elastin forms an encompassing matrix that binds the collagen fibrous bundles throughout the heart valve, thereby creating an elastin-collagen hybrid network of interconnected collagen fibers that provides greater mechanical strength [4]. The centrally located spongiosa is composed of glycosaminoglycans (GAG) and loose collagen fibers. The GAG side-chains of proteoglycans make a gelatinous substance in which other matrix molecules are able to form covalent crosslinks that support other components of the ECM [5]. Human heart valve GAGs are composed predominantly of hyaluronic acid, with smaller amounts of dermatan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate [6].

Cellular biology of the heart valve

VALVULAR ENDOTHELIAL CELLS

The two most prevalent cell types in heart valves are valvular endothelial cells (VECs) and valvular interstitial cells (VICs). VECs line the surface of valve cusps on both the aortic and ventricular surfaces. They provide a non-thrombogenic blood-tissue interface and maintain a semipermeable membrane that regulates the transfer of large and small molecules through the vascular wall [2]. Endothelial cells are also critical in the control of inflammatory and immune reactions, in controlling the proliferation of other cell types, and play a role in metabolism and protein synthesis [7].

The role of VECs in the heart valve is not passive. VECs can respond to external stimuli by phenotypically changing and acquiring new, inducible properties through a process known as endothelial activation. Both cytokines and hemodynamic forces have been shown to induce this process [2]. Once activated, VECs produce cytokines, chemokines, adhesion molecules, growth factors, and vasoactive molecules, all of which contribute to valve adaptation in response to the initial stimuli. Most often, this process is beneficial and allows for maintenance of valve homeostasis. However, a form of endothelial activation can occur that is known as endothelial dysfunction, which results in a surface that is adhesive to inflammatory cells or thrombogenic and can be a source of valve pathology [2].

Although VECs are often grouped together, there is in fact heterogeneity among these cells, as there is among endothelial cells in general. Their source, be it arterial or venous tissue, can affect cell phenotype, as can their location (aortic or pulmonary) [8]. Moreover, there is an increasing appreciation for the substantial data suggesting that VECs differ in many ways from

other types of endothelial cells. For example, in response to mechanical stress, VECs align perpendicularly to blood flow, whereas vascular endothelial cells in the aorta align in parallel with flow [9]. In a separate study, investigators demonstrated significantly different transcriptional gene profiles of VECs on aortic and ventricular sides of porcine aortic valves [10].

VALVULAR INTERSTITIAL CELLS

VICs are the most abundant cell type in heart valves and are primarily responsible for heart valve stability by the synthesis and remodeling of valve extracellular matrix (ECM). This process maintains the appropriate structural and functional relationship that allow for long-term heart valve competence. VICs contain varying characteristics of myofibroblasts, fibroblasts, and smooth muscle cells. There are five subtypes of VICs: embryonic, progenitor, quiescent, activated, and osteoblastic. Each subtype is unique and VICs can change phenotypically from one to another depending on the mechanical environment, chemical cellular or hormonal signals, or in response to injury.

While five subtypes do exist, the two most important distinct populations of VICs are those that are quiescent and those that are activated [5]. In the quiescent state, VICs most closely resemble fibroblasts in that they have low levels of α-smooth muscle actin [11]. Conversely, VICs are activated by a variety of different stimuli and conditions, including during embryonic valve development and with exposure to TGF-β or cyclic stretch. Additionally, various valvular pathologies can induce VIC activation. Importantly, studies have shown a distinct transition from quiescent to active VICs after implantation of a tissue-engineered heart valve. Similar results were seen after implantation of a pulmonary autograft as an aortic valve replacement [12].

VICs are of central importance to heart valve repair and maintenance operations. The constant and rigorous mechanical movement of valves results in persistent low level valvular damage and an ongoing extracellular matrix synthesis, remodeling, and degradation process. VICs regulate and perform this process by expressing ECM components and the proteins involved in matrix remodeling like matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Importantly, over-expression of MMPs has been observed in several heart valve pathologies, indicating a possible target for therapeutic intervention [13]. More investigation is needed to better understand the mechanism of action of MMPs and TIMPs and their importance in valve tissue morphogenesis, repair and remodeling.

Even though ample data exists regarding the reparative properties of VICs, their function is still not fully understood [5]. Important to understanding and potentially manipulating VIC phenotype and cellular activity is knowing how these cells send and receive signals, how mechanical information is transmitted from the ECM, and what specific factors can induce a variety of cellular responses like cell migration, adhesion, growth and differentiation [14].

Heart valve dysfunction and valvular repair and remodeling

HEART VALVE DYSFUNCTION

The American Heart Association has estimated that over 300,000 heart valve replacement procedures were performed in the United States between 1998 and 2005 [15]. Population growth, population aging, and improvements in longevity will fuel demand for heart valve replacements in the future, with a projected need of 850,000 in year 2050 [16]. The pathology of heart valve disease manifests itself in two ways, the first being a reduction in forward flow caused by failure of a valve to completely open, known as stenosis. The second is back flow during diastole caused by failure of a valve to completely close, known as insufficiency or regurgitation.

Aortic stenosis is a common valvular pathology. When severe, this disease can cause dyspnea on exertion, chest pain, and syncope. The most frequent cause of aortic stenosis is dystrophic calcification of the aortic valve cusps and ring (annulus). Overall prevalence of aortic stenosis

is 2% and is increasing with time as the American population ages. Bicuspid aortic valves are another cause of aortic stenosis, and represent one of the most frequent congenital cardiovascular malformations in humans. Bicuspid aortic valves are also sometimes associated with aortic insufficiency and increase the risk of infective endocarditis. The main source of chronic aortic insufficiency is aortic root dilation. This results in distended and outwardly bowed commissures as well as impaired cuspal coaptation.

Mitral valve prolapse is defined as the displacement of valve leaflets into the left atrium during systole and is the most common indication for surgical repair or replacement of this valve. Mitral valve prolapse is most commonly caused by myxomatous degeneration, and is also seen in patients with Marfan syndrome. Patients with mitral valve prolapse are exposed to increased risk of a variety of different complications including heart failure, bacterial endocarditis, mitral regurgitation, atrial fibrillation, and thromboembolic events [7]. The most common cause of mitral stenosis worldwide is history of rheumatic fever [2].

The leading cause of dysfunction of the tricuspid and pulmonary valves is congenital heart disease. Approximately 20,000 infants are born in the United States each year with a congenital heart defect, of which many involve absence or malformation of the pulmonary valve and pulmonary artery [17]. Major congenital heart disease diagnoses of the right ventricular outflow tract (which includes the pulmonary valve and pulmonary artery) include truncus arteriosus, pulmonary atresia with ventricular septal defect, severe tetralogy of Fallot, transposition with ventricular septal defect and pulmonary atresia, and double-outlet right ventricle. It is these children who would benefit most from tissue-engineered heart valves, which offer the potential to grow with the child thereby preventing need for re-operation.

VALVULAR REPAIR AND REMODELING

When valvular injury occurs, it stimulates VIC proliferation, migration, and apoptosis. The initial events in valve repair of a linear superficial denuding wound have been modeled *in vitro* and are characterized by prominent migration and proliferation of VICs [18]. The migration of VICs is governed by a sequence of processes including activation of integrins, which are cell surface heterodimeric receptors that control cell-ECM and cell-cell adhesion [19]. Changes in macroscopic mechanical stimuli like shear and solid stresses are also propagated via signal transduction through a complex cell-ECM network, which allows small external changes in valvular mechanics to ultimately affect cellular activity and function [7].

Activation of VICs results in a change of phenotypic subtype to activated myofibroblasts, as demonstrated in leaflets from patients with myxomatous mitral valve degeneration [13]. These phenotypic changes resemble the evolution of physiological wound healing in mitral valves associated with the phenotypic modulation of interstitial valvular cells from fibroblasts to myofibroblasts [2]. That said, when equilibrium is reached after injury, cells revert back to a quiescent phenotype like that of a fibroblast. However, in cases in which there is persistent injury or where a steady state equilibrium cannot be achieved, VICs will remain persistently activated, contributing to ECM remodeling and initiation of valvular pathologies [20].

Important to understanding VIC function and activation in response to injury is the interaction between VICs and the ECM. VICs takes cues from and act upon ECM by secreting ECM components and their proteolytic enzymes, and by promoting avenues of cellular migration. Interstitial collagenases, gelatinases and other MMPs are involved in the degradation and remodeling of connective tissue and are primarily secreted by activated VICs [21]. As in tissue throughout the body, MMPs in heart valves are critical in tissue morphogenesis, wound healing, and other tissue remodeling processes. The interaction between MMPs, their inhibitors (TIMPs), and their regulators are particularly important in cardiac and vascular remodeling [2]. In the degradation of ECM, interstitial collagenases MMP-1 and MMP-13 mediate the preliminary phase of collagen breakdown by disassembling the native helix of the

fibrillar collagen. The resulting collagen fragments are then accessible to further proteases, like gelatinases [22]. These interstitial collagenases are secreted by activated VICs in a similar manner as they are secreted by inflammatory cells in a multitude of systemic diseases [2]. That is, VICs are being stimulated by signal transduction, be it mechanical, chemical, hormonal, or otherwise, to produce and secrete soluble ECM proteases that allows for remodeling of ECM in response to changes in external environment. One means of signal transduction is cardiac catabolic factor, which is derived from porcine heart valves and found to stimulate collagen and proteoglycan breakdown *in vitro* [23]. While MMPs are a necessary component to normal remodeling of heart valves, excessive levels of MMP activity can lead to excess collagen and elastin breakdown, thereby weakening heart valve leaflets and predisposing to disease [13].

Heart valve replacement

Standard treatment for end-stage valvular dysfunction is heart valve replacement. The first successful implantation of a human valve was performed in 1952 [24]. Since then, more than 80 different designs of prosthetic heart valves have been developed [25]. Heart valve substitutes have undergone a progressive evolution, as newer models are developed to remedy the deficiencies of older devices. Prosthetic heart valves are either mechanical and composed entirely of synthetic material, or bioprosthetic and therefore fashioned from biological components. Slightly more than half of the world's implanted valves are mechanical, while the remainder are bioprosthetic [26]. While each type of heart valve is used successfully to improve the quality and length of life, each valve type also has its own unique set of problems [27].

The overall rate of complications is similar for mechanical prostheses and bioprostheses [28]. Four categories of valve-related complications predominate:

1) Thromboembolism, thrombosis, and secondary anticoagulation-related hemorrhage;
2) Prosthetic valve endocarditis;
3) Structural dysfunction including failure or degeneration of the prosthetic biomaterials; and
4) Non-structural dysfunction including complications arising from technical problems during surgical implantation such as perivalvular leak and biological integration (tissue overgrowth) [1].

Each valve type is associated with its own unique set of advantages and disadvantages.

The mechanical heart valve is characterized as having excellent durability due to the mechanical properties of the synthetic materials from which it is constructed. Unfortunately, these synthetic materials also give rise to poor biocompatibility. Specifically, mechanical prosthetic valves are associated with a substantial risk of thromboembolism and thrombotic occlusion caused by the lack of an endothelial lining and the flow abnormalities that result from a rigid outflow structure [29]. To minimize this risk, chronic anticoagulation therapy is required for all mechanical valve recipients. However, systemic anticoagulation renders patients vulnerable to potentially serious hemorrhagic complications. Thus, the combined risk of thromboembolic complications and hemorrhage secondary to anticoagulation constitute the principal disadvantage of mechanical prosthetic valves. A meta-analysis found an incidence of major embolism in the absence of antithrombotic therapy of 4 per 100 patient years, which was decreased to 2.2 per 100 patient years with anti-platelet therapy and to 1 per 100 patient years with anticoagulant therapy (e.g., warfarin) [30]. Disadvantages of long-term warfarin use include increased risk of bleeding complications, lifetime need for blood tests and therapy to maintain a therapeutic international normalized ratio (INR), and high rates of non-compliance [31]. In a review of randomized trials of anticoagulation therapy in patients 65 years and older, incidence of major bleeding ranged from 0% to 4.6% per year, with incidence of minor bleeding as high as 10.5% per year [32]. Indeed, patients over the age of 75 on anticoagulation for mechanical heart valves compared to patients who received a bioprosthetic valve assessed for bleeding rates had an odds ratio of 18.9 [33]. Other disadvantages of

mechanical heart valves include their ability to cause hemolysis and an increased susceptibility to endocarditis [34].

Bioprosthetic valve replacements such as glutaraldehyde-fixed xenografts and allografts are associated with a lower risk of thrombosis and hemolysis than mechanical heart valves [27]. Patients with glutaraldehyde-fixed xenograft valves do not require anticoagulation and therefore do not incur the risks of anticoagulation-associated bleeding. However, because of their mechanical properties and their composition of biologic material, the durability of a glutaraldehyde-fixed valve is more limited than that of the mechanical valve. The major disadvantage of tissue valves is progressive structural deterioration that eventually results in stenosis and/or regurgitation. The degradation mechanisms of bioprosthetic valves are progressive and the rate of failure is highly time dependent.

Alterations of their molecular composition and/or tissue structure of bioprosthetic valves during manufacturing can lead to valve failure [1]. One such example is the permanent fixture of one or more valve cusps in a specific configuration during manufacture which is associated with only one specific phase of the cardiac cycle [2]. In this case, normal cyclic rearrangements in the valve sub-architecture cannot appropriately occur and irregular tissue stress is created. Other examples include process-induced destruction to the endothelial coating which then allows penetration of inflammatory cells and plasma into the cusp after implantation, leading to valve inflammation-induced thickening and possible valve deterioration [2].

The principal problems for bioprosthetic valve durability after implantation are cuspal mineralization and non-calcific mechanical fatigue. Calcification occurs when calcium from plasma binds with residual organic phosphates of the crosslinked, non-viable cells of the preserved valve [7]. Further weakening can occur from proteolytic degradation of the collagenous ECM [35]. MMP activity has been demonstrated in explanted valve tissue that has undergone structural degradation, which signifies a degree of inflammation and ECM remodeling within damaged valves [36]. Similar findings have been found in *in vitro* models of valve function, suggesting that the mechanical stress that bioprosthetic valves are subject to may alone contribute to ECM degradation and prolonged remodeling [37].

Despite improvements over the last several years in bioprosthetic valve durability, structural valve deterioration is a significant problem particularly in patients less than 65 years old. In one study looking at patients having had a bioprosthetic aortic valve replacement, 60% of patients younger than 65 years required re-operation at 18 years post-implantation [38]. Indeed, the risk of structural failure is strongly age dependent, with individuals less than 35 years of age, and especially children and adolescents, having the highest rate. Nearly uniform failure occurs by 5 years in those less than 35 years old but 8–27% fail in 20 years in those older than 65 [39]. The major cause of bioprosthetic valve dysfunction is structural deterioration of the cuspal tissue [40]. Two distinct yet potentially synergistic processes are causal: [1] calcific degradation and [2] non-calcific degradation. Both eventually lead to failure of the connective tissue matrix of the tissue valve [1].

Another type of bioprosthetic valve is the cryopreserved homograft, which is particularly advantageous in those patients requiring aortic valve replacement or those needing congenital heart reconstruction with right-sided conduits [41]. However, the use of the cryopreserved homograft is a form of transplantation and thus subject to many transplant-associated problems, including the implanted tissue invoking a host immune response, thereby potentially increasing the risk and rapidity of structural valve deterioration. Cryopreserved homografts are currently the most biocompatible replacement heart valve and the treatment of choice in most pediatric cardiothoracic applications [42]. However, used in this application, they are severely limited by their inability to grow and by a significant incidence of structural deterioration resulting in limited durability and frequent need for re-operation. Overall, the poor long-term durability of currently available heart valves in young patients (children and

adults <35 years) makes clear the clinical need for an improved replacement heart valve, one which tissue engineering can help fulfill.

THE APPLICATION OF TISSUE ENGINEERING TOWARDS THE CONSTRUCTION OF A REPLACEMENT HEART VALVE

Tissue-engineering theory

The ideal heart valve replacement would be perfectly biocompatible, readily available, durable, and have the potential for growth. The construction of an autologous, tissue-engineered heart valve could potentially fulfill all of these requirements by utilizing natural mechanisms for repair, remodeling, and regeneration. The central paradigm underlying tissue engineering involves combining cells with a platform matrix to create neotissue [43]. The matrix acts as a three dimensional scaffold until proliferating cells produce sufficient ECM *in vitro* to permit *in vivo* implantation [2]. This process is followed by scaffold degradation, neotissue formation, and growth.

Tissue engineering is currently limited by the inability to construct microvasculature *de novo*, the inability to control innervation of neotissue, and the difficulties surrounding culturing certain cell types [44]. Although the semilunar heart valve is not totally avascular, oxygen and nutrients needed to sustain its function are supplied via two complementary pathways: diffusion from the blood stream and via a capillary network. Additionally, semilunar heart valves are composed of cells that are readily grown in culture. This allows for the isolation and expansion of autologous cells for construction of tissue-engineered heart valves. The blueprint for constructing an ideal tissue-engineered heart valve (TEHV) has evolved from a large body of research in bioprosthetic valves, diseased heart valves, and other tissue valve substitutes. Although these investigations have largely been clinical in nature, they have identified useful markers of cell function, matrix physiology, and matrix structure. This was summarized by five key concepts of functionally adaptive valvular remodeling/regeneration:

1) The highly specialized arrangement of collagen and other ECM;
2) Structural deterioration of native and substitute valves;
3) The quality of valvular ECM depends on valvular interstitial cell viability, function, and ability to adapt to different environments;
4) Cell viability in nearly all current bioprosthetic tissue valve substitutes is compromised or completely eliminated during processing;
5) The long-term success of a tissue-engineered valve replacement will, therefore, depend on the ability of its living cellular components to assume normal function with the capacity to repair structural injury, remodel the ECM, and potentially grow [2].

Tissue engineering offers the potential to create a non-thrombogenic, biomimetic, immunologically compatible tissue valve substitute that is capable of providing ongoing remodeling and repair which would allow growth in maturing recipients (Fig. 40.2) [2]. Such a technology would have dramatic implications in a clinical setting and greatly contribute to improving patient outcomes.

Biomaterials and scaffolds

Over the last several decades, an exhaustive variety of biomaterials have been explored to serve as scaffolds for tissue-engineered heart valves. These biologic scaffolds have the primary responsibility of promoting tissue regeneration and must be biocompatible, biodegradable into safe byproducts, easily manufactured and handled, highly porous to facilitate cell attachment, and yet mechanically stable enough to appropriate function [2].

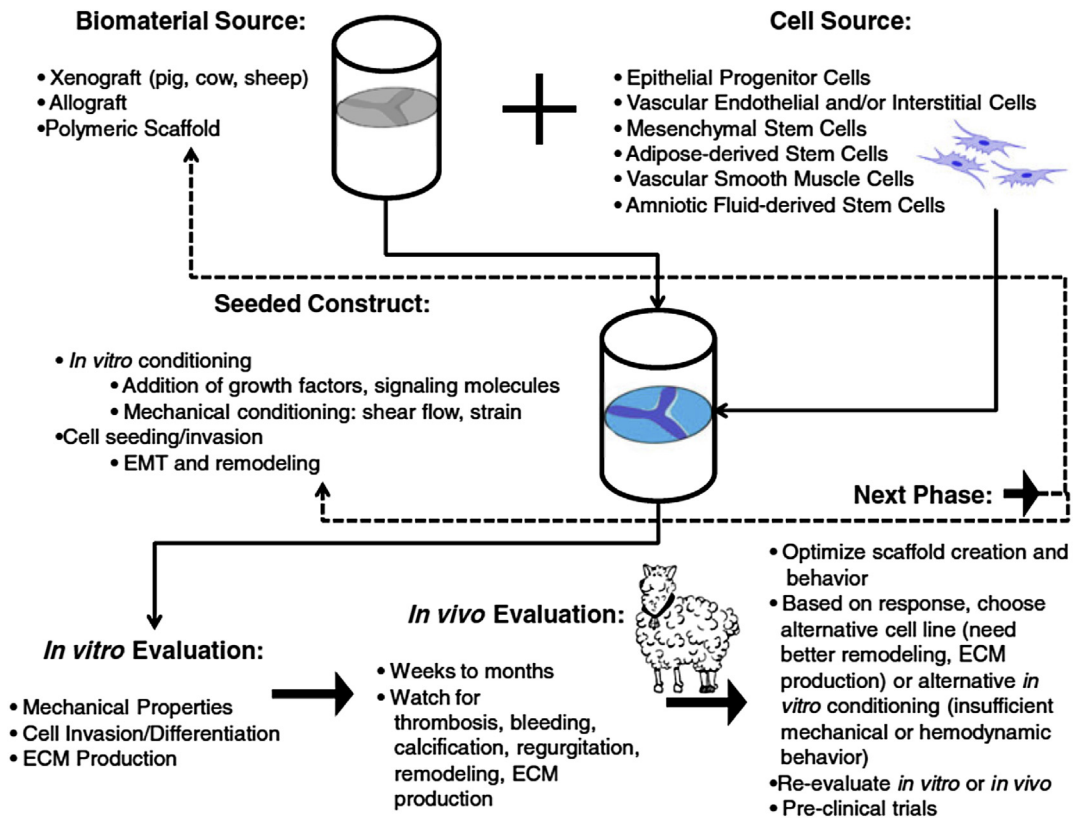

FIGURE 40.2
Overview of tissue engineering heart valves [46].

Scaffolds can be manufactured from either synthetic or natural materials. Natural biomaterials include ECM components such as collagen, fibrin, elastin, glycosamino-glycans, or decellularized tissues, such as heart valve, pericardium, arterial wall or small intestinal submucosa [45]. Synthetic polymers have the advantage in that they have predictable chemistry and their properties can be well-controlled [2]. Additionally, these materials are FDA (Food and Drug Administration) regulated and many have been approved in clinical applications [46]. The most commonly utilized synthetic polymers in tissue engineering include poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), copolymer poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), and polyhydroxyalkanoate (PHA).

A number of natural biodegradable polymers have been explored for use as scaffolds. They have the advantage of offering a more biocompatible template on which cells grow [5]. Decellularized small intestinal submucosal (SIS) matrix has been isolated to use in tissue engineering [47]. Other investigators have used porcine SIS scaffold as a resorbable matrix to make pulmonary valve leaflet replacement in porcine models [48]. When the explanted constructs were evaluated they revealed resorption of the submucosal matrix, fibrous connective tissue growth and formation of neo-vasculature.

Much of a heart valve's mechanical and tensile strength originates from its collagen composition and alignment [49]. Due to their highly conserved genetic code across species, collagens are weakly immunogenic and have been used widely for tissue-engineered heart valve constructs seeded with both human and porcine-derived cells [50,51]. Another natural scaffold that has been explored is fibrin gel. It has been used as an autogeneic scaffold in heart valve tissue engineering [52]. Some advantages of using fibrin gel as a material for scaffold construction include that it is made from the patient's own blood, and also that it is readily

biodegradable. However, early attempts at utilizing fibrin gel were limited by its tendency to shrink and its inability to withstand surgical implantation [5].

The process of removing cells from a biological matrix can reduce the immune response to the bioprosthetic while maintaining the potential for endothelialization by host VECs and VICs, either through cell ingrowth or via cell seeding [53]. Methods of tissue decellularization range from enzymatic digestion, detergent treatment, sonication and hypo-/hypertonic immersion [54]. These treatments effectively remove antigenic components such as nucleic acids, cell membranes, cytoplasmic structures, lipids, and soluble matrix molecules but retain elastin, collagen and GAG components of the scaffold ECM [55]. Decellularization has been utilized in porcine matrices that were subsequently seeded with human endothelial cells. The resulting construct had a confluent and viable monolayer cell surface, an important feature in reducing thrombogenic risk [56]. Further experimentation *in vivo* seeded a decellularized pulmonary valve with endothelial cells and carotid artery myofibroblasts followed by implantation in an ovine model [57]. After one month, the valve leaflets were completely endothelialized with dense infiltration by myofibroblasts by three months. However, enthusiasm was tempered by the finding of subvalvular calcification and inflammation resulting in increased thickening of the valve leaflets [5].

The commercially available SynerGraft valve (CryoLife Inc., USA) is based on the decellularized model of tissue engineering [58]. The SynerGraft decellularization process involves cell lysis in sterile water, enzymatic digestion of nucleic acids and a multi-day isotonic washout period. The method was developed as an alternative to glutaraldehyde crosslinking to decrease xenograft antigenicity. Histological evaluation 150 days post-implantation in porcine models showed intact leaflets with local myofibroblasts ingrowth and no calcification [5]. Early failure of the valve, however, was reported in human trials [59]. The SynerGraft matrix initiated an early strong inflammatory response followed by a lymphocyte response. Rapid structural degeneration of the valves occurred within 12 months. Furthermore, the valve scaffold was not repopulated with host cells, and calcific deposits were detected.

Proponents of synthetic matrices argue that customized polymers can be designed to exact specifications in a reproducible fashion. Also, by utilizing biodegradable polymers, complications associated with biocompatibility can be minimized. Initial work focused on polymers composed of polyglycolic (PGA) and polylactic acid (PLA) [60]. These 'off the shelf' polymers were selected because they are biocompatible, biodegradable, well characterized, and already FDA approved for human implantation. Cells readily attach to and grow on these synthetic polymers. The resulting neotissue construct possesses adequate biomechanical properties to be used for surgical application. However, the biomechanical profile of the construct is substantially different from that of a native heart valve. Tissue-engineered heart valves using polyglycolic acid and polylactic acid copolymer-based matrices are thicker, stiffer, and less pliable than native valves.

Early experiments used matrices made of a PLGA woven mesh sandwiched between non-woven PGA mesh sheets. The scaffolds were seeded with arterial myofibroblasts, followed by arterial endothelial cells and were then transplanted into the pulmonary position as a single leaflet in sheep. The seeded cells were seen in the structure after six weeks, and a post-mortem evaluation revealed a native tissue-like architecture [61]. Furthermore, there was confirmation of elastin and collagen production in the leaflets, which had mechanical properties similar to native leaflets [61]. This approach, however, resulted in only limited success, as the PGA and PLA polymers were too stiff for use as flexible trileaflet valves.

Polyhydroxyoctanoate (PHO), a more flexible synthetic polymer has been used to create a composite heart valve scaffold in a low-pressure pulmonary system [62]. The valve consisted of a layer of PHO film sandwiched between two layers of non-woven PGA felt. The leaflets were composed of a monolayer of porous PHO that was sutured to the conduit wall with

polydioxanone. Post-implantation examination revealed a uniformly organized tissue with large amounts of collagen and proteoglycans, but with no elastin. The PHO scaffold, however, had not completely degraded after 24 weeks, suggesting a longer degradation profile than glycolic or lactic acid polymers. Additional studies revealed smooth flow surfaces but could not demonstrate a confluent endothelium which could affect the long-term durability of the structures [63]. Additionally, although collagen and GAG deposition was seen, the constructs were devoid of elastin and demonstrated mild stenosis and regurgitation.

Hoerstrup and colleagues developed a novel composite scaffold material consisting of PGA coated with a thin layer of poly-4-hydroxybutyrate (P4HB) — a flexible, thermoplastic polymer with a more rapid degradation time than PHO [64]. Trileaflet heart valve scaffolds were fabricated from the composite material using a heat-application welding technique. Autologous myofibroblasts and endothelial cells from ovine carotid artery were seeded onto the scaffolds, cultured in a bioreactor for 14 days and then implanted in an ovine model [65]. When explanted, they showed increased ECM synthesis, more organized internal structure and improved mechanical properties over static controls.

It is not clear whether natural or synthetic scaffolds will ultimately prove to be more appropriate for tissue-engineered heart valve development, as there are still certain limitations associated with both. The design of a replacement aortic valved-conduit, for example, will require the inclusion of three dilated pouches alongside the 'cusp-like' leaflets to approximate the sinuses of Valsalva; atrioventricular (AV) valve designs may require the addition of other components of the AV valve apparatus, including chordae tendineae and papillary muscles [66]. Using stereolithography, Sodian and colleagues fabricated plastic models with an exact spatial representation of human aortic and pulmonary valves [67]. These models were then used to fabricate heart valve cell scaffolds using poly-3-hydroxyoctanoate-co-3-hydroxyhexanoate (PHOH) and P4HB, which were shown to function well in a pulsatile flow bioreactor under both normal and supranormal flow and pressure conditions. The choice and design of scaffolds will be integral to the successful clinical outcome of tissue-engineered heart valves. As well as conforming to a suitable anatomical shape, heart valve scaffolds will be required to possess both tensile and elastic properties. The scaffolds must be biocompatible, bioabsorbable or remodelable, and should provide a suitable template for facilitating development of new tissue. Scaffold permeability is also vital for the control of cell nutrition and removal of waste products [5].

The search for appropriate cell sources

An ideal cell source for tissue-engineered heart valves would demonstrate phenotypic plasticity and be able to change biomechanical properties in response to dynamic alterations in flow. It is an area of continued investigation. The use of autologous cells as opposed to xenograft or allograft tissue has the advantage of avoiding an immunological response that could result in rejection [68]. In an early attempt at tissue engineering a heart valve in 1995, valve leaflets were constructed by seeding cells from both autogeneic and allogeneic sources onto biodegradable polymeric scaffolds which were then implanted in an ovine model [61]. As anticipated, the autologous scaffold provoked less of an inflammatory response in the host, resulting in better performance and a higher success rate compared to allogeneic comparators.

A logical cell source for a tissue-engineered heart valve would be autologous VICs and VECs harvested from a patient's own heart valve leaflet. The use of these cells would eliminate the risk of rejection while imparting the requisite phenotypic profile [5]. Preliminary studies using an ovine model utilized valve biopsy samples as a VIC source [69]. In this study, the biopsy procedure generally did not appear to compromise leaflet function, with 9 of 13 animals showing intact valves and normal leaflet anatomy post-mortem. However, for human patients, it would be challenging to isolate and culture enough cells from a small biopsy to be of clinical use. It is also known that the ability to culture cells decreases with age, making this technique

less feasible in older patients. Additionally, patients requiring valve replacement have diseased VECs and VICs which may not be ideal for tissue engineering a replacement heart valve. Thus, the risks involved in valve biopsy are seemingly too high to make this a feasible technique in human trials. Recent studies have shown that decellularized human pulmonary valves seeded with autologous VICs and cultured *in vitro* demonstrated significant cell proliferation by four days, which suggests that such a construct can be manufactured in a shorter timeframe than initially thought [70].

Subsequent studies have utilized ovine femoral artery-derived cells as an autologous source [60]. This has failed to gain much favor given its potential for limb ischemia from disruption of the lower limb arterial blood supply. Myofibroblasts harvested from the carotid artery have also been investigated, but it was concluded by the investigators that the sacrifice of an intact tissue structure and the potential for injury introduced prohibitive risks [62]. In search of a more practical source, Shinoka et al. performed a study comparing dermal fibroblasts to arterial myofibroblasts as cells of origin for a tissue-engineered heart valve [71]. Unfortunately, leaflets derived from dermal fibroblasts were much thicker, more contracted and less organized than those derived from arterial myofibroblasts. It was therefore surmised that cells of mesodermal origin, such as arterial myofibroblasts, provide more specialized phenotypic properties than ectodermally derived skin fibroblasts.

Myofibroblasts derived from human saphenous vein have similar phenotypic properties to VICs but represent a more realistic source for clinical applications of tissue-engineered heart valves (TEHV) [72]. Unlike arterial cells, these cells can be harvested without the risk of limb ischemia [5]. These myofibroblasts were cultured on polyurethane scaffolds and were shown to be viable and confluent at six weeks. Moreover, compared to neotissue derived from aortic myofibroblasts, collagen production and mechanical stability were found to be higher in saphenous vein-derived structures. Follow-up studies have compared myofibroblasts isolated from ovine tricuspid heart valve leaflets to cells from jugular vein and carotid artery [73]. Interestingly, cells from jugular veins also demonstrated higher initial collagen production, but the study showed that all cell lines had a marked drop-off in collagen, elastin and GAG synthesis with time. This finding suggests that there is a critical timeframe for seeded cells to be placed on scaffolds while they are still able to perform necessary protein synthesis and organization [46]. In the first long-term follow-up of a clinical application of a TEHV, Dohmen et al. seeded a decellularized pulmonary allograft with cells isolated from saphenous vein and cultured in a bioreactor for two weeks. They cite 100% survival with adequate pressure gradients and no incidence of calcification at 10 year follow-up [74].

In the search for an alternative cell source, different stem cell sources have been investigated. In particular, umbilical cord-derived cells, amniotic fluid-derived cells, and chorionic villi-derived cells appear to carry great promise [14]. One group demonstrated the possibility of using autologous umbilical cord cells [75]. The isolated cells represented a mixed population of cells derived from umbilical cord artery, vein, and the surrounding Wharton's jelly. It was found that the cells demonstrated features of myofibroblast-like differentiation, such as expression of α-smooth muscle actin, vimentin, and deposition of collagen types I and III. More importantly, the cells successfully attached to scaffolds and formed a layered tissue-like structure comparable to scaffolds seeded with vascular cells [65]. Initial enthusiasm was tempered when it was observed that elastin was not being produced and GAGs were present only in low levels. Much more work is needed in characterization of the mixed cell population before the suitability of this source can be properly evaluated. Likewise, mesenchymal stem cells show promise, though many of the details remain to be elucidated. Their ability to develop into a variety of connective tissues, including bone, cartilage, muscle and fat, as well as easy collection via bone marrow puncture make these cells an attractive possibility [76]. In one experiment, human bone marrow stromal cells were collected and partially characterized using a number of myofibroblast markers [77]. Like umbilical cord cells, they were shown to express

α-smooth muscle actin and vimentin, and produce collagen types I and III. Biodegradable polymeric scaffolds cultured with mesenchymal stem cells *in vitro* demonstrated an organized internal structure and mature tissue development. Despite the encouraging results, it is not clear whether bone marrow stromal cells reliably differentiate into appropriate cell types in the scaffold or if they continue to remain differentiated *in vitro*, ensuring long-term function and durability of the replacement heart valve [78]. Investigations utilizing circulating endothelial and smooth muscle progenitor cells are at a similar stage [79]. However, given their remarkable differentiation potential, embryonic and adult stem cells may become valuable resources for heart valve tissue engineering.

The search for an ideal replacement for both VICs and VECs is ongoing, but the underlying goal across all these techniques is the same: transplanted cell populations in a scaffold can achieve the same distribution and differentiation pattern of cells in the native valve [80]. It is hard to prove this assertion, however, as there is limited data regarding the phenotypic profile of these cells once they are implanted into the scaffolds, especially when it comes to long-term follow-up data [5].

Cell seeding techniques

Growing valvular neotissue under *in vitro* and *in vivo* conditions remains a challenge in the development of reliable and reproducible tissue-engineered heart valves, and much of the difficulty is rooted in challenges of cell seeding and attachment to scaffolds. Indeed, cell attachment is a critical step in initiating cell growth and neotissue development [81].

Whereas relying on adjacent autologous myocardial cell growth into unseeded valve scaffolds has proven to be an unreliable and inconsistent technique, directed cell seeding either *in vitro* or *in vivo* has been successful. In seeding scaffolds under *in vitro* conditions, early strategies for improving cellular attachment included increasing seeded cell number or density, increasing scaffold porosity, or increasing scaffold surface area. More sophisticated techniques sought to enhance cell attachment by coating the matrix prior to seeding with various cell adhesion molecules like laminin [82]. Similarly, prior to seeding a pulmonary allograft for a Ross operation, Dohmen and colleagues covered the decellularized valve with a commercially available synthetic extracellular matrix protein to enhance cell attachment [74]. Cell seeding was then performed using a sedimentation technique, with excellent cellular attachment and eventual neotissue ingrowth [83].

Indeed, covering valvular scaffold with molecules that can target or attract specific circulating cells offers an avenue for *in vivo* autologous cell seeding which precludes the need for *in vitro* cell culture and all the microbial risks therein. Jordan and colleagues utilized this principle in conjugating a decellularized porcine pulmonary valve with CD133 antibodies as a means of promoting scaffold seeding by circulating endothelial progenitor cells [84]. This strategy led to increased endothelial cell attachment, increased interstitial cell number, increased structural proteins, and improved biomechanical properties when compared with unconjugated or traditional cell-seeded valves [84].

The method of cell seeding also influences efficiency of cellular attachment to scaffold. Traditional methods of seeding polymer scaffolds employed static cell culture techniques, in which a concentrated cell suspension is pipetted onto polymer scaffolds and left to incubate for a variable period of time, during which cells adhere to the scaffold. Dynamic cell seeding methods employ a seeding method in which either the medium or both the medium and scaffold are in constant motion during the incubation period. Dynamic cell seeding is often used in combination with a bioreactor and offers improved cellular attachment than static cell seeding [85]. Nasseri concluded that dynamic cell seeding onto tissue-engineering scaffolds increased cell adhesion, alignment in the direction of flow, cell infiltration, and seeding density [86]. There are various factors that can be further modulated in cell seeding, including

use of mixed versus pure cell populations, interval cell seeding, and single-step versus sequential seeding of different cells.

Adequate, uniform and reproducible cell seeding of both natural and synthetic polymeric scaffolds remains a challenge in the field. Optimization of rapid seeding techniques will be important in the development of a tissue-engineered heart valve, as it maximizes the use of donor cells, hastens the proliferation and subsequent differentiation of cells, decreases the time in culture, and provides a uniform distribution of cells [87]. Advances in the modification of scaffold surfaces to enhance cell adhesion and subsequent function offers a strategy to augment *ex vivo* valve seeding in a timely fashion [5]. This strategy also offers the potential to direct autologous circulating cell seeding of heart valves, which if successful precludes the need for *ex vivo* cell culturing and allows for an 'off the shelf' tissue-engineered heart valve.

Bioreactors

The use of bioreactors for tissue-engineering heart valves has increased over the last several years due to both improvement and complexity of available systems and the encouraging results that have been published with their use. Simplistically, a bioreactor is a biomimetic system used to optimize *in vitro* neotissue development. Ideally, the bioreactor system is designed to mimic the physiologic or pathophysiologic condition that is to be corrected with the engineered tissue. Factors such as shear stress, flow rate, flow profile, pressure, and media should be easily manipulated to change experimental conditions as researchers see fit [16]. Additionally, bioreactors should be easy to access, easy to exchange media, remain sterile under long culture times, accommodate multiple samples to facilitate adequate sample sizes for statistically significant comparisons, and should be arranged to allow for direct visualization of the samples [35]. In mirroring physiologic conditions in the heart, an ideal bioreactor for tissue-engineered cardiac valves consists of pulsatile flow and cyclic flex in order to generate the complex biomechanical environment that implanted valves must withstand [88]. Exposure to pulsatile flow modulates and the biomechanical properties of the neotissue, which is especially important in the development of a tissue-engineered heart valve in order to prevent premature valve deterioration [89].

One specific type of bioreactor used in the construction of a tissue-engineered heart valve is the pulse duplicator. This pulsatile bioreactor provides physiological pressure and flow to the developing tissue-engineered heart valve and promotes both the development of mechanical strength and the modulation of cellular function [90]. In using human umbilical cord blood-derived progenitor cells to seed heart valves made from biodegradable polymer, Sodian and colleagues used a pulse duplicator bioreactor wherein pulsatile flow (300–500 mL/min) and pressure (5 to 15 mmHg) were gradually increased over the course of seven days [91]. This technique led to tissue-engineered heart valves with similar connective tissue and extracellular matrix composition as that seen in native heart valves.

Other approaches to bioreactors for tissue-engineered heart valves include bioreactors that can provide cyclic strain or dynamic flexural strain. Exposure of seeded valve tissue *in vitro* to mechanical strain results in more pronounced and organized tissue formation with superior mechanical properties over unstrained controls and results in tissue-engineered heart valve cusps that are significantly less stiff than static controls [92]. This technique also yields substantial increases in DNA and net collagen content [93]. Cyclic flexure, which is a significant source of heart valve deformation, was used as the mechanical stimuli in a bioreactor into which cell-seeded and unseeded valves were incubated for three weeks. Cell-seeded valves undergoing cyclic flexure had >400% increase in effective stiffness compared to the unseeded controls and also had increased amounts of extracellular matrix [94].

Importantly, there is no uniform standard bioreactor that is used in tissue-engineering heart valves, in part because of the complexities of the mechanical environment *in vivo*. Further

understanding of the relationship between embryonic valve development and biomechanical signals may offer insight into designing an ideal bioreactor for *in vitro* development of heart valves.

Neotissue development in tissue-engineered heart valves

The process of neotissue development is poorly understood and appears to be controlled by a multitude of factors. In some ways it mimics embryonic development whereas in other ways it appears to be governed by the rules underlying tissue repair. In fact, it is probably a unique process governed by its own set of laws. The type of cells that are implanted and their interaction with the surrounding environment determine the type of tissue that ultimately develops from the cell-scaffold complex [43].

The environment in which the construct grows will influence the histological structure and extracellular matrix formed. Researchers have approached this phenomenon from several perspectives. In one approach, the formation of tissue begins *in vitro* by seeding cells onto a biodegradable scaffold and then maturing the tissue in a bioreactor prior to implantation *in vivo*. Using this approach, the scaffold is used as a cell delivery system and implanted *in vivo* shortly after cell attachment has taken place [44]. This approach uses a cell-scaffold construct to provide the initial structural integrity necessary to provide temporary physiological function until neotissue grows and remodels. The technique proposes that appropriate environmental signals for tissue repair and remodeling are inherently present in the *in vivo* milieu. In addition, *in vivo* biomechanical environmental forces provide important stimuli that affect the formation of extracellular matrix and direct the biomechanical properties of the developing neotissue. The key steps in this process include:

a) Cell proliferation and migration,
b) ECM production and organization,
c) Scaffold degradation, and
d) Tissue remodeling [1].

The mechanical and biological signals underlying neotissue formation remains an area of focused research. Recent studies of tissue-engineered vascular grafts in murine and ovine models have demonstrated that seeded mononuclear cells stimulate a robust infiltration of host macrophages during early timepoints [95]. Additional evidence suggests that circulating host bone marrow-derived cells are active in the acute inflammatory phase but do not represent a source of mature neotissue. Rather, macrophages appear to initiate an inflammatory cascade to drive vascular remodeling and neotissue ingrowth from the surrounding native vessel [96]. Other pathways specific to heart valve physiology may also be vital to understanding neotissue formation. One key process in the development of the heart valve is epithelial-to-mesenchymal transformation (EMT). It has been demonstrated that EMT pathways involving TGF-β3, BMP2 and VEGFA are activated and localized in TEHV remodeling [97]. Many groups believe that the identification of biomaterials that can induce EMT and direct cellularization of tissue-engineered heart valves will be invaluable to the field [46].

Tissue-engineered heart valves have been evaluated biochemically, molecularly, histologically, physiologically, biomechanically, and morphologically (Fig. 40.3). In each type of analysis, the tissue-engineered valve has been compared with the native valve [78]. These data can then be used to identify shortcomings in the tissue-engineered heart valve and aid in the rational design of an improved version. One challenge that remains is the identification of good biomarkers or methodologies for the non-invasive study of the structural remodeling and functionality of *in vivo* constructs so that a tissue-engineered valve can be followed over time [98].

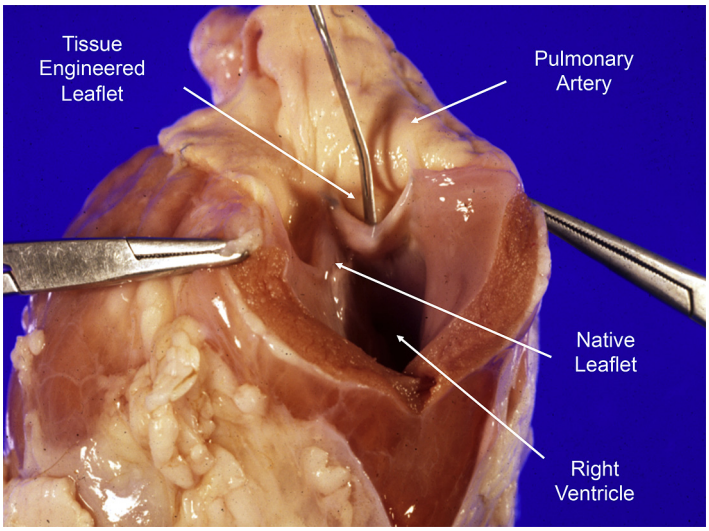

FIGURE 40.3
Photograph of a tissue-engineered valve in a sheep heart.

Clinical applications of the tissue-engineered heart valve

Dohmen et al. reported the first successful use of a tissue-engineered heart valve using a decellularized cryopreserved pulmonary allograft [74]. Since then, Dohmen and colleagues have published long-term data on a series of 11 patients who underwent the Ross operation with a tissue-engineered heart valve to surgically reconstruct the right ventricular outflow tract. In each case, the allograft was seeded in a bioreactor with autologous vascular endothelial cells that had been isolated from a segment of forearm vein two to four weeks prior to the operation. At ten years follow-up, the tissue-engineered heart valves showed excellent hemodynamic function. All patients remained in New York Heart Association class I heart failure and computed tomography showed no evidence of calcification or valve degeneration (Fig. 40.4) [99]. Despite the lack of a control group, the small sample size, and the lack of long-term biochemical or tissue samples, this study is a significant advance in the clinical application of tissue-engineered heart valves. Re-analysis of the group of patients at 15 and 20 years post-implantation will offer insight into the effect that autologous cell seeding has on allografts in preventing long-term complications that are seen in unseeded allografts and homografts like calcification and valve degeneration.

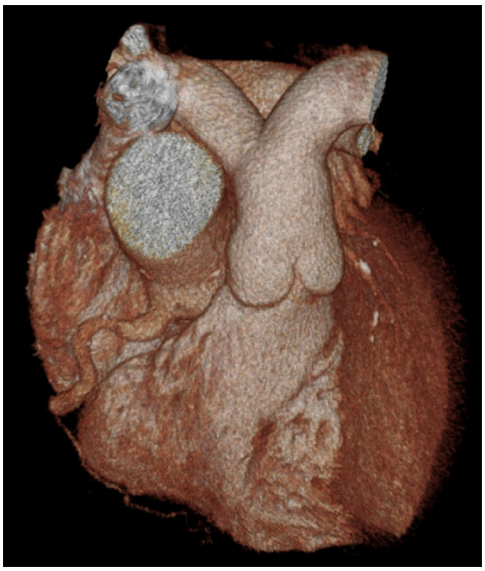

FIGURE 40.4
Multi-slice computed tomography shows a normal tissue-engineered pulmonary valve, without evidence of calcification or degeneration after 10 years in a male patient who was operated on at age 46 [99].

Other investigators have used decellularized scaffold implantations with autologous cell seeding to tissue-engineer cardiac valves. While not technically tissue-engineered valves, as that would require some degree of *ex vivo* cell seeding, this method holds the promise of valve implantation of allogenic or xenogenic tissue with a decreased host response to donor antigens. In lieu of data that host antigen recognition and antibody development may be linked to early tissue calcification and degeneration, the advantage of decellularizing a scaffold prior to implantation is that the cellular antigen burden decreases while the extracellular architecture and material properties are maintained [100].

Use of xenografts in this fashion was halted in 2003 after Simon et al. reported failure of SynerGraft decellularized porcine valves implanted in four children as pulmonary valves for right ventricular outflow tract reconstruction. In this study, two valves were severely degenerated at six weeks and one year after implantation, and one valve ruptured seven days after implantation, ultimately resulting in three deaths and the fourth child's valve explanted prophylactically [59]. Since then, focus has turned to allogenic decellularized pulmonary valves using the SynerGraft process with results so far showing promise for this technique over standard cryopreserved allogenic valves. Studies published to date comparing SynerGraft decellularized allogenic valves with standard cryopreserved valves show in the SynerGraft valves decreased short-term stenosis or regurgitation, decreased clinically significant insufficiency, lower peak valve gradients, and fewer interventions [101]. Brown and colleagues performed a multicenter retrospective cohort study of 342 patients undergoing right ventricular outflow tract reconstruction with SynerGraft or standard cryopreserved valves and showed decreased regurgitation in the SynerGraft group and overall safety and efficacy at four years post-implantation [41].

To date, synthetic scaffolds have not been used to fabricate a replacement heart valve for clinical use. However, the principles of tissue engineering outlined thus far and the approach of identifying and isolating a cell source, choosing a scaffold, and seeding the cells on to the scaffold were applied by Shinoka et al. in the first human clinical trial investigating tissue-engineered vascular grafts (TEVG) in children with complex congenital heart disease. The study began in 2001 and included 25 patients with an average age of five years undergoing extracardiac total cavopulmonary connection using the TEVG as a conduit. The synthetic scaffold used was a copolymer of polyglycolic acid and ε-caprolactone reinforced with poly-L-lactide. The scaffolds were seeded with autologous bone marrow-derived mononuclear cells. At one month post-implantation, all patients were alive and symptom free, and radiographic studies demonstrated no cases of TEVG aneurismal dilatation, thrombosis, or stenosis. Patients were followed serially with angiography, computerized tomography, or magnetic resonance imaging examinations. At one year there was one patient diagnosed with partial mural thrombus who was treated successfully with anticoagulation [102]. At late-term follow-up (average 5.8 years post-implantation), four patients had died of causes unrelated to the TEVG and four patients had developed TEVG stenosis requiring balloon angioplasty or stenting [103]. Overall, the tissue-engineered vascular conduits had reduced incidence of calcification, no risk of rejection because of autologous cell seeding, minimal risk of infection, and potential for growth [104]. Following the excellent results of Shinoka and colleagues, we are currently performing the first FDA-approved human clinical trial investigating use of TEVGs in children with congenital heart disease. Shinoka's results prove that the application of tissue engineering principles to vexing clinical problems has the potential to dramatically improve outcomes and lessen the morbidity and mortality of disease. All of these potential benefits would hold true for a tissue-engineered heart valve.

As the field of cardiovascular tissue engineering continues to expand, so too will the desire to apply methods and models in human clinical trials. It is essential for investigators to have thoroughly established the efficacy of techniques in large animal and pre-clinical models prior to translation into humans. Issues of bacterial contamination, insufficient cell seeding, and rapid degeneration of scaffold are common complications of tissue-engineered devices and

therefore strict protocols must be in place and adhered to in order for this promising field to expand clinically in an effective and responsible manner.

That randomized clinical trials are the gold standard for evaluating efficacy of new drugs and devices should not prevent the rationale translation of tissue-engineering technology to man. In most clinical circumstances in which tissue-engineered vessels or valves are to be applied, patients have poor prognoses, and therefore the benefit of tissue-engineered devices will outweigh the risks inherent in a new technology. For this reason, it behooves the FDA and other regulatory agencies to utilize the humanitarian exception for these devices in instances where pre-clinical data is promising and patient disease is severe. Most importantly, though, for physicians, scientists, regulators, and engineers, is to apply this promising technology in a responsible manner, placing the safety and well being of the patients as the top priority in all instances.

CONCLUSION

Successful development of a tissue-engineered replacement heart valve holds the key to better treatment and improved clinical outcomes for end-stage valvular disease. Although significant progress has been achieved since its inception in the early 1990s, the field is young and many key issues have yet to be resolved. We are still exploring the cellular and ECM biology that govern the maintenance of a normal valve. Better characterization of valve cells like VECs and VICs may offer clues to optimize cell seeding. Moreover, advances in other fields of tissue engineering and stem cell biology may provide new techniques and cell types that could transform either the cell source or cell seeding technique used in engineered heart valves. Similarly, growth in other fields like 3D printing or quantification of flow using magnetic resonance imaging may eventually find clinical applications of their respective technologic advancements in engineering heart valves. To that point, it is clear that tissue engineering is a multi-disciplinary, multi-faceted field that requires cooperation, coordination, and collaboration between experts in a variety of different specialties. Fostering these types of relationships using unique funding mechanisms and programs will help move this field forward and will ultimately benefit tissue engineering as a field and the patients that benefit from its growth.

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PART 10

Endocrinology and Metabolism

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CHAPTER 41

Generation of Pancreatic Islets from Stem Cells

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INTRODUCTION

Diabetes mellitus is one of the most prevalent chronic diseases. Glucose homeostasis disruption occurs when β-cells fail to secrete the insulin necessary to maintain the homeostasis of glucose in the blood flow. Over time, diabetes can lead to the rise of different long-term complications, such as diabetic foot, retinopathy, neuropathy, nephropathy and arteriosclerosis. Nowadays, the only treatments for diabetes consist of exogenous insulin supply or pancreas/islet transplantation, but the inability to achieve a tight control over glucose regulation by exogenous insulin administration and the shortage of pancreatic islets donors have motivated recent efforts to develop renewable sources and protocols for effective β-cell replacement.

Embryonic stem cells are non-specialized cells that share two important characteristics: *self-renewal*, which allows them to expand indefinitely while maintaining the undifferentiated state; and *pluripotency*, which is the capacity to differentiate into almost all specialized cell types. Proof-of-concept experiments demonstrate that embryonic stem cells have the ability to differentiate into insulin-producing cells, even if at a very low frequency.

In this chapter, we review the attempts that have been made thus far to convert embryonic stem cells into pancreatic endocrine cell types of potential use in the treatment of type I diabetes.

FIRST ATTEMPTS TO OBTAIN B-CELL LIKE CELLS BY DIFFERENTIATION

The first attempt to promote the differentiation of insulin-producing cells was carried out using a combination of directed differentiation and cell selection methods. Mouse embryonic stem cells (ESCs) expressing antibiotic resistance under control of either the insulin or the Nkx6.1 promoter [1,2] were driven to differentiate into nutrient-induced insulin-secreting cells which rescue streptotozotocin-diabetic mice from hyperglycemia when transplanted either into the spleen or under the kidney capsule. Furthermore, the cell type selection protocol allowed no tumor formation by the presence of non-differentiated ESCs. In contrast, most of the initial differentiation techniques that relied upon embryoid body (EB) formation appeared to be successful using either mouse ESCs [3–5] or human ESCs [6–7], but the absence of C-peptide, tumor formation and lack of demonstration of any rescue of diabetic animals revealed

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that the observed intracellular insulin in these cells did not originate from *de novo* synthesis, but rather from uptake from the culture media [7–11].

Although pioneering results [1–2] showed that pancreatic β-cell-like cells may be obtained from ESCs, new differentiation strategies that can be used with human stem cells needed to be developed. Differentiation strategies were based on knowledge of early mouse development, the sequential expression of the transcription factors [12–14] and the signaling pathways [15] involved in β-cell formation. The application of such developmental principles to stem cell biology seem to be the key to obtaining a successful differentiation process, thus recent approaches chosen by the majority of investigators working on human ESC differentiation to produce insulin-secreting cells are based in a multi-stage protocol attempting to mimic all phases of *in vivo* pancreas development. Thus, the aim is to induce human ESCs to transition sequentially through mesendoderm, definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor stages, resulting in a final, functional, insulin-expressing cell.

STEPS TOWARDS β-CELLS: PROTOCOL COMPARISON

Obtaining mesendoderm and definitive endoderm

In order to make human ESCs differentiate into insulin-producing cells, the first goal is the efficient generation of definitive endoderm, which has been readily achieved by D'Amour et al. [16] using a combination of a TGFβ family member, Activin A, to activate Nodal signaling, and low serum concentration of media to avoid the activation of PI3K. Furthermore, to improve the yield of definitive endoderm cells, the activity of PI3K could be inhibited using two different inhibitors; LY 294002 [4,17] or wortmannin [18]. Wnt3a-mediated Brachyury expression is also important for the migration of precursor cells through the anterior region of the primitive streak (PS) and the formation of a mesendoderm population from which both endoderm and mesoderm will be generated depending on the magnitude and duration of Nodal signaling [19–20]. Hence, the efficiency of definitive endoderm generation can be further improved by exposure of human ESCs to a combination of Activin A and Wint3a in the absence of serum on the first day, followed by one day of culture in a medium supplemented with Activin A and 0.2% serum, and then three days in a medium supplemented with Activin A and 2% serum [21]. In contrast to Wnts, bone morphogenic proteins (BMPs) inhibit endoderm induction. Therefore, inhibition of BMP signaling using the BMP antagonist, Noggin, resulted in increased expression of PS/endoderm markers and in a rapidly reduced expression of PS/mesoderm markers, thus demonstrating the cooperative intertalk of canonical Wnt/β-catenin, Activin/Nodal and BMP signaling pathways during ESCs specification of PS, mesoderm and endoderm [22]. A different approach to inducing definitive endoderm has been recently published [23], and uses two small molecules identified as endoderm inducers (IDE1 and IDE2) with an efficiency similar to that obtained with the Activin A treatment described above.

Obtaining foregut patterning

Once definitive endoderm has been obtained, the next step is to trigger DE to foregut patterning, which results from the complex crosstalk between mesoderm and endoderm, involving gradients of fibroblast growth factors (FGFs), BMPs, retinoic acid (RA) and sonic hedgehog (SHH) [24]. During foregut patterning, high concentrations of FGF4 promote a posterior/intestinal endoderm cell fate, whereas lower FGF4 levels induce a more anterior/pancreas-duodenal cell fate [24]. In the same way, it has been shown that FGF2 specifies human ESCs-derived definitive endoderm into different foregut lineages in a dose-dependent manner. Specifically low doses of FGF2 promote a hepatic cell fate, intermediate FGF2 levels induce a pancreatic cell fate and high concentrations of FGF2 induce midgut endoderm small intestinal progenitors [25]. Retinoids are known as morphogens and differentiation inducers

during embryonic development and ESC differentiation, as reported by many investigators. These studies often used retinoic acid to induce pancreatic endoderm commitment in the definitive endoderm obtained by Activin A treatment [18,21,26–29]. In early development, SHH is highly expressed in the stomach and duodenal endoderm, but not in the pancreatic endoderm; hence specific inhibition of sonic hedgohog signaling has been shown to promote pancreatic differentiation by expanding the endoderm population of Pdx1-expressing cells [30]. In the same way, inhibition of SHH prevents stomach and duodenal endoderm specification, and the inhibition of BMP signaling pathway by Noggin has been shown to block hepatic commitment. Hence, to promote pancreatic endoderm specification at the expense of other foregut endoderm lineages, Cyclopamine, firstly introduced by León-Quinto et al. [2] for *in vitro* differentiation, and Noggin are often used in conjunction with RA treatment in human ESC differentiation protocols [18–29]. Curiously, anti-sonic hedgehog, which displayed better results than the non-specific small molecule cyclopamine, is not used.

Obtaining endocrine precursors

Notch signaling regulates the consecutive cell fate decisions required for the formation of specialized tissues, including β-cell generation. Once pancreatic endoderm is obtained, Notch inhibition seems to be critical for further differentiation towards an endocrine fate. This is consistent with the fact that Notch signaling represses transcription of Ngn3, a critical transcription factor for the formation of pancreatic endocrine cells. Notch activity serves to expand the pool of pancreatic progenitors preventing premature endocrine differentiation. During *in vitro* differentiation, it has been recently proved that EGF treatment can be used to expand the Pdx1-positive population of progenitor cells, which is necessary to obtain a large number of endocrine cells [18]. After EGF or FGF10 treatment, inhibition of Notch signaling using N-[N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester (DAPT) a γ-secretase inhibitor had only a slight impact on promoting endocrine differentiation [21,31,32] probably due to a non-specific inhibition of Notch.

Maturation of pancreatic endocrine cells

The last step of human ESC differentiation into insulin-producing cells consists of directing the maturation of endocrine precursors into specialized and functional hormone-secreting cells. However, despite of the great number of biologically active compounds that have been used in published endocrine pancreas differentiation protocols, as yet an *in vitro* differentiation of ESCs into functional β-cells has not been achieved. D'Amour et al. [21] used a mix of different 'maturation factors' such as IGF1, Exendin-4, HGF and B27 supplement during terminal differentiation stages, but observed only minor effects on differentiation when these factors were omitted. On the other hand, Cho et al. [32] demonstrated that the application of betacellulin and nicotinamide to D'Amour's protocol resulted in sustained Pdx1 expression and led to subsequent insulin production. Nevertheless, insulin-producing cells obtained by *in vitro* differentiation protocols are commonly immature and non-functionally glucose-responsive. As a consequence, *in vitro* terminal differentiation steps were omitted from the protocol published by Kroon et al. [29], where pancreatic progenitors were allowed to mature into functional β-cell by *in vivo* maturation after transplantation in streptozotocin-induced hyperglycemic mice.

ALTERNATIVE STRATEGIES FOR PROTOCOL OPTIMIZATION

All the signaling pathways and factors described above are the result of more than ten years of research into ESCs differentiation with the aim of obtaining functional insulin-secreting cells. The fact that this aim has still not been achieved demonstrates the complexity of the differentiation process (Fig. 41.2). New factors and different culture conditions will be probably required to induce the complete differentiation and maturation of ESC-derived β-cells.

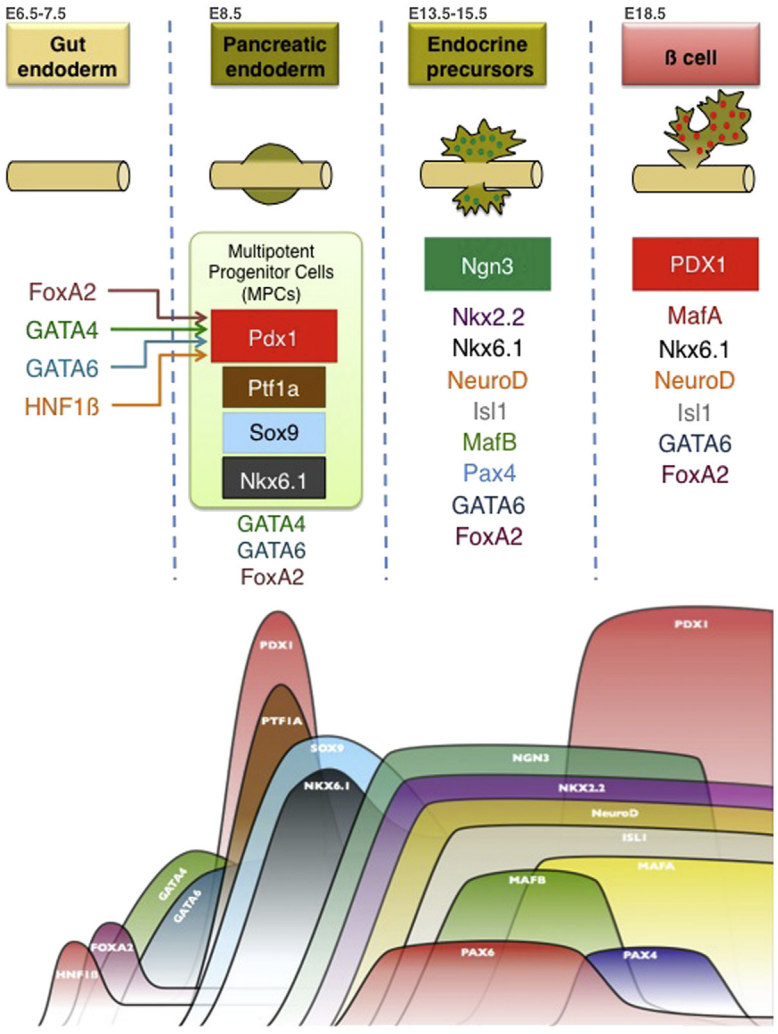

FIGURE 41.1
Schematic representation showing the transcription factors and signaling pathways identified in the development of mouse pancreatic β-cells.

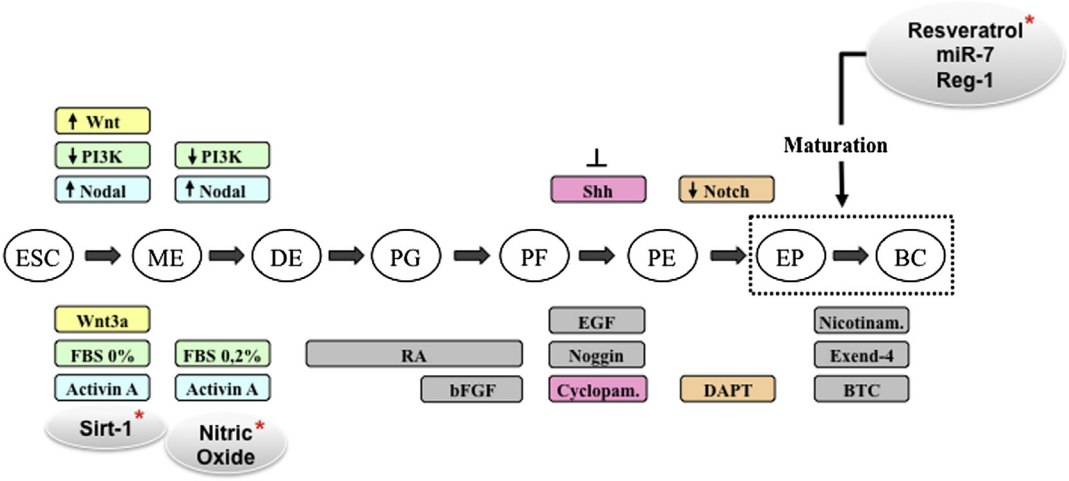

FIGURE 41.2
Overview of the signaling pathways and factors that have been shown to efficiently differentiate ESCs to a β-cell fate. Resveratrol, miR-7 and Reg-1 are proposed factors to improve the maturation stage. ESC: embryonic stem cells; ME: mesendoderm; DE: definitive endoderm; PG: primitive gut; PF: posterior foregut; PE: pancreatic endoderm; EP: endocrine precursors; BC: beta cells; DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester; BTC: betacelulin. *Adapted from Champeris Tsaniras S et al. (2010). (*) Differentiation factors contributed by our group.*

Here we mention some novel approaches that could be useful in improving definitive endoderm generation, and the final maturation of the endocrine precursors, resulting in a more efficient insulin-secreting cell differentiation strategy. In addition, stem and somatic cells other than ESCs have been used to obtain a β-cell phenotype. Some of these strategies are overviewed below.

Increase of the glucose-stimulated insulin secretion pathway

Recent studies have shown the impact of Resveratrol (RSV) on insulin secretion and the way that this compound potentiates glucose-stimulated insulin secretion. RSV (3,5,4′-trihydroxy-trans-stilbene) is a polyphenol that has been shown to activate Sirt-1, a NAD+-dependent deacetylase [33]. Sirt-1 plays a role not only in the maturation process, but also in the initial differentiation process [34]. The effect of RSV on insulin secretion was studied for the first time by Zhang Y et al. [35] and recently by Vetteli et al. using the INS-1E cell line and human islets [36]. Bordone et al. demonstrated that Sirt-1 represses transcription of the mitochondrial uncoupling protein 2 (UCP2) by binding directly to its promoter [37]. Lower levels of UCP2, induced by Sirt-1 overexpression, result in increased ATP production and enhanced insulin secretion in INS-1E [38]. In humans, the transcriptional pathway regulating β-cell UCP2 gene expression is activated by the transcriptional cofactor peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1 α) [39] which is another target of both Sirt-1 and RSV (Fig. 41.3). Hence RSV could be considered as a good candidate for improving the maturation process of human, ESC-derived, insulin-secreting cells.

Effects of new soluble factors on the maturation process

Of all the differentiation steps described above, the most difficult one to promote seems to be the maturation stage. Despite the large number of factors and their combinations that have been used in current protocols, functional β-like cells have not been produced. Screening for new active molecules to be used as 'maturation factors' could be helpful. In this context, a previous study described a fetal soluble factor, released by pancreatic buds, that has been used to induce *in vitro* endocrine pancreatic differentiation in mouse ESCs [40]. Subsequent proteomic studies (data not published) have demonstrated that one of the most abundant proteins present in the soluble factors released by pancreatic buds was Regenerating 1 (Reg-1). Reg-1 is normally induced in pancreatic β-cells and acts as an autocrine/paracrine growth factor for β-cell regeneration [41,42]. Based on this information, Reg-1 could be used in differentiation protocols to induce human ESC-derived β-cell maturation.

Nitric oxide and definitive endoderm induction

The relevant role of nitric oxide (NO) in developmental processes in the embryo has been previously described [43,44]. NO has also been reported to play a role in the induction of ESCs

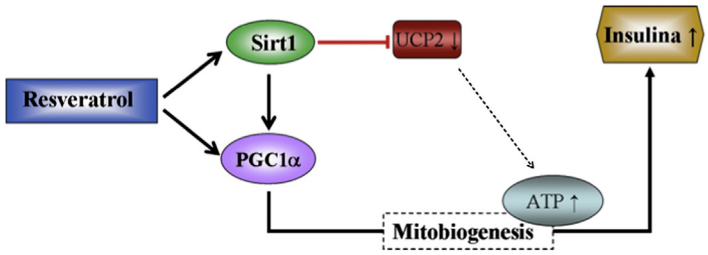

FIGURE 41.3

Proposed links beteen Resveratrol, Sirt1, UCP2 and insulin secretion. The indirect activation of Sirt1 by Resveratrol regulates insulin signaling pathways via repression of UCP2 transcription and through phosphorylation and subsequent deacetylation of PPARγ coactivator 1α (PGC1α), leading to mitochondrial function modulation, ATP increase and insulin secretion.

to differentiate into cardiomyocytes [45,46]. Recently the mechanism by which NO induces ESC differentiation has been described, and it has been observed that the exposure of ESCs to exogenous donors of NO like 1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) for a short time induces early differentiation towards a definitive endoderm phenotype. Treatment with DETA-NO induced the expression of endoderm markers such as Pdx1 and GATA4 after a very short period of exposure (19 hrs): this is an interesting approach which could offer a suitable alternative for the generation of endoderm to the 3–5 days of Activin/Wnt3a treatment [47].

Endothelial cell co-culture

A less studied area in the field of pancreatic differentiation is the effect of endothelial cell signaling in β-cell maturation; this is increasingly being appreciated as an important contributing factor in *in vivo* pancreatic islet maturation. Recent studies have shown the effect of endothelial cell signaling in the maturation of human ESC-derived pancreatic progenitor cells into insulin-producing islet-like cells [48–50].

Microenvironment considerations

Despite the great influence of oxygen tension and extracellular matrix (ECM) on islet survival, the physiological environment has been largely ignored in β-cell differentiation protocols. Islet cells are extremely sensitive to both hypoxia and hyperoxia, and at the same time they need a three-dimensional (3D) cell-to-cell interaction structure to achieve a functional phenotype [51]. Unfortunately the *in vitro* condition used until now could not reproduce an optimal *in vivo* microenvironment, thus the 3D islet-like structure is not compatible with physiological oxygen distribution because of the lack of capillary vessels in the cluster structure. Novel differentiation approaches that take into account the role of both the ECM and oxygen distribution could be important for improving the maturation process of the endocrine precursors derived from human ESC differentiation. For these reasons, studies of the use of ECM components, such as laminins, and silicone rubber membranes to control oxygen tension are being carried out [52,53].

MicroRNAs

MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression by post-transcriptional interference with specific messenger RNAs (mRNA). Study of miRNAs as regulators of complex gene expression networks is an emerging field that could be of great impact in both the differentiation and maintenance of cell phenotype. Studies in β-cell development demonstrate that miR-7 is highly expressed in both mouse and human developing pancreas [54], in the same way it contributes to *in vivo* β-cell development, consequentially miR-7 could be considered as an important player for the achievement of a complete differentiated human ESCs-derived β-cell.

ALTERNATIVE CELL SOURCES

Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are a new source of embryonic-like stem cells obtained by reprogramming somatic cells. Similar to ESCs, iPSCs can differentiate into many different cell types, including insulin-secreting cells [55], suggesting that patient-specific functional β-cells might be generated [56]. Furthermore, β-cells generated from iPSCs were able to reverse hyperglycemia after transplantation into diabetic mice [57]. One of the most important advantages of using patient-specific β-cells is that they avoid the risk of immunological rejection [58]. However, unexpectedly, rejection of autologous iPSCs transplanted in genetically identical mice has been observed [59] hence, further studies are required to ensure consistency and safety of iPSCs before they can be used in future cell regenerative therapy.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic progenitor cells that are being explored as a promising new treatment for tissue regeneration. However, the ability of MSCs to differentiate into insulin-producing cells when treated with different soluble factors is still under question [60]. Nevertheless the efficacy of MSCs in the treatment of diabetes could derive from different abilities of these cells, such as their immunomodulatory properties or their capability to differentiate into endothelial cells, thus providing environmental support for pancreatic regeneration [61]. Actually several lines of evidence have demonstrated that cotransplantation of islets and MSCs exhibits a better outcome then islet transplantation alone, by promoting vascularization of the graft and hence preventing rejection [62,63].

Transdifferentiation

In addition to studies using stem cells and iPSCs, recent reports suggest that pancreatic duct cells, liver cells, acinar cells, and other mature cell types (Fig. 41.4) have the ability to trans-differentiate into insulin-producing cells [64–66]. Even adult monocytes retain this capability [67]. However is difficult to demonstrate that these 'insulin-producing cells' possess the criteria to be considered pancreatic β-cells. The difficulties of differentiating adult cells into insulin-producing pancreatic cells have been bypassed by using *in vivo* gene transfer of the pdx-1 gene into liver cells to induce hepatocyte transdifferentiation [68,69]. Experiments are not conclusive because cells generated in this manner are not true β-cells, but rather hybrids of hepatic and pancreatic cells. To avoid the problem of viral infection, a very recent study shows that is possible to induce liver transdifferentiation by using a hydrodynamic approach to deliver genes such as pdx1, ngn3 and mafA [70]. On the other hand, a more efficient attempt at transdifferention of non-endocrine tissue into β-cells has been achieved using exocrine cells as the starting material. In this context, it was feasible to reprogram pancreatic exocrine tissue into islet cell types using a combination of three genes (Pdx1, Ngn3, and MafA) [71,72]. Unlike in other transdifferentiation settings, the original exocrine phenotype appeared to be completely abrogated, and diabetic mice subjected to transplantation of these cells showed a significant and permanent improvement in blood glucose levels, even if their diabetes was not completely reversed.

De novo organ formation

While it has been shown that insulin-producing cells may benefit glucose homeostasis, in human physiology the actual micro-organs controlling blood glucose are the pancreatic islets.

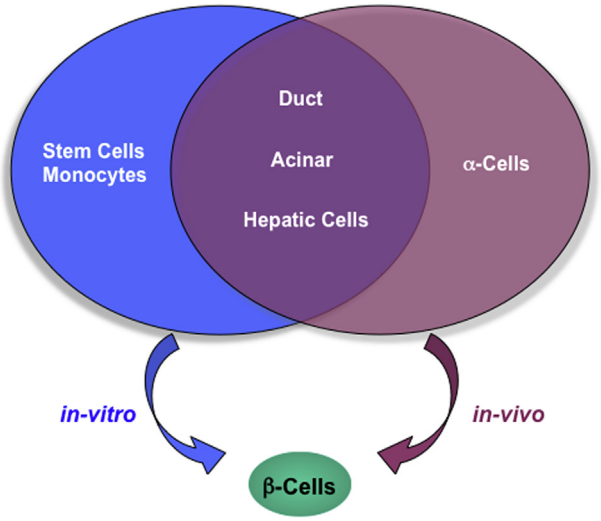

FIGURE 41.4
Cell sources that have been proved able to transdifferentiate into insulin-producing cells under in vitro and/or in vivo conditions.

Pancreatic islets are well-vascularized and innervated structures in which endocrine and non-endocrine cells are responsible for an integral response to blood glucose oscillations. Insulin-secreting β-cells respond in synchrony to stimulatory nutrient increases within the islet building a well-tuned response [73–75], simultaneously glucagon- and somatostatin-secreting cells modulate their activity to other nutrient ranges [76,77]. It is generally accepted that pancreatic islets better represent the overall physiology than isolated β-cells, but so far no-one has been able to produce a pancreatic islet *in vitro*. A complex and exciting strategy is appearing in the field of regenerative medicine which consists of the generation of entire transplantable organs. The complex cellular interactions among and within tissues that are required for organogenesis are extremely difficult to recapitulate *in vitro* so, as an alternative, promising new studies on blastocyst complementation are being undertaken [78]. The aim is to generate pluripotent stem cell (PSC)-derived donor organs *in vivo* by injection of PSCs into blastocysts obtained from mutant mice in which the development of a certain organ was precluded by genetic manipulation. The PSC-derived cells would developmentally compensate for the defect and form the missing organ [78]. Nakauchi's group has shown proof-of-principle findings for pancreas generation through the injection of PSCs into pancreas-deficient Pdx1(−/−) mouse blastocysts [79]. This innovative approach poses not only technical but ethical questions; for example, could the production of human organs in human-pig chimeras be an alternative approach? Obviously, we are still far from such an attempt.

CONCLUSION

Development of human ESC-based therapy for diabetes represents one of the most challenging areas of stem cell research. Mimicking the complex developmental processes of β-cell formation has been demonstrated to be the most promising and effective approach to obtaining insulin-secreting cells. However, our understanding of the signals which are important in the final phase of pancreatic endoderm specification is still incomplete. Study of endothelial cell-released factors and cell matrix interactions during pancreatic differentiation will be required to generate functionally competent insulin-secreting pancreatic cells. On the other hand, direct or indirect reprogramming of somatic cells through transdifferentiation or iPSCs derivation has been shown to be an effective strategy to produce β-cell surrogates. However, the problem of genetic manipulation that characterizes both these two techniques represents a safety concern that is still unlikely to be acceptable for clinical applications. In conclusion, despite all the investigations efforts and promising progress reported in this review, further studies are required to generate new transplantable insulin-producing cells that are safe and able to mimic extremely closely the complex functions of an endogenous β-cell.

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CHAPTER 42

Bioartificial Pancreas

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INTRODUCTION

Diabetes is a significant health problem affecting an estimated 25.8 million people (or 8.3% of the population) in the United States. Of these, 5–10% have autoimmune, or juvenile Type 1 diabetes, which results from the destruction of the insulin-producing β cells in the pancreas. Adult onset Type 2 diabetes has a more complicated etiology, which includes development of resistance to insulin, chronically elevated levels of insulin, and eventual damage to the pancreatic β cells. Although initially controlled by diet, exercise and oral medication, Type 2 diabetes often progresses towards insulin dependence. It is estimated that the diabetics who receive insulin either by itself or in combination with oral medication comprise 26% of the total population of diabetics, or approximately 6.7 million people in the US (CDC National Diabetes Fact Sheet, 2011; http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011.pdf).

Although insulin-dependent diabetes (IDD) is considered a chronic disease, even the most vigilant insulin therapy cannot reproduce the precise metabolic control present in the non-diseased state. The poor temporal match between glucose load and insulin activity leads to a number of complications, including increased risk of heart disease, kidney failure, blindness, and amputation due to peripheral nerve damage. Providing more physiological control would alleviate many of the diabetes-related health problems, as suggested by findings from the Diabetes Control and Complications Trial and its continuation study [1,2]. Cell and tissue-based therapies, which provide continuous regulation of blood glucose through the physiologic secretion of insulin, have the potential to revolutionize diabetes care.

Several directions are being considered for cell-based therapies of IDD, including the transplantation of immunoprotected allogeneic or xenogeneic islets, of continuous cell lines, or of cells engineered to acquire a β phenotype genetically or through differentiation. For allogeneic islet transplantation, a protocol developed by physicians at the University of Edmonton [3–6] has dramatically improved the survivability of grafts. The protocol uses human islets from cadaveric donors, which are transplanted in the liver of carefully selected diabetic recipients via portal vein infusion. The success of the Edmonton protocol is attributed to two modifications relative to earlier islet transplantation studies: the use of a higher number of islets and the implementation of a more benign, steroid-free immunosuppressive regimen. However, two barriers prevent the widespread application of this therapy. The first is the limited availability of human tissue, as generally more than one cadaveric donor pancreas is needed for the treatment of a single recipient. The second is the need for life-long immunosuppression, which, even with the more benign protocols, results in long-term side effects in the patients and may also negatively affect the transplanted islets themselves.

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00042-2

A tissue-engineered pancreatic substitute aims to address these limitations by using alternative cell sources, relaxing the cell availability limitation, and by reducing or eliminating the immunosuppressive regimen necessary for survival of the graft. A number of significant challenges are facing the development of such a substitute, however. These include procuring cells at clinically relevant quantities; the immune acceptance of the cells, which is exacerbated in Type 1 diabetes by the resident autoimmunity in the patients; and the fact that diabetes is not an immediately life-threatening disease, so that any other therapy will have to be more efficacious and/or less invasive than the current standard treatment of daily blood glucose monitoring and insulin injections or infusions by a pump.

In general, developing a functional living tissue replacement requires advances and integration of several types of technology [7]. These are:

1) Cell technology, which addresses the procurement of functional cells at the levels needed for clinical applications.
2) Construct technology, which involves combining the cells with biomaterials in functional three-dimensional configurations. Construct manufacturing at the appropriate scale and preservation, as needed for off-the-shelf availability, also fall under this set of technologies.
3) Technologies for *in vivo* integration, which address the issues of construct immune acceptance, *in vivo* safety and efficacy, and assessing construct integrity and function post-transplantation.

The same three types of technology need also be developed for a pancreatic substitute. It should be noted, however, that the critical technologies differ depending on the type of cells used. With allogeneic or xenogeneic islets or beta cells, the major challenge is the immune acceptance of the graft. In this case, encapsulation of the cells within semipermeable barriers, which allow passage of low molecular weight nutrients and metabolites, including insulin, but exclude larger antibodies and cytotoxic cells of the host, assist the immune acceptance of the graft. With cell therapies based on potentially autologous non-pancreatic cells, targeted by gene expression vectors, transdifferentiated *in vivo*, or retrieved surgically, engineered *ex vivo* and returned to the host, the major challenge is achieving insulin secretion to the necessary level and in precise response to physiologic stimuli. Lastly, with stem or progenitor cells, the primary hurdle is their reliable, reproducible differentiation into cells of the pancreatic β phenotype. Fig. 42.1 shows schematically the two general therapeutic approaches based on allo- or xenogeneic cells (Fig. 42.1a) or autologous cells (Fig. 42.1b).

This chapter is therefore organized as follows. We first describe the types of cells that have been used or are of potential use in engineering a pancreatic substitute. We then discuss issues of construct technology, specifically encapsulation methods and the relevant biomaterials, manufacturing and preservation of the constructs. The challenges of *in vivo* integration and results from *in vivo* experiments with pancreatic substitutes are presented next. We conclude by offering a perspective on the current status and the future challenges in developing an efficacious, clinically applicable bioartificial pancreas.

CELL TYPES FOR PANCREATIC SUBSTITUTES

Islets

Despite several efforts, the *in vitro* expansion of primary human islets has been met with limited success. Adult human islets are difficult to propagate in culture, and their expansion leads to de-differentiation, generally manifested as loss of insulin secretory capacity. Although reports exist on the re-differentiation of expanded islets [8,9] and of non-islet pancreatic cells, which are discarded after islet isolation [10], the phenotypic stability and the *in vivo* efficacy of these cells remain unclear. Additionally, with expanded and re-differentiated islets, it remains unknown whether the insulin-secreting cells arose from

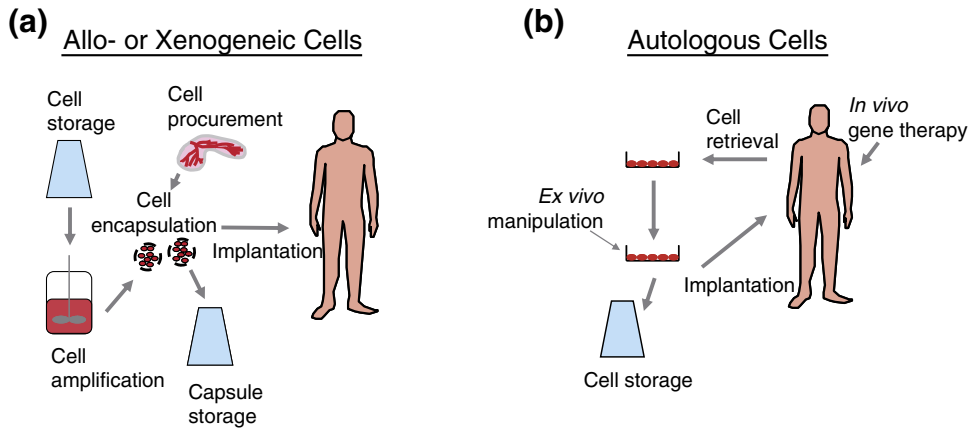

FIGURE 42.1

Approaches for bioartificial pancreas development using allo- or xenogeneic cells (a) and autologous cells (b). In (a), islets are procured from pancreatic tissue, or cells are thawed from cryostorage and expanded in culture; cells are encapsulated for immunoprotection before they are transplanted to achieve a therapeutic effect; encapsulated cells may also be cryopreserved for inventory management and sterility testing. In (b), cells are retrieved surgically from the patient; manipulated *ex vivo* phenotypically and/or genetically in order to express β cell characteristics, and in particular physiologically responsive insulin secretion; the cells are implanted for a therapeutic effect either by themselves or, preferably, after incorporation in a three-dimensional substitute; some of the cells may be cryopreserved for later use by the same individual. In *in vivo* gene therapy approaches, a transgene for insulin expression is directly introduced into the host and expressed by cells in non-pancreatic tissues.

the re-differentiation of mature endocrine cells or from an indigenous stem or progenitor cell population in the tissue isolate [10].

Animal, such as porcine, islets are amply available, and porcine insulin is very similar to human, differing only by one amino acid. Furthermore, use of closed, porcine endogenous retroviruses (PERV)-free herds is reasonably expected to alleviate the possibility of PERV transmission to human hosts. A difficulty with porcine tissue is the strong xenograft response elicited by the transplant. A combination of less immunogenic islets, islet encapsulation in semipermeable barriers, and host immunosuppression may accomplish long-term survival of the graft. Use of transgenic pigs that do not express the α-Gal (α[1,3]-galactose) epitope is one possible approach for reducing the immunogenicity of the islets. Studies also indicate that neonatal pig islets induce a lower T cell reactivity than adult islets [11], even though the α-Gal epitope is abundant in neonatal islets as well [12]. Furthermore, it is possible that the primary antigenic components in islet tissue are the ductal epithelial and vascular endothelial cells, which prominently express the α-Gal epitope; on the other hand, β cells express the epitope immediately after isolation, but not after maintenance in culture [13]. In spite of difficulties related to delayed insulin secretion as they undergo maturation, neonatal porcine islets are also receiving increased attention due to their natural resistance to hypoxia [14]. Lastly, it should be noted that the large-scale isolation of porcine islets under the conditions of purity and sterility that are necessary for their eventual clinical application pose some major technical hurdles, which have not been fully addressed yet.

β cell lines

Recognizing the substantial difficulties involved with the procurement and amplification of pancreatic islets, several investigators have pursued the development of continuous cell lines,

which can be amplified in culture, yet retain key differentiated properties of normal β cells. One of the first successful developments in this area was the generation of the βTC family of insulinomas derived from transgenic mice carrying a hybrid insulin-promoted simian virus 40 tumor antigen gene; these cells retained their differentiated features for about 50 passages in culture [15]. The hypersensitive glucose responsiveness of the initial βTC lines was reportedly corrected in subsequent lines by ensuring expression of glucokinase and of the high K_m glucose transporter Glut2, and no or low expression of hexokinase and of the low K_m transporter Glut1 [16,17]. A similar approach was used to develop the murine MIN-6 cell line that exhibits glucose-responsive secretion of endogenous insulin [18]. Subsequently, Efrat and co-workers developed the βTC-tet cell line, in which expression of the SV40 T antigen (Tag) oncoprotein is tightly and reversibly regulated by tetracycline. Thus, cells proliferate when Tag is expressed, and shutting-off Tag expression halts cell growth [19]. Such reversible transformation is an elegant approach in generating a supply of β cells via proliferation of an inoculum, followed by growth arrest when the desirable population size is reached. In any case, when retained in capsules, proliferating cells do not grow uncontrollably, since the dissolved oxygen concentration in the surrounding milieu supports up to a certain number of viable, metabolically active cells in the capsule volume. This number of viable cells is maintained through equilibration of cell growth and death processes [20–22]. Thus, growth arrest is primarily useful in preventing the growth of cells that have escaped from broken capsules *in vivo* and in reducing the cellular turnover in the capsules. The latter reduces the number or accumulated dead cells in the transplant and thus the antigenic load to the host due to antigens shed by dead and lysed cells, which pass through the capsule material.

In a different approach, Newgard and co-workers [23] carried out a stepwise introduction of genes related to β cell performance into a poorly secreting rat insulinoma (RIN) line. In particular, RIN cells were iteratively engineered to stably express multiple copies of the insulin gene, the glucose transporter Glut2, and the glucokinase gene, which are deemed essential for proper expression of β cell function. Although this is an interesting methodology, identifying and expressing all the genes necessary for reproducing β cell function in a host cell is a daunting task. Also, significant progress has been made towards establishing a human pancreatic β cell line that appears functionally equivalent to normal β cells [24]. This was accomplished through a complicated procedure involving retroviral transfection of primary β cells with the SV40 large T antigen and cDNAs of human telomerase reverse transcriptase. This resulted in a reversibly immortalized human β cell clone, which secreted insulin in response to glucose, expressed β cell transcriptional factors, prohormone convertases 1/3 and 2 that process proinsulin to mature insulin, and restored normoglycemia when transplanted in diabetic immunodeficient mice [24].

With regard to β cell lines capable of proliferation under appropriate conditions, key issues that remain to be addressed include their long-term phenotypic stability, their potential tumorigenicity, especially if cells escape from the encapsulation device, and their possible recognition by the immune system of the host.

Engineered non-β pancreatic cells

The use of non-β pancreatic cells from the same patient, engineered for insulin secretion, relaxes both the cell availability and immune acceptance limitations that exist with other types of cells. Experiments with an autoimmune diabetic mouse model indicate that such cells may not be recognized by autoimmunity [25]. The A-chain/C-peptide and B-chain/C-peptide cleavage sites on the proinsulin gene can be mutated so that the ubiquitous endopeptidase, furin, recognizes and completely processes proinsulin into mature insulin absent of any intermediaries [26]. Based on this concept, several non-endocrine cell lines have been successfully transfected to produce immunoreactive insulin, including hepatocytes, myoblasts and fibroblasts [27]. In a different approach, Lee and coworkers [28] expressed in hepatocytes

a synthetic single-chain insulin analog, which does not require post-translational processing. Although recombinant insulin expression is relatively straightforward, a key remaining challenge is achieving the tight regulation of insulin secretion in response to physiologic stimuli, which is needed for normoglycemia in higher animals and, eventually, humans.

One approach for regulating insulin secretion is through glucose control of biosynthesis at the gene transcription level, as realized in hepatocytes by Thulé et al. [29,30] and Lee et al. [28]. Besides the ability to confer transcriptional level regulation, hepatocytes are particularly attractive producers of recombinant insulin due to their high synthetic and secretory capacity and their expression of glucokinase and Glut2 [31,32]. Hepatic delivery by viral vectors and expression of the glucose-responsive insulin transgene in diabetic rats controlled the hyperglycemic state for extended periods of time [28,30,33]. Nevertheless, transcriptional regulation is sluggish, involving long time lags between stimulation of cells with glucose and induced insulin secretion, as well as between removal of glucose and downregulation of the secretory response [34]. The latter is physiologically more important, as continued insulin secretion following glucose downregulation results in potentially serious hypoglycemic excursions. Increasing the number of stimulatory glucose elements in a promoter enhances the cellular metabolic responsiveness *in vitro* [29]. With regard to secretion downregulation, Tang and Sambanis [34] hypothesized that the slow kinetics of this process following removal of the transcriptional activator are due to the stability of the preproinsulin mRNA, which continues to become translated after transcription has been turned off. Using a modified preproinsulin cDNA that produced an mRNA with two more copies of the insulin gene downstream of the stop codon resulted in preproinsulin mRNA subjected to nonsense mediated decay and thus destabilized. This significantly expedited the kinetics of secretion downregulation upon turning off transcription [34]. Thus, the combination of optimal transcriptional regulation with mRNA destabilization promises further improvements in insulin secretion dynamics from transcriptionally regulated hepatic cells. It should be noted, however, that despite the time delays inherent in transcriptional regulation, hepatic insulin gene therapy is sufficient to sustain vascular nitric oxide production and inhibit acute development of diabetes-associated endothelial dysfunction [35]. Hence, many promising aspects of the therapeutic potential of hepatic insulin expression remain to be explored.

Another appealing target cell type is endocrine cells, which possess a regulated secretory pathway and the enzymatic machinery needed to process authentic proinsulin into insulin. Early work in this area involved expression of recombinant insulin in the anterior pituitary mouse AtT-20 cell line [36], which can be subjected to repeated episodes of induced insulin secretion using non-metabolic secretagogues [37]. Co-transfection with genes encoding the glucose transporter Glut-2 and glucokinase resulted in glucose-responsive insulin secretion [38]. Limitations of this approach include possible instabilities in the cellular phenotype and secretion of endogenous hormones, such as adrenocorticotropic hormone from AtT-20 cells, which are not compatible with prandial metabolism.

In this regard, endocrine cells of the intestinal epithelium, or enteroendocrine cells, are especially promising. Enteroendocrine cells secrete their incretin products in a tightly controlled manner that closely parallels the secretion of insulin following oral glucose load in human subjects; and incretin hormones are fully compatible with prandial metabolism and glucose regulation [39,40]. As with β cells, enteroendocrine cells are polar, with sensing microvilli on their luminal side and secretory granules docked at the basolateral side, adjacent to capillaries. Released incretin hormones include the glucagon-like peptides (GLP-1 and GLP-2) from intestinal L cells and glucose-dependent insulinotropic polypeptide (GIP) from K cells, which potentiate insulin production from the pancreas after a meal [41]. The importance of enteroendocrine cells (and in particular, L cells) was first put forward by Creutzfeldt whose primary interest in these cells was for the prospect of using GLP-1 for the treatment of Type 2 diabetes [42]. Furthermore, ground-breaking work by Cheung et al. demonstrated that insulin

secreted by genetically modified intestinal K cells of transgenic mice prevented the animals from becoming diabetic after injection with streptozotocin (STZ), which specifically kills the β cells of the pancreas [43]. This is an important proof-of-concept study, which showed that enteroendocrine cell-produced insulin can provide regulation of blood glucose levels. Subsequent work with human and murine intestinal L cell lines demonstrated that these cells can be effectively transduced to express recombinant human insulin, which co-localizes in secretory vesicles with endogenous GLP-1 and is secreted with identical kinetics to GLP-1 in response to stimuli [44–47]. Furthermore, a combination of enteroendocrine L and hepatic cells, both engineered to express recombinant insulin, appears to better mimic the secretion kinetics of normal β cells than either cell type alone [48]. Although the intestinal tract is an attractive target for gene therapy because of its large size making it the largest endocrine organ in the body, enteroendocrine cell gene therapy faces several challenges. These are caused by anatomic complexity, with L cells being located at the base of invaginations of the gut mucosa called crypts, the harsh conditions in the stomach and intestine, and the rapid turnover of the intestinal epithelium.

Transdifferentation of adult non-β cells towards the β phenotype offers another promising approach towards replacing β cell function. This is generally accomplished by expressing β cell-related transcription factors in target cells. For example, expressing the pancreatic and duodenal homeobox gene 1 (PDX-1) in adult human liver cells reprogrammed them to express insulin, secrete it in response to glucose, and function *in vivo* in immunodeficient diabetic mice [49]. Reprogramming adult exocrine pancreatic cells towards the β phenotype was achieved *in vivo* in mice by adenoviral delivery of three transcription factors [50]. One of the challenges with cellular reprogramming is to express the requisite β cell function while stopping short of fully converting the target cell to a β cell that is then recognized by autoimmunity in Type 1 diabetics.

Contrary to direct *in vivo* gene delivery, *ex vivo* gene therapy involves retrieving the target cells surgically, culturing them and possibly expanding them *in vitro*, genetically engineering them to express the desired properties, then returning them to the host either as such or in a three-dimensional tissue substitute. It is generally thought that the *ex vivo* approach is advantageous, as it allows for the thorough characterization of the genetically engineered cells prior to implantation, possibly for the preservation of some of the cells for later use by the same individual, and, importantly, for localization and retrievability of the implant. However, the challenges imposed by the *ex vivo* approach, including the surgical retrieval, culturing and *in vitro* genetic engineering of the target cells, are significant, so such methods are currently under study.

Differentiated stem or progenitor cells

Naturally, throughout life, islets turn over slowly and new small islets are continually generated from ductal progenitors [51]. There exists also evidence that adult pluripotent stem cells may be a possible source of new islets [52–54]. Differentiation of human stem cells towards the b phenotype is actively being pursued by several groups, and recently success has been reported in deriving cells that secrete insulin in response to glucose from human embryonic stem cells [55,56]. Interestingly, these cells do not function well *in vitro*, but mature towards the β phenotype after transplantation into mice. Also, mouse embryonic stem cells transfected to constitutively express Pax4, a transcription factor essential for β cell development, differentiated into insulin-producing cells and normalized blood glucose when transplanted in STZ diabetic mice [57]. Overall, the existing results are quite promising but also point towards considerable additional work that needs to be done before stem or progenitor cells can be reliably differentiated into β cells in a safe way, ensuring absence of teratogenesis *in vivo*, and at a clinically relevant scale. Harnessing the *in vivo* regenerative capacity of the endocrine pancreas may also constitute a promising approach [58].

Engineering cells for enhanced survival *in vivo*

As islets or other insulin-secreting cells experience stressful conditions during *in vitro* handling and *in vivo* post-transplantation, several strategies have been implemented to enhance islet or non-islet cell survival in pancreatic substitutes. These generally focus on improving the immune acceptance of the graft, enhancing its resistance to cytokines, and reducing its susceptibility to apoptosis. Phenotypic manipulations include extended culturing of neonatal and pig islets at 37°C, which apparently reduces their immunogenicity, possibly by downregulating the major histocompatibility class 1 antigens on the islet surface; islet pretreatment with TGF-β1; and enzymatic treatment of pig islets with α-galactosidase to reduce the a-galactosyl epitope on islets [59]. However, the permanency of these modifications is unknown. For instance, a-galactosyl epitopes reappear on islets 48 hours after treatment with α-galactosidase. Surface engineering of islets by grafting of functional groups is also pursued to reduce procoagulation and proinflammatory responses post-transplantation by hepatic portal vein injection [60]. With proliferative cell lines engineered for recombinant insulin expression, selection of clones resistant to cytokines appears feasible [61].

Genetic modifications for improving survival *in vivo* may offer prolonged expression of the desired properties relative to phenotypic manipulations, but also presents the possibility of modifying the islets in additional, undesirable ways. Examples include the expression of the immunomodulating cytokines IL-4 or a combination of IL-10 and TGF-β, which promoted graft survival by preventing immune attack in mice [62]; the overexpression of the anti-apoptotic bcl-2 gene, which enhanced protection of islets when the adaptive immune response was inhibited by locally produced CTLA4Ig [63]; and the engineering of murine βTC3 insulinomas and of murine islets to express high levels of stromal cell derived factor-1 (SDF-1), which resulted in increased survival of the allografts in mice [64].

CONSTRUCT TECHNOLOGY

Construct technology focuses on associating cells with biocompatible materials in functional three-dimensional configurations. Depending on the type of cells used, the primary function of the construct can be one or more of the following: to immunoprotect the cells post-transplantation; to enable cell function; to localize insulin delivery *in vivo*; or, to provide retrievability of the transplanted cells.

Encapsulated cell systems

Encapsulation for immunoprotection involves surrounding the cells with a semipermeable barrier, in essence an ultrafiltration membrane, which allows passage of low molecular weight nutrients and metabolites, including insulin, but excludes larger antibodies and cytototoxic cells of the host. Fig. 42.2 summarizes the common types of encapsulation devices, which

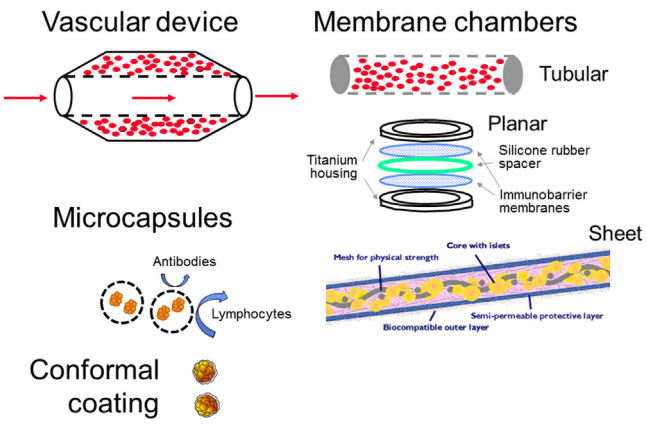

FIGURE 42.2
Schematics of commonly used encapsulation devices for islets and other insulin-secreting cells. Vascular devices and membrane chambers of tubular, planar or sheet architectures are generally referred to as macrocapsules, in distinction from the much smaller microcapsules.

include spherical microcapsules, tubular or planar diffusion chambers, thin sheets, and vascular devices.

Although several methods exist, encapsulation is generally pursued by one of two major approaches. With capsules fabricated using water-based chemistry, cells are first suspended in un-crosslinked polymer, which is then extruded as droplets into a solution of the cross-linking agent. A typical example here is the very commonly used alginate encapsulation. Alginate is a complex mixture of polysaccharides obtained from seaweeds, which forms a viscous solution in physiologic saline. Islets or other insulin-secreting cells are suspended in sodium alginate and droplets are extruded into a solution of calcium chloride. Calcium cross-links alginate instantaneously, trapping the cells within the gel. The size of the droplets, hence also of the crosslinked beads, can be controlled by flowing air parallel to the extrusion needle so that droplets detach at a smaller size than if they were allowed to fall by gravity; or, by using an electrostatic droplet generator, in which droplets are detached from the needle by adjusting the electrostatic potential between the needle and the calcium chloride bath. Capsules generated this way can have diameters from few hundred micrometers to a little over one millimeter. Alginate by itself is relatively permeable, and to generate the semipermeable barrier, beads are treated with a polycationic solution, such as poly-L-lysine or poly-L-ornithine. The reaction time between alginate and the polycation determines the molecular weight cutoff of the generated membrane. Poly-L-lysine is highly inflammatory *in vivo*, however, beads are coated with a final layer of alginate to improve their biocompatibility. Hence, calcium alginate/poly-L-lysine/alginate (APA) beads are finally formed. The permanency of the last alginate coat, however, is questionable. Treating the beads with a calcium chelator, such as citrate, presumably liquefies the inner core forming APA membranes. Other materials that have been used for cell microencapsulation include agarose, photocrosslinked poly(ethylene glycol), chitosan, and various copolymers [58,65,66]. The advantages of hydrogel microcapsules include a high surface-to-volume ratio and thus good transport properties, as well as ease of handling and transplantation. Small beads can be transplanted in the peritoneal cavity of animals simply by injection; without the need for incision. Other transplantation sites include the subcutaneous space and the kidney capsules. Disadvantages include the fragility of the beads, especially if the crosslinking cation becomes chelated by compounds present in the surrounding milieu or released by lysed cells, and the difficult retrievability once the beads have been dispersed in the peritoneal cavity of a host. Earlier problems caused by the variable composition of alginates and the presence of endotoxins have been resolved through the development and commercial availability of ultrapure alginates of well-defined molecular weight and composition [67,68].

Hydrogels impose little diffusional resistance to solutes, and indeed effective diffusivities in calcium alginate and agarose hydrogels are in the range of 50–100% of the corresponding diffusivities in water [69,70]. However, with conventional microencapsulation, the volume of the hydrogel contributes significantly to the total volume of the graft. For example, with a 500 μm microcapsule containing a 300 μm islet, the polymer volume constitutes 78% of the total capsule volume. Additionally, conventional microcapsules are not appropriate for hepatic portal vein infusion as, besides their higher graft volume relative to the same number of naked islets, they result in higher portal vein pressure and more incidences of blood coagulation in the liver. To address this problem, methods have been developed for islet encapsulation in thin conformal polymeric coatings. Materials that have been used for conformal coating include photopolymerized poly(ethylene glycol) (PEG) diacrylate [71,72], hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) [73,74], and poly(L-lysine)-poly(ethylene glycol)(biotin) (PPB) and streptavidin (SA) in PPB/SA multilayers [75]. Conjugation of glucagon-like peptide 1 (GLP-1) to PEG hydrogels has been shown to reduce islet cell death due to photopolymerazation [76]; the antiapoptotic and insulinotropic effects of GLP-1 may also help islet survival and function *in vivo*. Conformal coatings offer a promising approach for islet encapsulation, however, the stability, continuity for complete islet coverage, and permeability

of the coatings remain to be addressed. Ultimately, the *in vivo* efficacy of conformal-coated islets needs to be established, as such data are mostly lacking.

Encapsulated cell systems can also be fabricated by preforming the semipermeable membrane in a tubular or disk-shaped configuration, filling the construct with a suspension of islets or other insulin-secreting cells in an appropriate extracellular matrix, and then sealing the device. This approach is particularly useful when organic solvents or other chemicals harsh to the cells are needed for the fabrication of the membranes. Membrane chambers can be of tubular or planar geometry (Fig. 42.2). The cells are enclosed in the semipermeable membrane and can be transplanted intraperitoneally, subcutaneously, or at other sites. Membrane materials used in fabricating these devices include polyacrylonitile-polyvinyl chloride (PAN-PVC) copolymers, polypropylene, polycarbonate, cellulose nitrate, and polyacrylonitrile-sodium methallylsulfonate (AN69) [65,67,77,78]. Typical values of device thickness or fiber diameter are 0.5 to 1 mm. Advantages of membrane chambers are the relative ease of handling, the flexibility with regard to the matrix in which the cells are embedded, and retrievability after transplantation. A major disadvantage is their inferior transport properties, since the surface-to-volume ratio is smaller than that of microcapsules and diffusional distances are longer.

Constructs connected to the vasculature via an arteriovenus (AV) shunt consist of a semipermeable tube surrounded by the cell compartment (Fig. 42.2). The tube is connected to the vasculature, and transport of solutes between the blood and the cell compartment occurs via the pores in the tube wall. A distinct advantage of the vascular device is the improved transport of nutrients and metabolites, which occurs both by diffusion and convection. However, the major surgery that is needed for transplantation and problems of blood coagulation at the anastomosis sites has reduced the enthusiasm felt for these devices.

Other construct systems

A common approach for improving the oxygenation of cells in diffusion chambers is to encourage the formation of neovasculature around the graft. This will be discussed in the 'In vivo transplantation' section. Other innovative approaches that have been proposed include the electrochemical generation of oxygen in a device adjacent to a planar immunobarrier diffusion chamber containing the insulin-secreting cells [79]; the co-encapsulation of islets with algae, where the latter produce oxygen photosynthetically upon illumination [80]; and the use of biomaterials that are hydrolytically activated to generate oxygen [81]. More work is clearly needed to evaluate whether these approaches can be translated into effective and clinically usable *in vivo* configurations.

In a different design, Cheng et al. combined constitutive insulin-secreting cells with a glucose-responsive material in a disk-shaped construct [82,83]. As indicated earlier, it is straightforward to genetically engineer non-β cells for constitutive insulin secretion; the challenge is engineering appropriate cellular responsiveness to physiologic stimuli. In this proposed device, a concanavalin A (con A)-glycogen material, sandwiched between two ultrafiltration membranes, acted as a control barrier to insulin release from an adjacent compartment containing the cells. Con A-glycogen forms a gel at low concentrations of glucose, which is reversibly converted to sol at a high glucose concentration as glucose displaces glycogen from the gel network. Since insulin diffusivity is higher through the sol than through the gel, insulin secreted by the cells during low glucose periods diffused slowly through the gel material; when switched to high glucose, insulin that had accumulated in the cell compartment during the previous cycle was released at a faster rate through the sol-state polymer. Overall, this approach converted the constitutive secretion of insulin by the cells to a glucose-responsive insulin release by the device [82]. However, these were *in vitro* studies, and the *in vivo* efficacy of such hybrid devices remains to be evaluated.

Construct design and *in vitro* evaluation

Design of three-dimensional encapsulated systems can be significantly enabled using mathematical models of solute transport through the tissue and of nutrient consumption and metabolite production by the cells. Beyond the microvasculature surrounding the construct, transport of solutes occurs by diffusion, unless the construct is placed in a flow environment, in which case convective transport may also occur. Due to its low solubility, transport of oxygen to the cells is the critical issue. Models can be used to evaluate the dimensions and the cell density within the construct so that all cells are sufficiently nourished and the capsule as a whole is rapidly responsive to changes in the surrounding glucose concentration [70]. Experimental and modeling methods for determining transport properties and secretion kinetics have been described [84]. Furthermore, models can be developed to account for the cellular reorganization that occurs in constructs with time as a result of cell growth, death, and possibly migration processes [20]. Such reorganization is significant when the encapsulated cells proliferate in the capsule environment [20,68,85] and it may result in changes in the secretory properties of capsules as the intracapsular environment remodels [86].

Pancreatic tissue substitutes should be evaluated *in vitro* prior to transplantation in terms of their ability to support the cells within over prolonged periods of time and to exhibit and maintain their overall secretory properties. Long-term cultures can be performed in perfusion bioreactors under conditions simulating aspects of the *in vivo* environment. In certain studies, the bioreactors and support perfusion circuits were made compatible with a nuclear magnetic resonance spectrometer. This allowed measurement of intracellular metabolites, such as nucleotide triphosphates, as a function of culture conditions and time without the need to extract the encapsulated cells [21,22,87,88]. Such studies produce a comprehensive understanding of the intrinsic tissue function in a defined and controlled environment prior to introducing the additional complexity of host-graft interactions in *in vivo* experiments.

The secretory properties of tissue constructs can be evaluated with low time resolution in simple static culture experiments by changing the concentration of glucose in the medium and measuring the secreted insulin. In general, a square wave of insulin concentration is implemented, from basal to inducing to basal conditions for insulin secretion. To evaluate the secretory response at a higher time resolution, perfusion experiments need to be performed, in which medium is made to flow around the tissue and secreted insulin is assayed in the effluent. Again, a square wave of glucose concentration is generally implemented. By comparing the secretory dynamics of free and encapsulated cells, one can ensure that the encapsulation material does not introduce excessive time lags compromising the secretory properties of the construct. Indeed, properly designed hydrogel microcapsules introduce only minimal secretory time lags [70,86,89]. Secretion measurements by perfusion on explanted capsules also shed light on changes that occur to the graft *in vivo*.

Manufacturing considerations

Fabrication of pancreatic substitutes of consistent quality requires the use of cells which are also of consistent quality. Although with clonal, expandable cells this is a straightforward issue, with islets isolated from human and animal tissues there can be significant variability in the quantity and quality of the cells in the preparations. With islets from cadaveric human donors, the quality of the isolates is assessed by microscopic observation, viability staining, and possibly a static insulin secretion test. It is generally recognized, however, that a quantitative, objective assessment of islet quality would help improve the consistency of the preparations. Assays evaluating mitochondrial function and oxygen consumption rate, especially when conducted with intact islets, are promising in evaluating their quality and are being established as standard tests prior to transplantation [90].

It is conceivable that encapsulated cell systems could be fabricated at a central location from which they would be distributed to clinical facilities for transplantation. In this scheme, preservation of the constructs for long-term storage, inventory management and, importantly, sterility control would be essential. Cryopreservation appears to be a promising method for maintaining fabricated constructs for prolonged time periods. Although there have been significant studies on the cryopreservation of single cells and some tissues, the problems pertaining to cryopreserving artificial tissues are in the process of being addressed. Cryopreservation of murine insulinomas and islets in microcapsules appears feasible [91–95], however, cryopreservation of macroencapsulated systems is expected to be particularly challenging.

IN VIVO TRANSPLANTATION

This section highlights results from *in vivo* experiments using the different configurations outlined above. Results with encapsulated cell systems are presented first. Since *in vivo* experiments with non-β cells engineered for insulin secretion are at present based mostly on *in vivo* gene therapy approaches, these are described next. Technologies for the *in vivo* monitoring of cells and constructs and graft retrievability are then discussed.

Encapsulated cell systems

In vivo experiments with pancreatic substitutes are numerous in small animals, limited in large animals, and few in humans. Allogeneic and xenogeneic islets in hydrogel microcapsules transplanted in diabetic mice and rats have generally restored normoglycemia for prolonged periods of time. In the early study of O'Shea et al. [96], islet allografts encapsulated in APA membranes were transplanted intraperitoneally in STZ-induced diabetic rats. Of the five animals which received transplants, three remained normoglycemic for more than 100 days, and one of these three remained normoglycemic 368 days post-transplantation. Microencapsulation in alginate crosslinked with barium without a traditional semipermeable barrier allowed prolonged survival of syngeneic and allogeneic transplanted islets in diabetic BALB/c and non-obese diabetic (NOD) mice for more than 350 days [97]. Interestingly, adult porcine islet (API) xenografts encapsulated in the same type of capsule also restored normoglycemia in STZ-induced diabetic mice for at least one month [98] or, in another study [99], for up to 190 days, the latter in spite of the presence of an antibody response.

On the other hand, with the NOD mouse model, it was found that encapsulation alone prolonged *in vivo* survival of APIs from less than one week to 35 ± 14 for microencapsulated APIs [100]. With continuous costimulatory blockade comprised of CTLA4-Ig and anti-CD154 monoclonal antibodies, non-encapsulated API functioned for 27 ± 4 days, whereas microencapsulated API functioned for more than 450 days [100]. Thus, microencapsulation and costimulatory blockade acted synergistically, at least for APIs in the NOD mouse model. Normoglycemia has also been restored in rodents transplanted with encapsulated cell lines, such as APA-encapsulated βTC6-F7 insulinomas in diabetic rats, where diabetes was reversed for up to 60 days [101], and APA-encapsulated βTC-tet insulinomas in NOD mice, where normoglycemia was restored for at least eight weeks [102]. With a human cell line [24], experiments were performed with un-encapsulated cells transplanted into STZ-induced diabetic severe combined immunodeficiency mice. Control of blood glucose levels started within two weeks post-transplantation, and mice remained normoglycemic for longer than 30 weeks [24]. Besides rodents, long-term restoration of normoglycemia with microencapsulated islets has been demonstrated in large animals, including pancratectomized dogs, where normalization of fasting blood glucose levels was achieved with encapsulated islet allografts in nine out of nine dogs for up to 214 days [103]. Also, in one study with porcine islet xenografts transplanted in spontaneously diabetic non-human primates (NHPs), animals became insulin independent for periods ranging from 120 to 804 days [104]. These results with NHPs, however, have not

been reproduced. Successful results with NHPs usually involve reduction of exogenous insulin requirements to maintain normoglycemia, as in the study of Elliott et al. with encapsulated neonatal porcine islets in cynomolgus recipients [105]. Also, in a study with non-diabetic NHPs transplanted with sub-therapeutic levels of alginate-encapsulated adult pig islets in NHPs, islet survival was reported for up to six months, even in the presence of antibody response [106]. Overall, in spite of certain promising results, correcting diabetes in NHPs by encapsulated islet grafts without exogenous insulin injections remains a daunting task.

Human studies with encapsulated islet transplants are few and far in between. In an earlier study involving encapsulated allogeneic islets, the graft reportedly provided tight glycemic control for nine months [107]; however, there is general skepticism about the validity of these results. In another study, also with encapsulated allografts transplanted intraperitoneally, Type 1 diabetic patients remained non-immunosuppressed, but exogenous insulin had to be provided [108]. Encapsulated neonatal porcine islets transplanted intraperitoneally in a Type 1 diabetic did not render the patient insulin independent, but reduced the exogenous insulin requirement and improved glycemic regulation, as evidenced by a reduction in total glycated hemoglobin [109]. Currently, Phase I and II clinical trials are ongoing in New Zealand and Russia by Living Cell Technologies Limited; in these, encapsulated neonatal porcine islets are transplanted intraperitoneally with laparoscopy.

In vivo results with vascular devices are mixed. Implantation of devices containing allogeneic islets as arteriovenous shunts in pancreatectomized dogs resulted in 20–50% of the dogs becoming normoglycemic for up to 10 weeks post-transplantation without exogenous insulin administration. When xenogeneic bovine or porcine islets were used, only 10% of the dogs remained normoglycemic 10 weeks post-transplantation. All dogs were reported to be diabetic or dead after 15 weeks [110]. A hollow fiber device composed of polyethylene-vinyl alcohol fibers and a poly-amino-urethane-coated, non-woven polytetrafluoroethylene fabric seeded with porcine islets provided normalization of the blood glucose levels in totally pancreatectomized pigs when connected to the vasculature of the animals [111]. It should be noted however, that the overall interest in vascular devices has faded due to the surgical and blood coagulation challenges that they pose.

Although several hypotheses exist, the precise cause of the eventual *in vivo* failure of encapsulated cell systems remains unclear. There exists increasing evidence that two possible causes for the gradual attrition and eventual failure of encapsulated grafts are small molecular weight cytotoxic compounds, including cytokines and nitrogen oxide, and hypoxia. Indeed, encapsulation does not completely prevent the immune recognition of the graft, and antigens shed by the encapsulated cells as a result of secretion and, more importantly, lysis eventually pass through the semipermeable barrier and are recognized by the antigen-presenting cells of the host. Passage of low molecular weight molecules cannot be prevented by immunoprotective membranes imposing a molecular weight cutoff on the order of 50 kDa. Overall, optimal properties of immunoprotective barriers remain largely unknown and may actually depend on the type of the host and the transplanted encapsulated cells. Transplantation sites, including the peritoneal cavity, are also intrinsically hypoxic, and transplantation of encapsulated insulin-secreting cells further reduces the dissolved oxygen concentration at the transplantation site, apparently as a result of the additional metabolic load to the host [112,113], to levels that affect secretion from pancreatic islets [114]. Sustained hypoxia also results in viability loss.

Non-specific inflammation may also occur around the graft and develop into a fibrous capsule, so reducing the oxygen available to the cells within. The fibrotic layer has been found to consist of several layers of fibroblasts and collagen with polymorphonuclear leukocytes, macrophages, and lymphocytes. The surface roughness of the membrane may also trigger inflammatory responses. In one study, membranes with smooth outside surfaces exhibit a minimal fibrotic reaction 10 weeks post-transplantation, regardless of the type of encapsulated cells, whereas

rough surfaces elicited a fibrotic response even one week post-transplantation [115]. Use of high purity materials also helps minimize inflammatory reactions. If a material is intrinsically inflammatory, such as poly-L-lysine, it can be coated with a layer of non-inflammatory material, such as alginate, to minimize the host's reaction. Such coverage may not be sufficiently permanent, though, resulting in the eventual fibrosis of the graft. Indeed, several investigators report improved results with plain alginate beads without a poly-L-lysine layer, especially when allogeneic cells are used in the capsules.

Provision of nutrients to and removal of metabolites from encapsulated cells can be especially challenging *in vivo*. Normal pancreatic islets are highly vascularized and thus well oxygenated. There exists evidence that un-encapsulated islets injected in the portal system of the liver become re-vascularized, which enhances their engraftment and function. On the other hand, encapsulation prevents re-vascularization, so the transplanted tissue is nourished by diffusion alone. Promotion of vascularization around the immunoprotective membrane increases the oxygenation of the transplanted islets [116]. Interestingly, transformed cells, such as the βTC3 line of mouse insulinomas, are more tolerant of hypoxic conditions than intact islets; such cells may thus function better than islets in transplanted capsules [117]. However, with transformed cells, too, enhanced oxygenation increases the density of functional cells that can be effectively maintained within the capsule volume. Vascularization is dependent on the microarchitecture of the material, which should have pores of 0.8–8.0 μm in size allowing permeation of host endothelial cells [118,119]. Vascularization is also enhanced by the delivery of angiogenic agents, such as Fibroblast Growth Factor-2 (FGF-2) and Vascular Endothelial Growth Factor (VEGF), possibly with controlled release devices [120]. Although vascularization can be promoted around a cell-seeded device, improved success has reported if a cell-free device is first implanted and vascularized, then the cells are introduced. An example of this procedure involved placing a cylindrical stainless steel mesh in the subcutaneous space of rats with the islets introduced 40 days later [121]. Replacement of a vascularized graft is challenging, however, due to the bleeding that occurs. A solution to this problem may entail the design of a device that can be emptied and refilled with a suspension of cells in an extracellular matrix without disturbing the housing and the associated vascular network.

Gene and cell-based therapies

In vivo efficacy studies with gene therapy and non-β cells genetically engineered for insulin secretion are generally limited to small animals. Intraportal injection of recombinant adenovirus expressing furin-compatible insulin under the control of a glucose-responsive promoter containing elements of the rat liver pyruvate kinase gene restored near-normal glycemia in STZ diabetic rats for periods of one to twelve weeks [30]. With hepatic delivery of a recombinant adeno-associated virus expressing a single-chain insulin analog under the control of an L-type pyruvate kinase promoter, Lee and coworkers [28] controlled blood glucose levels in STZ diabetic rats and NOD mice for periods longer than 20 weeks. However, transiently low blood glucose levels observed 3–5 hours after glucose loading indicated a drawback of the transcriptional regulation of insulin expression, which may result in hypoglycemic episodes [28]. Possible approaches towards ameliorating this problem include optimizing the number of glucose-regulatory and insulin-sensing elements in the promoter [122] and destabilizing the preproinsulin mRNA; the latter has been shown to significantly expedite the downregulation of secretion dynamics from transcriptionally controlled cells upon removal of the secretory stimulus [44].

In vivo gene therapy with small animals has also shown success when the target cells for insulin expression were intestinal endocrine K or gastric G cells. Using a transgene expressing human insulin under the control of the GIP promoter, Cheung et al. expressed insulin specifically in gut K cells of transgenic mice, which protected them from developing diabetes following STZ-mediated destruction of the native β cells [43]. Similarly, use of a tissue-specific promoter to

express insulin in gastric G cells of mice resulted in insulin release into circulation in response to meal-associated stimuli, suggesting that G cell insulin expression is beneficial in the amelioration of diabetes [123]. Translation of these approaches to adult and large animals and, eventually, humans, requires the development of effective methods of gene delivery to intestinal endocrine or gastric cells *in vivo* or the development of effective *ex vivo* gene therapy approaches.

In vivo monitoring

Monitoring of the number and function of insulin-secreting cells *in vivo* would provide valuable information directly on the transplant and possibly offer early indications of graft failure. Additionally, in animal experiments, the ability to monitor a transplant non-invasively reduces the number of animals that are needed in the experimental design and helps establish a critical link between transplantation and end-point physiologic effects, the latter commonly being blood glucose levels and animal weight and general well being.

Imaging techniques can provide unique insight into the structure/function relationship of a construct *in vitro* and *in vivo*. There are several imaging modalities that have been applied to monitor tissue-engineered constructs, including computed tomography (CT), positron emission tomography (PET), optical techniques and nuclear magnetic resonance (NMR) imaging and spectroscopy. Among these, NMR offers the unique advantage of providing information on both construct integrity and function without the need to genetically modify the cells (e.g., through the expression of green fluorescent protein or luciferase, used in optical methods) or the introduction of radioactive labels (e.g., PET agents). Furthermore, since magnetic fields penetrate uniformly throughout the sample, NMR is ideally suited to monitor constructs transplanted at deep-seated locations. Its disadvantage is its low sensitivity. Whereas optical and radionuclide techniques can detect tracer quantities, NMR detects metabolites that are available in the millimolar or, in some cases, sub-millimolar range. Nonetheless, NMR has been used successfully to monitor *in vivo* agarose disk-shaped constructs containing mouse insulinoma cells and transplanted in the peritoneal cavity of mice. Construct integrity was visualized by MR imaging and the metabolic activity of the cells within by water-suppressed ^{1}H NMR spectroscopy [124]. Labeling of cells or of the encapsulation matrix with magnetic nanoparticles which can be detected by magnetic resonance [125], and genetically engineering cells so that they express a fluorescent or luminescent marker that can be optically detected [126], are other methods being pursued to track the location and possibly viability and function of transplanted cells *in vivo*. Incorporation of a perfluorocarbon emulsion in the encapsulation matrix may also help assess the metabolic activity of a transplant through measurement of dissolved oxygen levels by ^{19}F NMR [113]. It is expected that development of robust monitoring methodologies will be helpful not only in experimental development studies but also in eventual clinical applications.

Retrievability

The issue of construct retrievability needs to be considered for all pertinent applications. Useful lifetimes of constructs are limited, so repeated transplantation of cells will likely be required. Although guidelines have not been established, it can be reasonably expected that failed constructs will need to be removed periodically. Hence, technologies for graft retrieval under minimally invasive conditions are also likely to be needed.

Concluding Remarks

Tight glycemic regulation in insulin-dependent diabetics significantly improves their overall health and reduces the long-term complications of the disease. A pancreatic substitute holds significant promise at accomplishing this in a relatively non-invasive way. However, to justify the improved outcome, a substitute needs to be not only efficacious in terms of insulin

secretion, but also immunologically acceptable. A number of approaches are being pursued to address this obstacle and additionally develop constructs that can be manufactured at a clinically relevant scale. However, a number of challenges remain to be addressed before reproducible success in large animal models can be claimed and constructs can be moved to clinical trials. Encapsulation in semipermeable barriers improves the immune acceptance of allo- and xenografts, but it is quite doubtful that encapsulation will, by itself, ensure long-term survival and function of xenografts in non-immunosuppressed hosts. The development of specific, benign immune suppression protocols that work in concert with encapsulation appears necessary. Reducing the immunogenicity of transplanted cells and modifying them so that they better withstand the encapsulation and *in vivo* environment are appropriate strategies. To ensure that substitutes can be fabricated at the necessary scale, methods to expand pancreatic islets in culture, to produce β-like cells from stem or progenitor cells, or to generate expandable β cell lines with appropriate phenotypic characteristics need to be pursued. In alternative approaches involving genetic engineering of non-β cells towards the β phenotype, or transdifferentiation of adult non-β cells, the major problem is ensuring precise regulation of insulin secretion by glucose or other physiologic stimuli and phenotypic stability of the engineered cells. This poses a different set of problems which, however, are equally challenging to those of β cell procurement and immune acceptance. Methods for the preservation of substitutes and for the non-invasive monitoring of their integrity and functionality *in vivo* are integral parts of construct development and characterization in regards to construct manufacturing and assessment of *in vivo* efficacy, respectively.

As in many aspects of life, with challenges come opportunities. It is essential that multiple approaches be pursued in parallel, as it is currently unclear which ones will eventually develop into viable therapeutic procedures. If more than one method evolves into a clinical application, this would be welcome news, as it may allow flexibility in the personalization of therapy.

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CHAPTER 43

Thymus and Parathyroid Organogenesis

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STRUCTURE AND MORPHOLOGY OF THE THYMUS

The mature thymus is a highly dynamic cellular environment, comprised of developing T lymphocytes [1–3] (thymocytes) – which make up over 95% of its cellularity – and a range of stromal elements that includes mesenchymal cells, bone marrow (BM) derived cells, vasculature, and the uniquely specialized thymic epithelium (TE) [4]. This complex cellularity, and the functional importance of the spatial organization of the different cellular components of the organ, present challenges for strategies aiming to generate a functional, self-tolerant T cell repertoire *in vivo* or to develop a functional thymus for transplantation.

The thymus is an encapsulated and lobulated organ, and contains three principal histologically defined regions, the cortex, the medulla and the subcapsule (Fig. 43.1). The capsule and trabeculae consist of a thick layer of connective tissue, and are separated from the cortex by a thin layer of simple epithelium, the subcapsule [4]. The cortex and medulla each contain open networks of epithelial cells, which are densely packed with thymocytes [4–6], and each of the cortical and medullary regions contains several different morphologically and phenotypically distinct epithelial subtypes (see below). These thymic epithelial cells (TEC) are a key element of the thymic stroma, providing many of the organ's specialist functions. The outer cortex also contains fibroblasts, and the organ as a whole is heavily vascularized. BM-derived stromal cells also exist and are found in both compartments, macrophages being distributed throughout the organ, while thymic dendritic cells – which are required for imposition of tolerance on the emerging T cell repertoire – are found predominantly at the cortico-medullary junction (CMJ) and in the medulla [4].

Thymus structure is intimately linked to its function, which is to support T cell development. This encompasses the linked processes of T cell differentiation and T cell repertoire selection, which together ensure that the peripheral T cell repertoire is predominantly populated by T cells that respond to antigens in the context of self-major histocompatibility antigens (MHC), but which are not activated by binding self-peptides. T cell development has been extensively reviewed elsewhere [7–14] and is not discussed in detail herein.

In brief, thymocyte progenitors enter the postnatal thymus at the CMJ, and subsequent T cell development is then regulated such that thymocytes at different stages of development are

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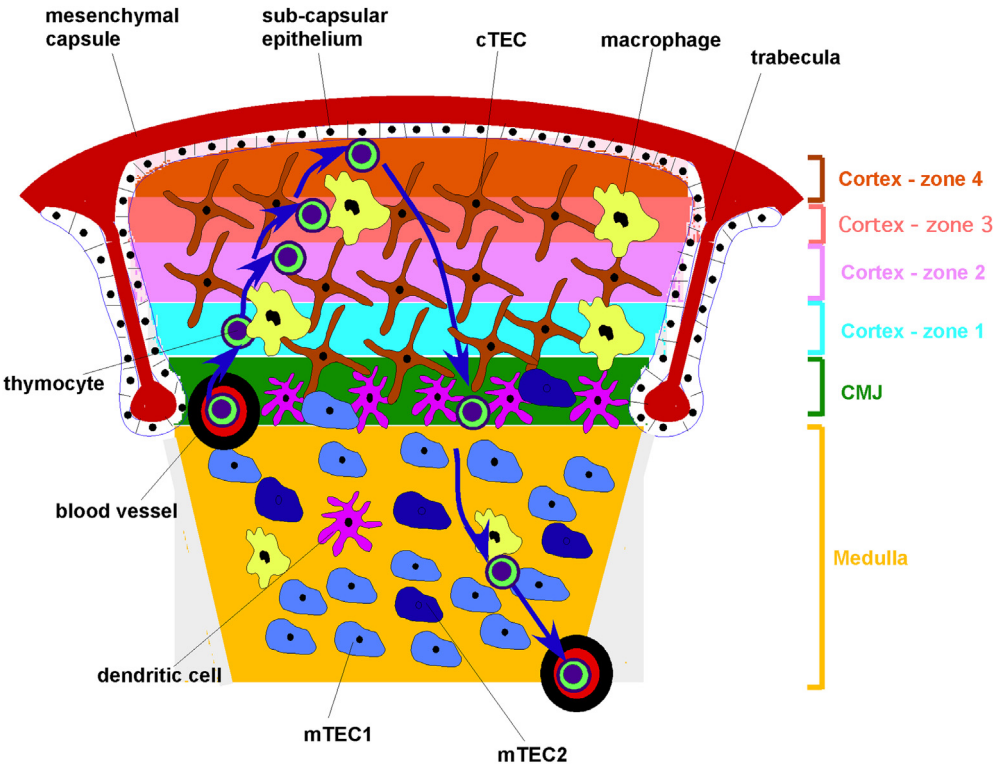

FIGURE 43.1

Histology of the postnatal thymus. The postnatal thymus is surrounded by a capsule consisting of mesenchymal cells and connective tissue, which penetrates into the thymus at regular intervals to form trabeculae. Underlying the capsule and trabeculae is the sub-capsular epithelium consisting of a layer of simple epithelium, which overlies the outer cortex. The cortex is populated with cortical thymic epithelial cells (cTEC), macrophages and developing thymocytes at the double negative (DN) and double positive (DP) stages of development. Thymocytes enter the thymus at the cortico-medullary junction (CMJ) via the vasculature and migrate through the cortex to the subcapsule as they differentiate. The cortex can be divided into four zones based on the differentiation status of thymocytes that reside within it. Thus, zone 1 contains the most immature DN1 thymocytes and zone 4 contains thymocytes undergoing the DN4-DP transition. DP thymocytes are then screened for propensity to recognize self-MHC, a process termed 'positive selection', and those selected to mature into $CD4^+$ or $CD8^+$ single positive (SP) cells migrate into the medulla, where they undergo the final stages of maturation before being exported to the periphery. Central tolerance is established by deletion of self-reactive thymocytes in a process termed 'negative selection', and occurs principally at the cortico-medullary junction and in the medulla: negative selection is mediated both by thymic dendritic cells and medullary TECs. Medullary TECs are also required for the generation of $CD4^+CD25^+$ T regulatory (Treg) cells and natural killer T cells, both of which actively repress self-reactive T cells.

found in different intrathymic locations. The earliest thymocyte progenitors do not express the CD4 or CD8 co-receptors and are referred to as double negative (DN) cells. DN thymocyte progenitors undergo a highly ordered series of maturation stages in the thymic cortex and upregulate expression of CD4 and CD8 to become the major double positive (DP) thymocyte subset. The cortex itself can be subdivided into four regions based on the localization of thymocyte populations at different stages of development. Thus, zone 1 contains the colonizing population of thymocyte progenitor cells; these early thymocytes undergo proliferative expansion in zone 2; T cell lineage commitment is completed in zone 3; and in zone 4, thymocytes differentiate to the DP stage of development, characterized by expression of both CD4 and CD8 co-receptors [15,16](see Fig. 43.1). Only DP thymocytes expressing T cell receptors that mediate low affinity interactions with self-peptide/MHC complexes, presented on cortical epithelial cells, are positively selected. These positively selected thymocytes survive and mature into $CD4^+$ or $CD8^+$ single positive (SP) cells. Thymocytes that have been positively selected then migrate into the medulla [17,18]. The remaining DP thymocytes, which fail positive selection, die by apoptosis [19].

In the medulla, a wide array of self-antigen/MHC complexes presented by epithelial cells and/or dendritic cells induce apoptosis of those SP thymocytes that express T cell receptors with high affinity for self-antigens [20,21]. This process, termed 'negative selection', is essential to reduce the frequency of autoreactive T cells in the peripheral T cell repertoire. SP thymocytes proliferate and undergo the final stages of T cell maturation in the medulla before emigrating from the thymus to enter into the peripheral immune system. Some high affinity self-reactive thymocytes escape negative selection and instead differentiate into T regulatory (Treg) cells, which suppress autoreactive T cells in the periphery and are therefore an important component of self-tolerance [22–24].

Intrathymic migration of thymocytes is at least partly a regulated process. Both the outward migration of thymocytes from the CMJ to the outer cortex [25], and the migration of positively selected cells from the cortex into the medulla [14,17,26–29] are controlled by chemokines.

Thymic epithelial cells

MORPHOLOGICAL AND PHENOTYPIC CLASSIFICATION

The TE within the sub-capsular/sub-trabecular, cortical and medullary regions is highly heterogeneous in terms of immnophenotype, morphological characteristics, and function. Ultrastructural and immunohistochemical analyses have revealed six different subtypes of TEC [30], assigned as 'clusters of thymic epithelial staining' [31–34]. Ultrastructural analysis also revealed large complexes of TECs and developing thymocytes [34], termed 'thymic nurse cells' (TNCs) [35,36]. In addition some epithelia identified in small isolated clusters at the corticomedullary junction were classified as undifferentiated cells [34] which were proposed to be precursors of differentiated epithelial cells [37]. These analyses give an indication of the complexity of the TE, but appear to underestimate the number of TEC subtypes present in the mature organ based on more recent immunophenotyping data.

The different TE subpopulations have now been defined by differential expression of cytokeratins (K) and other markers including MHC Class II. All TEC express MHC Class I, while MHC Class II expression is variable [38–41]. In both the cortex and the medulla, high levels of surface MHC Class II are thought to identify the most mature TECs [42–44].

Two cortical populations have been identified based on cytokeratin expression – a predominant $K5^{-}K14^{-}K8^{+}K18^{+}$ subset and a minor subset consisting of $K5^{+}K14^{-}K8^{+}K18^{+}$ cells that is found at the CMJ and scattered throughout the outer cortex [45]. Most if not all cortical (c) TECs also express the determinants recognized by mAbs Ly-51 [46], which recognizes the homodimeric cell surface glycoprotein BP-1, and CDR1 [47], the target of which is not yet biochemically defined. cTEC additionally express the endocytic receptor CD205 [48–50], and the TEC-specific catalytic proteasome subunit, β5t [51–53].

Most medullary (m) mTEC are $K5^{+}K14^{+}K8^{-}K18^{-}$ [45] and also bind mAb MTS10 [33], while a minor $K5^{-}K14^{-}K8^{+}K18^{+}$ $MTS10^{-}$ mTEC subset also exists [45]. mTECs also express high levels of epithelial cell adhesion molecule (EpCAM)[54], and bind the lectin *Ulex europeus agglutinin-1* (UEA-1)[55], with a subset of $K14^{-}$ mTECs binding UEA-1 at high levels. mTECs can be further subdivided based on CD80 expression, the level of which correlates with degree of functional maturation [42,56]. The lineage relationships between these different TEC subsets, and between cortical and medullary TECs are not well defined and this is a subject of much current investigation.

FUNCTIONAL DIVERSITY

The clear functional dichotomy between the cortical and medullary compartments of the mature thymus (see the section 'Structure and morphology of the thymus' above) is reflected in functional differences between the cortical and medullary TEC. cTEC express Delta-like 4 (Dll4), the Notch ligand required throughout the lifespan for commitment of thymocyte

progenitors to the T cell lineage [57–59], and the chemokine CXCL12 (also known as stromal cell-derived factor 1 alpha (SDF1α)), a regulator of the β-selection checkpoint which controls progression from the DN to the DP stage of thymocyte development [60–62]. Additionally, cTEC express ligands required for positive selection [63]. Notably, β5t is required for generating an optimally selected $CD8^+$ T cell repertoire. The β5t-containing 'thymoproteasome' generates a specific set of self-peptides for presentation by MHC Class I, and in the absence of β5t, a functionally deficient $CD8^+$ TCR repertoire is selected [52,64–66].

mTECs play an essential role in development central tolerance, by mediating both negative selection of the T cell repertoire and development of Tregs and natural killer T cells, both of which actively repress self-reactive T cells [24,67,68]. Additionally, they regulate migration of positively selected thymocytes into the medulla, via expression of CCL19 and 21 [27]. mTECs regulate central tolerance through expression of a broad repertoire of proteins, termed Tissue Restricted Antigens (TRAs), that are otherwise tissue or developmentally restricted [69–71]. Expression of a subset of these genes is regulated by the *Autoimmune Regulator, Aire*, which, within the thymus, is specifically expressed in mTEC [72–78]. In the absence of Aire, mice and humans develop the broad-spectrum autoimmune syndrome, APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) [75,76]. Aire is expressed in $CD80^{hi}$ mTEC, and most Aire-dependent and Aire-independent TRAs are induced in the $CD80^{hi}$ population [70].

The medulla is also distinguished by corpuscular bodies of epithelial cells known as Hassall's corpuscles. These are particularly prevalent in human thymus and have been shown to express thymic stromal lymphopoietin, a signaling molecule that instructs closely associated dendritic cells in the medulla to induce Treg cells [22,23]. In this regard, mTEC also regulate the accumulation and positioning of dendritic cells in the medulla via secretion of the chemokine XCL1 [79].

IN VITRO T CELL DIFFERENTIATION

The high level of phenotypic and functional heterogeneity demonstrated above presents a significant challenge for attempts to support full T cell development, including proper repertoire selection, *in vitro* and is also pertinent to cell replacement or regenerative strategies for enhancing thymus activity *in vivo*. Currently, it is not possible to reconstitute full thymus function *in vitro* or on transplantation except in cultures based on *ex vivo* thymus tissue. However, *in vitro* systems that fully or partly support T cell differentiation are widely used as a tool for investigating regulation of this process [80] and recently, the use of *in vitro* culture of hematopoietic progenitors to enhance lymphohematopoietic reconstitution following bone marrow transplantation has been investigated. Current approaches to *in vitro* T cell generation are summarized below.

Fetal thymic organ culture (FTOC) utilizes *ex vivo* thymic lobes, usually derived from E15.5–E16.5 mouse embryos or second-trimester human fetuses, to support the differentiation of T cell progenitors from endogeneous or exogenous sources [81–85]. The technique of reaggregating fetal thymic organ culture (RFTOC), in which defined TEC subpopulations are obtained by cell purification techniques, reaggregated with fibroblasts and defined lymphocyte populations, and then cultured further *in vitro*, was developed as an extension of FTOC and has proved invaluable for assessing the role of individual stromal components during specific stages of T cell maturation [63,86]. This approach has also been combined with grafting, typically under the kidney capsule, as grafted RFTOC typically exhibit better cortical and medullary patterning than *in vitro* cultured RFTOC, and has been adapted for testing the potency of different fetal and adult TEC subpopulations from mouse, rat and human [87–93]. A recently developed protocol for generating RFTOC has improved the reproducibility of the technique and has also incorporated the capacity to control the juxtaposition of the different cell types included in the cellular reaggregate [94].

A related approach demonstrated generation of an *in vitro* 'thymic organoid' by seeding a tantalum-coated carbon matrix with *ex vivo* murine thymic stromal cells [95]. When these structures were co-cultured with human $CD34^+$ hematopoietic progenitors, efficient generation of mature CD4 and CD8 SP T cells was observed after 14 days. The T cells generated in this system were functional, as demonstrated by their proliferative response to mitogenic stimuli, and demonstrated a diverse TCR repertoire comparable to that of peripheral blood T cells [95]. These findings demonstrate that the utilization of three-dimensional matrices in conjunction with thymic stromal cells can provide an efficient and reproducible method of *in vitro* T cell generation. However, this approach relies on seeding with *ex vivo* thymus tissue and therefore is not highly scalable.

The demonstration that transfection of the BM stromal cell line OP-9 with the Notch ligand Delta-like 1 conferred the capacity to support T cell differentiation from a variety of hematopoietic progenitors in monolayer culture represented a major breakthrough [96]. Using this system, DP thymocytes can be generated efficiently from mouse fetal liver-, adult bone marrow-, or Embryonic Stem (ES) cell-derived hematopoietic progenitors; $CD8^+$ SP T cells are also produced inefficiently although $CD4^+$ SP T cells are largely absent [96,97]. This system was also shown to support T cell development from human cord blood- and human bone marrow-derived $CD34^+$ cells [98–101]. OP9 cells stably transfected with Dll4 also efficiently support generation of DP cells [102], and in this system lower levels of Dll4 than DL1 are required for T lineage commitment [102], consistent with the non-redundant role of Dll4 in T cell lineage commitment *in vivo* [57,58].

While neither the OP9-DL1 nor the OP9-Dll4 system represents a scalable means of supporting the *in vitro* generation of mature T cells for transplantation [103], due to their limited capacity to support positive and negative selection of the T cell repertoire, these systems have been widely used to dissect cellular and molecular regulation of T cell differentiation in mouse and human (see e.g., references [16,104]). Additionally, in a powerful recent development, they have been used to produce expanded pools of hematopoietic progenitors for transplantation [105–109]. In particular, adoptive transfer of OP9-DL1-produced $CD4^-CD8^-$ DN thymocytes in a mouse model of hematopoietic stem cell transplantation resulted in improved T cell reconstitution [105]. Similarly, transplantation of human $CD34^+CD7^{hi}$ cells produced by *in vitro* culture of umbilical cord blood or adult hematopoietic stem cells resulted in the efficient engraftment of the thymus of immunodeficient mice [106,108]. Furthermore, in preliminary clinical trial data, transplantation of OP9-DL1-cultured hematopoietic progenitors, together with a single unit of cord blood, resulted in more rapid recovery of neutrophil numbers following a myeloablative preparative regimen than the currently standard procedure of transplantation of a double unit of cord blood [107].

Thus, *in vitro* approaches based on generating and/or expanding hematopoietic progenitor cell types appear poised for translation. Progress towards the goals of generating transplantable T cell repertoires *in vitro*, however, is likely to require a combination of stem cell-based approaches coupled with tissue engineering to pattern the resulting organoid. Furthermore, while proof of principle studies have demonstrated the feasibility of restoring adaptive immunity in patients by thymus transplantation [110–112], this approach currently relies on transplantation of human neonatal thymus tissue. Stem cell and tissue-engineering technologies may thus provide an alternative source of cells, allowing thymus transplantation to be more widely adopted. In the following sections, we summarize current knowledge of molecular and cellular control of thymus development and maintenance as related to these aims. We focus primarily on the TEC compartment, based on the critical function of these cells in the thymus. We also discuss our current understanding of parathyroid development, since parathyroid replacement is also a clinically important goal.

THYMUS ORGANOGENESIS

Cellular regulation of early thymus organogenesis

The thymus arises in the pharyngeal region of the developing embryo, from a common primordium with the parathyroid gland. This common primordium develops from the third pharyngeal pouch (3PP), one of a series of bilateral outpocketings of pharyngeal endoderm that form sequentially in a rostral to caudal manner.

In the mouse, outgrowth of the 3PP occurs from approximately E9.0 [113]. At this stage, the epithelium of the 3PP consists of a single layer of columnar epithelium surrounded by a condensing population of neural crest cells (NCC) that will eventually form the capsule [114–117]. Overt thymus organogenesis is evident from between E10.5 and E11.0, at which stage the epithelium begins to proliferate, assuming a stratified organization [118]. At E12.5, the primordia separate from the pharynx and begin to resolve into discrete thymus and parathyroid organs. The thymus primordium subsequently migrates to its final anatomical location at the midline, following the path of the carotid artery and vagus nerve, while the parathyroid primordium associates with the lateral margins of the thyroid [119,120]. In the case of the thymus, this migration is active, and is regulated by NCC through EphB-ephrin-B2 interactions [121–123].

Within the common primordium, the prospective thymus is located in the ventral domain of the third pouch, and the prospective parathyroid in the dorsal aspect. Patterning of these prospective organ domains occurs early in organogenesis, as the parathyroid domain is delineated by the transcription factor Gcm2 as early as E9.5 (see the section Specification of the thymus and parathyroid).

The mesenchymal capsule surrounding the thymus primordium is derived from the migratory neural crest, a transient population formed between the neural tube and the surface ectoderm. In the mouse, NCCs migrate into the pharyngeal region from E9.0. Elegant chick-quail chimera studies provided the first evidence that NCCs are the source of mesenchymal cells in the thymus [115], which has been confirmed in the mouse by heritable genetic labeling *in vivo* [114,117,124].

Colonization of the mouse thymus with hematopoietic progenitor cells occurs between E11.25 and E11.5 [125–127]. As vascularization has not occurred by this stage, the first colonizing cells migrate through the peri-thymic mesenchyme into the TE. These cells have been reported to exhibit comparatively low T cell progenitor activity, while a second colonizing wave which arrives between E12 and E14 appears to display much higher levels of T cell potential upon *in vivo* transfer [128].

Following the formation of the thymic primordium and the commitment of the epithelial cells to the TEC lineage, the thymus undergoes a period of expansion involving both the proliferation of stromal cells and an increase in thymocyte numbers. *In vitro* experiments have demonstrated that both Fgf7 and Fgf10, which are expressed by the perithymic mesenchyme [129], can stimulate the proliferation of fetal TECs [130,131]. Furthermore, thymi in mice lacking *Fgfr2IIIb*, the receptor for Fgf7 and Fgf10, are severely hypoplastic although able to support T cell differentiation, and *Fgf10*$^{-/-}$ mutants also exhibit hypoplastic thymi [129]. Similarly, expression of a soluble dominant negative FgfR2IIIb fusion protein by thymocytes resulted in reduced thymic size and cellularity, although thymocyte development was unperturbed [132].

Concomitantly with this proliferation TEC differentiation commences, with the first evidence of differentiation into cortical and medullary cell types appearing by E12.5 [44,89]. Development of the two compartments then proceeds in a lymphocyte independent manner until E15.5 [133,134]. The expression of MHC Class II and MHC Class I on the surface of TEC is first detected at E12.5, and ~E16 respectively [41,44,135,136] and is followed by the appearance

of $CD4^+$ and $CD8^+$ SP thymocytes at E15.5 and E17.5 [41,135]. Although a functional thymus is present in neonates, the full organization of the stroma is not achieved until 2–3 weeks postnatally in the mouse.

We note that the precise timings of developmental events is known to vary between mouse strains, and the Blackburn lab has observed that the thymic primordium in C57BL/6 embryos is consistently developmentally retarded by at least 12 hours compared to CBAxC57BL/6 F1 embryos at E12.5. Hence, the timings reported for particular events in thymus organogenesis may vary slightly due to variations in genetic background.

Origin of thymic epithelial cells

The precise embryonic origins of the TE were a matter of long-standing controversy [88,126,137–142]. However, definitive evidence for a single endodermal origin in mice was provided through histological, fate and potency analysis of the pharyngeal region [113]. These data demonstrated that although the 3PP endoderm and third pharyngeal cleft ectoderm make contact at E10.5, these germ layers subsequently separate, with apoptosis occurring in the contact region. Furthermore, lineage tracing of pharyngeal surface ectoderm of E10.5 mouse embryos failed to find evidence for an ectodermal contribution to the thymic primordium and transplantation of pharyngeal endoderm isolated from E8.5–E9.0 embryos (i.e., prior to initiation of overt thymus organogenesis) indicated that the grafted endoderm was sufficient for complete thymus organogenesis [113], similar to previous results obtained using chick-quail chimeras [139]. Thus, pharyngeal endoderm alone is sufficient for the generation of both cortical and medullary thymic epithelial compartments.

Thymic epithelial progenitor cells (TEPC)

The phenotype of TEPC has been of considerable interest, and remains an area of on-going investigation. Analysis of a subset of human thymic epithelial tumors provided initial evidence supporting the existence of a common TEPC activity; these tumors contained cells that could generate both cortical and medullary subpopulations, thus suggesting that the tumorigenic targets were epithelial progenitor/stem cells [143]. In addition, ontogenic studies suggested that the early thymus primordium in both mouse and human might be characterized by co-expression of markers that later segregated to either the cortical or medullary epithelium [144].

The first genetic indication of a TEPC phenotype was provided by a study addressing the nature of the defect in *nude* mice [145], which fail to develop a functional thymus due to a single base deletion in the transcription factor Foxn1 [146,147]. Analysis of allophenic *nude*-wild-type aggregation chimeras demonstrated that cells homozygous for the *nude* mutation were unable to contribute to the major TE subsets, establishing that the *nude* gene product (Foxn1) is required cell-autonomously for the development and/or maintenance of all mature TEC [145]. However, a few *nude*-derived cells were present in the thymi of adult chimeras and phenotypic analysis indicated that these cells expressed determinants reactive to mAbs MTS20 [33] and MTS24, but did not express markers associated with mature TEC including MHC Class II. These findings suggested that in the absence of *Foxn1*, TE lineage cells underwent maturational arrest and persisted as $MTS20^+24^+$ progenitors [145]. This hypothesis was confirmed by an elegant study in which clonal reactivation of a conditional null allele of *Foxn1* in the neonatal thymus was shown to result in generation of functional thymus tissue containing organized cortical and medullary regions [148] – demonstrating unequivocally that in the absence of Foxn1, a common TEPC persists in the thymic rudiment.

Further data regarding the phenotype of TE progenitors came from analysis of mice with a secondary block in thymus development resulting from a primary T cell differentiation defect. The thymi of postnatal *CD3e26tg* mice, in which thymocyte development is blocked at the $CD44^+CD25^-$ TN1 stage [149,150], principally contain epithelial cells that

co-express K5 and K8 [45] — which, in the normal postnatal thymus, are predominantly restricted to the medulla and cortex respectively and are co-expressed by only a small population of TEC at the CMJ. In this study, Klug and colleagues demonstrated that transplantation of *CD3e26tg* thymi into *Rag1*$^{-/-}$ mice, which sustain a later block in T cell differentiation, resulted in the development of K5^{-}K8^{+} cells, suggesting that the K5^{+}K8^{+} cells are progenitors of cTEC [45].

The phenotypic and functional properties of MTS20^{+}24^{+} cells within the fetal mouse thymus were addressed directly in several studies. Analysis of the functional capacity of isolated MTS20^{+}24^{+} cells and MTS20^{-}24^{-} cells via ectopic transplantation demonstrated that MTS20^{+}24^{+} TEC were sufficient to direct establishment of a functional thymus containing both cortical and medullary TEC populations [89,90]. These studies clearly identified fetal MTS20^{+}24^{+} TEC as TEPC, but lacked clonal evidence for the existence of a common TEPC. Expression profiling subsequently identified the target of MTS20 and MTS24 as the orphan cell surface protein Plet1 [151].

Evidence for a common TEPC has been presented in two independent studies. A short-term retrospective clonal lineage analysis demonstrated the presence of both a common TEPC able to generate both cTEC and mTEC, and cTEC-restricted TEPC, in the neonatal thymus [148]. In an elegant extension of these experiments, clonal reversion of a *Foxn1* null allele resulted in the generation of small regions of thymus tissue that contained both cortical and medullary TEC [148], providing conclusive evidence for the existence of a common progenitor in *Foxn1*$^{-/-}$ mice, as discussed above. In a complementary study, the existence of a common TEPC in the E12.5 thymus was demonstrated by transplantation of single E12.5 Plet1^{+} TEC [92], establishing that the Plet1^{+} TEC population originally identified as TEPC indeed contains a common TEPC.

The view that Plet1 is a marker for TEPC was challenged in a recent report demonstrating, in contrast to initial conclusions [90], that at E14.5 both the Plet1^{+} and Plet1^{-} TEC compartments can form a functional thymus upon transplantation [152]. However, a key difference between these studies is that Gill and colleagues transplanted limiting numbers of Plet1^{+} TEC [90], whereas in the later study cell number was not limiting [152]. Furthermore, Rossi and colleagues did not provide phenotypic analysis of the input populations and therefore could not determine precursor:progeny relationships — a caveat of particular importance since the Plet1-negative TEC population at E14.5 and subsequent developmental stages is highly heterogeneous [44]. Rossi's findings are thus consistent with the existence of intermediate progenitor populations restricted to cortical and medullary TEC fates downstream of Plet1^{+} TEC in the thymic epithelial differentiation hierarchy, which can together generate a fully functional thymus. No information yet exists regarding the reversibility or otherwise of the early steps in thymic epithelial lineage differentiation, and therefore other alternative explanations for these findings are also possible.

Taken together, the available genetic and functional analyses strongly support Plet1 as a marker of the founder cells of the thymic epithelial lineage, and demonstrate loss of Plet1 expression with the onset of differentiation in the fetal thymus [44]. The functional relationship between fetal Plet1^{+} TEPC and the population of Plet1^{+} mTEC of the postnatal thymus is however not clear. While the Plet1^{+} TEC population isolated from the fetal thymus at stages up to and including E16.5 could initiate *de novo* thymus organogenesis upon ectopic transplantation, this capacity is lost by E18.5 [152,153], indicating a clear functional difference between the fetal and postnatal Plet1^{+} TEC populations. At the present time, both the function of postnatal Plet1^{+} TEC and the phenotypic identity of postnatal TEPC remain to be determined. Nonetheless, the identification of Plet1^{+} as a marker enabling isolation of the earliest fetal progenitor cells for the TEC lineage [44,89,90,151], coupled with the demonstrations that these cells are common TEPC [92] and can generate an organized, functional thymus upon

transplantation [89,90], points to the possibility of using these Plet1$^+$ TEPC as a source of TEC in generation of *in vitro* thymus organoids and in thymus transplantation.

The existence of intermediate mTEC- and cTEC-restricted progenitors in the fetal and postnatal thymus has now been demonstrated by a number of studies. Analysis of MHC Class II mismatched chimeras showed that the medullary epithelium initially forms as individual clonally-derived islets, and that these coalesce later in development [88]. This study demonstrated the presence of mTEC sub-lineage-restricted progenitors (mTEPC) until at least E15.5 [88]. Furthermore, medullary sub-lineage-restricted TEPC can be isolated from E13.5 fetal thymus based on expression of Claudin 3 (Cldn3) and Cldn4 [154], and the UEA1$^+$ subset of fetal Cldn3,4$^+$ TEC was shown to represent the precursor of the clinically important Aire$^+$ subset of mTECs [154]. Cldn4 co-localizes extensively with Plet1 at early developmental stages, and Plet1$^+$ cells are a minor subset of Cld4$^+$ cells in the postnatal thymus, potentially indicating a precursor-progeny relationship between Plet1$^+$ TEC and the broader Cld4$^+$ population [151]. A cTEC progenitor activity has also been identified within the CD205 TEC population of the fetal thymus [50], the descendants of which are suggested to acquire more differentiated characters in a stepwise fashion. CD205 is a broad marker of cTEC in the postnatal thymus and identification of cTEC progenitors within this population is therefore an area of current investigation.

Human thymus development

Early human thymus development closely parallels that of the mouse; the human thymus forms from the 3PP in a common primordium with the parathyroid gland. The 3PP is evident from early week 6 of human fetal development, and initially develops as a tube-like lateral expansion from the pharynx, which makes contact with the ectoderm of the third pharyngeal cleft [155,156]. A single endodermal origin has not been demonstrated directly for the human TE, however, since the thymus has a single endodermal origin in mice and avians [113,139] it is reasonable to assume that this is also the case in humans. Within the human common thymus/parathyroid primordia, the thymus and parathyroid domains are located ventrally and dorsally, and are surrounded by condensing NCC-derived mesenchyme from the onset of development [155]. The thymus component of this primordium begins to migrate ventrally from week 7 to mid-week 8, forming a highly lobulated, elongated, cord like structure. The upper part of this structure normally disappears at separation of the two organ rudiments, leaving the parathyroid in the approximate location in which it will remain throughout adulthood [155]. The bilateral thymic primordia continue to migrate towards the midline, where they eventually meet and attach at the pericardium — the permanent location of the thymus into adulthood — by mid-week 8 [155]. As in the mouse [89], the human early thymus primordium appears to contain undifferentiated epithelial cells which express some markers that are later restricted to either cortical or medullary compartments [144]. Nascent medullary development is evident from week 8, and by week 16 distinct cortical and medullary compartments are present. Other cell types penetrate the thymus from week 8, including mesenchymal, vascular and lymphoid cells, and mature lymphocytes begin to leave the thymus to seed the peripheral immune tissues between weeks 14 and 16 [157,158].

Cervical thymus in mouse and human

The presence of a cervical thymus in both animals [159] and humans has been recorded for some time [157,160,161], and recent publications indicate that an ectopic cervical thymus is also a common occurrence in at least some mouse strains [162,163]. In terms of size and cellularity, the cervical thymus is much smaller than the thoracic thymus, however, the morphology of the two structures is very similar, with organized cortical and medullary regions and similar expression patterns of cytokeratin molecules [162,163]. Furthermore, the cervical thymus expresses the transcription factors Foxn1 and Aire and can produce functional T cells

that are tolerant to self-antigens [162,163], although the range of self-antigens present may be more restricted than in the thoracic thymus [162].

The origin of the cervical thymus is at present unclear. A plausible hypothesis is that it may arise from remnants of the thymus domain of the 3PP that become detached from the organ during separation of the thymus and parathyroid domains. Several alternative explanations exist, and the identification of cervical thymi in mice will allow the embryonic origins of these structures to be addressed experimentally. It appears that in mice the cervical thymus may mature postnatally [163], while in humans, it is clearly present in second trimester of fetal development. The presence of Foxn1+ epithelial cells has not been reported in the cervical regions of developing mouse embryos. However, as it is now clear that cells specified to the TE lineage retain their identity in the absence of Foxn1 expression [148], it remains possible that thymus-fated cells that are Foxn1-negative may be present outside the thoracic thymus and serve as an origin for cervical thymi.

Molecular regulation of thymus and parathyroid organogenesis

Although the regulation of thymus organogenesis is incompletely understood, studies of classical and genetically-engineered mouse mutants have begun to reveal a network of transcription factors and signaling molecules that act in the pharyngeal endoderm and surrounding mesenchyme and mesoderm to regulate thymus and parathyroid organogenesis. Understanding of this network may facilitate the development of robust protocols to direct differentiation of embryonic stem cells or iPS cells *in vitro*. By recapitulating the stepwise restriction of differentiation potential observed *in vivo*, a highly enriched anterior foregut endoderm population can be obtained from pluripotent cell-derived definitive endoderm [164,165]. Further manipulation of these cells or cells isolated *ex vivo*, informed by the developmental events discussed below, may allow the controlled expansion and differentiation of functionally validated thymus and parathyroid progenitors isolated *ex vivo* or generated by differentiation of pluripotent stem cells *in vitro*. The principal components of this network are discussed below and summarized in Fig. 43.2.

MOLECULAR CONTROL OF EARLY ORGANOGENESIS

The T-box transcription factor Tbx1, retinoic acid (RA) signaling, and fibroblast growth factor 8 (Fgf8) signaling have been implicated as important regulators of the earliest events in thymus organogenesis, which occur prior to overt organ development and relate to molecular control of 3PP formation.

Tbx1 is the gene responsible for cardiovascular and glandular defects in *Df1* mice, which carry a large deletion of chromosome 16 [166]. *Df1* heterozygotes closely phenocopy a human condition known as 22q11.2 deletion syndrome (22q11.2DS, or DiGeorge Syndrome) in which a deletion in chromosome 22 covering an interval of approximately 30 genes [167], results in a range of defects including thymus aplasia or more frequently, hypoplasia [168,169].

During development, *Tbx1* is expressed in the pharyngeal endoderm and the core mesenchyme of the pharyngeal arches from approximately E7.5 and continues to be expressed in a variety of structures until E12.5 [170–172]. *Tbx1* mutants have severe defects throughout the pharyngeal region, including abnormal patterning of the first pharyngeal arch, hypoplasia of the second arch and absence of the third and fourth arches and pouches [173]. As a result, $Tbx1^{-/-}$ mutants lack both thymus and parathyroids and display a spectrum of cardiovascular abnormalities and craniofacial defects [173].

The phenotype of $Tbx1^{-/-}$ animals suggests an important role in segmentation of the pharyngeal region. Supporting evidence for this hypothesis was provided by an elegant study addressing the temporal requirement for *Tbx1* in the development of the pharyngeal region.

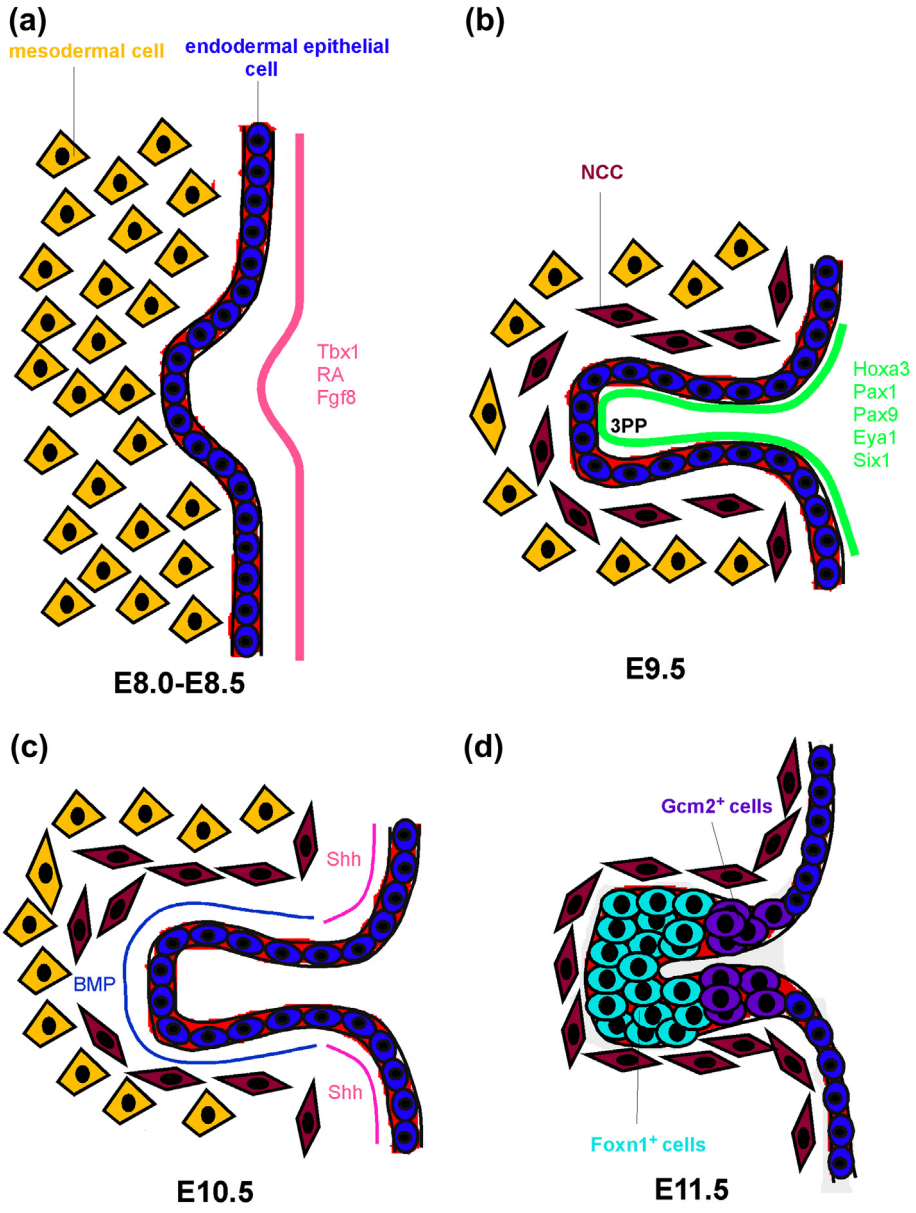

FIGURE 43.2

Molecular regulation of early thymus organogenesis. (a) At approximately E8.0-E8.5 the formation of the third pharyngeal pouch (3PP) is initiated in the pharyngeal endoderm and is dependent on the expression of Tbx1 and retinoic acid (RA), and on fibroblast growth factor 8 (Fgf8) signaling (pink). (b) At E9.5 the 3PP has formed and is surrounded by mesenchymal cells of mesodermal and neural crest cell (NCC) origin. Continued development is dependent on the expression of the transcription factors Hoxa3, Pax1, Pax9, Eya1 and Six1 (green). (c) Bone Morphogenetic Protein (Bmp) (blue) and Sonic Hedgehog (Shh) (pink) signaling occur at E10.5 in the 3PP endoderm in the ventral and dorsal aspects respectively. These factors may be involved in the specification of the 3PP into thymus and parathyroid specific domains. (d) At E11.5 epithelial cells in the ventral domain of the 3PP express the transcription factor Foxn1 (light blue) and will form the thymic epithelium. Epithelial cells in the dorsal domain express the transcription factor Gcm2 (purple) and will form the parathyroid gland. The differentiation and maintenance of both of these cell types are dependent on these factors. Note that throughout this figure, gene expression domains are annotated only for the endoderm. Many of the factors shown are also expressed in the mesoderm and/or mesenchyme of the pharyngeal arches, and/or in the pharyngeal ectoderm, as described in the text.

Deletion of *Tbx1* at E8.5, during the formation of the 3PP, resulted in complete absence of thymus and parathyroid, and complementary fate mapping experiments demonstrated that cells that express *Tbx1* at E8.5 contribute significantly to the thymic primordium [174]. However, although deletion of *Tbx1* at E9.5/E10.5 (after initial formation of the 3PP) caused

morphological defects in the thymus, these were not as severe as the aplasia seen after deletion at E8.5, and fate mapping of cells expressing *Tbx1* at E9.5/E10.5 revealed only a small contribution to the thymus [174].

Taken together, these data suggest that Tbx1 is required for establishment of the 3PP, but is not directly required for subsequent thymus development. Furthermore, although *Tbx1* is haplo-insufficient with respect to thymus development [175], the basis of this insufficiency remains to be determined and may result either from secondary effects resulting from mild defects in pouch formation, or dosage effects related to factor provision by non-NCC mesenchymal cells.

A role for RA in 3PP formation was suggested by experiments in which RA antagonist was administered to whole embryo cultures. Here, blockade of RA signaling at E8.0 resulted in the absence of the growth factors Fgf8 and Fgf3 in the 3PP endoderm, and impaired NCC migration to the third and fourth pharyngeal arches [176]. Expression of the transcription factor Pax9 (see below) was also absent in the 3PP, but was expanded in the 2PP endoderm. These data suggest that RA signaling is required for the specification of the 3PP, which confers subsequent competence to support NCC migration. *In vivo* evidence for a role for RA signaling was subsequently provided by the finding that fetal mice lacking RA receptors α and β display thymus agenesis and ectopia [177].

There is considerable evidence that Fgf8 is required during the early stages of thymus and parathyroid development. Fgf8 is expressed in the early gut endoderm and in the endoderm and ectoderm of the pharyngeal pouches and clefts. Mice carrying hypomorphic alleles of *Fgf8* show defects in thymus development ranging from hypoplasia to complete aplasia [178,179]: the initial impairment in thymus and parathyroid organogenesis is likely to occur at an early stage in development in these mice, as the third and fourth pharyngeal arches and pouches are usually hypoplastic/aplastic, in addition to other abnormalities in the pharyngeal region [178,179].

In terms of the cell types affected by impaired Fgf8 signaling, similarities between the phenotype of Fgf8 hypomorphs and the spectrum of abnormalities found during experimental NCC ablation [180,181] suggest that the glandular defects may result from defective NCC migration/differentiation or survival. In support of this, NCC of Fgf8 hypomorphs show increased levels of apoptosis [178,179] and reduced expression of *Fgf10*, which mediates expansion of the thymic primordium from E12.5 [129], in the neighboring NCC mesenchyme [179]. There is also a mild reduction in the expression of genes associated with differentiated NCC [178] suggesting that the maintenance of NCC is perturbed.

Taken together, these data implicate Fgf8 in maintaining a competent NCC population that can contribute to thymus organogenesis. However, Fgf8 may also act specifically on the 3PP endoderm, since ablation of Fgf8 in the endoderm and ectoderm, or ectoderm alone, results in different phenotypes. Ablation in the ectoderm alone causes vascular and craniofacial defects as seen in Fgf8 hypomorphs [182] whereas when Fgf8 is also deleted in the endoderm, glandular defects are evident, including thymus hypoplasia and ectopia [182]. The NCC defects were the same in both cases.

Further insight into the role of Fgf signaling in regulation of thymic primordium development was provided by a detailed study of Fgf signaling components during thymus organogenesis. This revealed a complex picture in which Fgf signaling is modulated by expression of Sprouty 1 and 2, such that although the 3PP expresses both Fgfs and FgfR, signaling in the endodermal component of the thymus is restricted by Sprouty 1 and 2 [183]. Thus, while the importance of Fgf8 signaling in regulating development of the 3PP is clear, further studies are required to understand the temporal requirements for this pathway and its interplay with other signaling pathways involved in thymus and parathyroid organogenesis.

TRANSCRIPTION FACTORS AND REGULATION OF 3PP OUTGROWTH

After initial 3PP formation, continued development of the common primordium is dependent on a complex networks of transcription factors, including Hoxa3, Pax1, Pax9, Eya1, Six1 and Six4; all of these factors are expressed in the 3PP endoderm from E9.5–E10.5 and, with the exception of Pax1 and Pax9, are also expressed in associated NCC and ectoderm.

Absence of functional Hoxa3 or Eya1 results in the failure to initiate overt thymus and parathyroid organogenesis once the 3PP has formed, revealing the essential roles of these factors [119,120,184,185]. *Tbx1* and *Fgf8* are both downregulated in the 3PP of *Eya1*$^{-/-}$ mice at E9.5 [185], indicating that Eya1 plays a role in regulation of these factors. Lack of Six1, Six1 and 4, Pax1 or Pax9 causes much less severe phenotypes. The common primordium begins to develop in *Six1*$^{-/-}$ mice, and patterning into thymus and parathyroid domains (see the section 'Specification of the thymus and parathyroid') is initiated. However, subsequent apoptosis of endodermally-derived cells in the common primordium leads to complete disappearance of the organ rudiment by E12.5 [185]. A similar phenotype is evident in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos, though the size of the primordium is further diminished in the double mutants indicating synergy between these gene products [185]. *Pax9* loss of function mutations result in formation of ectopically located thymic primordia, which form within the laryngeal cavity, rather than within the pharyngeal arch mesoderm. These primordia fail to migrate to the mediastinum, and exhibit severe hypoplasia from E14.5. However, the thymic lobes are vascularized, contain both thymocytes and mesenchymal cells, and express Foxn1 [186]. *Pax1*$^{-/-}$ mutants show relatively mild thymus hypoplasia and aberrant TEC differentiation [187–189]. Since *Pax1* and *Pax9* are highly homologous, these phenotypes may reflect functional redundancy, as demonstrated in other tissues [190,191].

Expression of *Hoxa3*, *Eya1* and *Six1* in multiple germ layers complicates interpretation of the respective null phenotypes for these genes. However, Hoxa3 appears to regulate *Pax1* and *Pax9* either directly or indirectly, since *Pax1* and *Pax9* expression is initiated normally in *Hoxa3*$^{-/-}$ mutants but fails to be maintained at wild-type levels beyond E10.5 [120]. Furthermore, *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ compound mutants show delayed separation of the thymus/parathyroid primordium from the pharynx, resulting in thymic ectopia and a more severe hypoplasia than that seen in *Pax1*$^{-/-}$ single mutants [121], indicating that these factors function in the same network.

It has been suggested that Eya1 and Six1 act downstream of the *Hox/Pax* genes, as *Eya1*$^{-/-}$ embryos show normal expression of *Hoxa3*, *Pax1* and *Pax9* but reduced expression of *Six1* in the endoderm of the third and fourth PP and ectoderm of the second, third and fourth pharyngeal arches [184]. However, while it is likely that Six1 acts downstream of Eya1, recent evidence indicates that Eya1 and Six1 do not act downstream of the Pax genes. This has been shown by analysis of *Pax9*$^{-/-}$ and *Pax1*$^{-/-}$*Pax9*$^{-/-}$ mutants, which show normal expression of *Eya1* and *Six1* in the 3PP, and *Eya1*$^{-/-}$;*Six1*$^{-/-}$ double mutants, which lack expression of *Pax1* in the E10.5 3PP [185]. Interestingly, *Pax9* expression is unaffected in *Eya1/Six1* double mutants [185]. However, it remains possible that *Eya1* and *Six1* may be regulated by Hoxa3 independently of Pax1 and Pax9 function.

A recent report has demonstrated that mice homozygous for a null mutation in *Ripply3*, a RA signaling target, have ectopic and hypoplastic thymi, with the thymic lobes being located in the oropharynx of *Ripply3*$^{-/-}$ embryos, similar to *Pax9* null thymi [192,193]. *Ripply3* was shown to repress *Tbx1* transcription in an *in vitro* Luciferase assay system, and the expression domain of *Pax9* was increased in the pharyngeal region of E9.5–E10.5 *Ripply3*$^{-/-}$ mice [192]. Furthermore, a 3.7kb promoter region of *Pax9* was shown to be Tbx1 responsive in a Luciferase assay. These studies together suggest that the expression of *Tbx1* may regulate *Pax9* expression.

The data reviewed above indicate the power of compound mutant analysis for unraveling genetic interactions. Overall, the complexity of these data suggests it is likely that all or some of these factors form a stable network, which is sustained by complex feedback and autoregulatory loops. Further work is required to elucidate exactly how these and other transcription regulators cooperate in the development of the 3PP endoderm, and to determine the precise role of each of these factors at the cellular level.

SPECIFICATION OF THE THYMUS AND PARATHYROID

Prior to the overt formation of the thymus and parathyroid primordia, the 3PP is specified into organ specific domains. At E9.5, epithelial cells within the anterior dorsal aspect initiate expression of Gcm2 [197], a transcription factor required for the development of the parathyroid [194]. Current evidence suggests that Gcm2 acts downstream of Eya1 and Hoxa3, as *Gcm2* is downregulated in *Hoxa3*$^{-/-}$, *Eya1*$^{-/-}$ and *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ compound mutant mice [121,184,140]. *Gcm2* is also directly regulated by Gata3, with the result that in E11.5 *Gata3*$^{+/-}$ mice, a lower proportion of cells in the 3PP expresses *Gcm2* [195]. The first known thymus specific transcription factor, Foxn1, is expressed at functionally relevant levels in the ventral domain of the 3PP from approximately E11.25, although low levels can be detected by polymerase chain reaction (PCR) from E10.5 [196,197]. Foxn1, a forkhead class transcription factor, is required for TEC differentiation and hair development, and is discussed in more detail in the section 'Foxn1 and regulation of TEC differentiation'.

The expression patterns of *Foxn1* and *Gcm2* clearly define the thymus and parathyroid domains of the 3PP. However, these factors do not appear to be responsible for specification of their respective organs, indicating that specification must be mediated by an upstream factor or factors. With respect to Foxn1, this model is supported by the finding that, epithelial cells in the ventral domain of the E11.5 3PP express interleukin 7 (IL-7), a cytokine required for thymocyte differentiation [198,199], making IL-7 one of the earliest currently identified markers of thymus identity. In the thymus primordium *Foxn1*$^{-/-}$ embryos show normal *IL-7* expression [199], indicating that TE identity is specified in the absence of Foxn1. Transplantation experiments also support this model, as E9.0 pharyngeal endoderm, which does not express functionally relevant levels of *Foxn1*, gives rise to a functional thymus when grafted ectopically [113], indicating that at this developmental stage some pharyngeal endoderm/3PP cells are already specified to the TE lineage.

Similarly, our recent studies conclude that GCM2 is not required for specification of the parathyroid, as other parathyroid-specific markers including *Tbx1*, *Ccl21* and *CaSR* are initiated in *Gcm2*$^{-/-}$ mice, although this domain undergoes rapid and coordinated apoptosis in the absence of GCM2 [200]. Thus, *Gcm2* may play a somewhat analogous role in parathyroid development to that of *Foxn1* in the thymus, although the thymus rudiment does survive in the absence of *Foxn1*.

Upstream regulation of *Foxn1* is currently not understood, and analyses of mutations in potential upstream regulators have not been informative in this regard. *Hoxa3*$^{-/-}$ and *Eya1*$^{-/-}$ embryos do not express *Foxn1*, but this is likely due to the block in primordium formation prior to the onset of *Foxn1* expression in each of these mutants. Although *Foxn1* expression is unaltered in *Pax9*$^{-/-}$ and *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ mutants [186,189], the possibility remains of functional redundancy between Pax1 and Pax9. Furthermore, while *Six1*$^{-/-}$ mutants display reduced *Foxn1* expression, the 3PP exhibits increased cell death in the absence of *Six1* [185], and therefore, the reduced expression of *Foxn1* may reflect poor survival of Foxn1^{+} cells rather than a direct interaction between *Foxn1* and Six1. Further studies are required to determine whether a regulatory relationship exists between these genes and *Foxn1*.

Several candidate regulators of thymus specification have, however, recently been identified based on their expression in the 3PP prior to initiation of *Foxn1* [201]. These include Nkx2.5,

Nkx2.6, Isl1, Gata3 and Foxg1. *Nkx2.5*, *Nkx2.6*, *Isl1* and *Gata3* are expressed in the developing 3PP at E9.5 and are restricted to the ventral portion of the 3PP endoderm at E10.5. *Foxg1* is not expressed in the 3PP at E9.5, but at E10.5 is expressed in two discrete regions, one dorsal and the other ventral. By E11.5, when *Foxn1* is first activated in the 3PP, *Foxg1* and *Isl1* are already restricted to the thymus domain. These factors continue to be expressed in TECs throughout late fetal and postnatal differentiation. However it remains unknown whether they transcriptionally activate *Foxn1* or instead regulate Foxn1-independent aspects of thymus development.

How then are the thymus and parathyroid domains within the 3PP established? Evidence suggests that opposing gradients of bone morphogenetic proteins (BMP) and sonic hedgehog (Shh) may play an important role in this process. During thymus development *Bmp4* expression is first detected at E9.5 when it is expressed by a small number of mesenchymal cells in the third pharyngeal arch [202]. By E10.5, the *Bmp4* expression domain has expanded to include the ventral 3PP endoderm and the adjacent mesenchyme, but remains absent from the dorsal 3PP. This expression pattern is maintained at E11.5 and by E12.5 *Bmp4* is expressed throughout the thymic primordium and the surrounding mesenchymal capsule [202].

In vivo evidence of a role for Bmps in thymus organogenesis was provided by a study in which expression of the Bmp inhibitor *Noggin* was driven by the *Foxn1* promoter, thus impairing Bmp signaling in the thymic stroma [203]. These mice developed hypoplastic and cystic thymi that failed to migrate to their normal position above the heart. It is highly likely that this is due to a direct effect on the thymic stroma, as impaired development is evident prior to lymphocyte immigration; mediators of Bmp signaling such as *Msx1* and phosphorylated Smad proteins were downregulated in both the epithelium and surrounding mesenchyme. Interestingly, *Foxn1* expression was only partially blocked in this transgenic model. However, *IL7* expression was also absent in the cells that had downregulated *Foxn1*, suggesting that thymus fate, rather than solely *Foxn1*, may have been affected [204]. A caveat for interpreting these data is that the inhibition of Bmp signaling was driven by the *Foxn1* promoter, and thus occurred after initiation of *Foxn1* expression. Furthermore, addition of a Bmp inhibitor did cause loss of *Foxn1* in a zebrafish model [204]. A more recent study showed that Bmp signaling is necessary for *Foxn1* initiation in co-cultures of qE2.5 quail pharyngeal pouch endoderm and chick mesenchyme. Bmp is required only during a narrow time window, however, as addition of Noggin has no effect on *Foxn1* initiation in co-cultures containing qE3 endoderm. *Gcm2*, in contrast to *Foxn1*, is initiated in the absence of mesenchyme and maintained in the presence of Noggin [205].

Conditional deletion of *Bmp4* in the pharyngeal endoderm (and, with variable efficiency, in the surrounding mesenchyme) using *Foxg1-Cre* indicates that Bmp4 is required for thymus and parathyroid morphogenesis. In E11.5 *Foxg1-Cre;Bmp4*$^{F/F}$ embryos, the thymus-parathyroid primordium is hypoplastic but expression of *Foxn1* and *Gcm2* is normal as detected by *in situ* hybridization, demonstrating proper specification and patterning of the thymus and parathyroid domains. However, delayed separation of the two organ primordia and reduced migration of the thymus primordia are evident at E12.5 and E13.5 [206]. *Wnt1-Cre* or *Foxn1-Cre* mediated deletion of *Bmp4* has no effect on thymus and parathyroid morphogenesis, suggesting that *Bmp4* signaling is required in the pharyngeal epithelium during a narrow time window prior to the onset of *Foxn1* expression. However, as the thymic primordium expresses Bmp2 and Bmp7 (CCB and NRM unpublished data), which signal through the same receptor as Bmp4, redundancy is an issue for interpreting these results.

During development of the 3PP, expression of the secreted glycoprotein sonic hedgehog (Shh) is restricted to the region of 3PP immediately adjacent to the pharyngeal endoderm at E10.5 and E11.5, although its receptor Patched1 is expressed by cells in close proximity to this region [207]. Analysis of *Shh*$^{-/-}$ embryos revealed expanded expression domains for both *Bmp4* and *Foxn1* in the 3PP, while the corresponding *Gcm2*$^{+}$ parathyroid domain was lost in these

mutants [207]. Consistent with these observations, cyclopamine treatment of chick embryos at HH14–16 blocked *Gcm2* initiation and led to enhanced *Bmp4* expression in the caudal pharyngeal pouches. However, loss of Shh signaling at a later stage (HH21) caused ectopic expression of *Gcm2* and *CaSR* in the anterior first ectodermal cleft and posterior second arch, and had no effect on *Gcm2* transcription in the 3PP [208]. Thus, at early developmental stages, the role of Shh in thymus organogenesis may be to oppose the action of Bmp4 to allow the specification and development of the parathyroid. At later stages, Shh signaling may also restrict parathyroid development to the caudal pharyngeal pouches.

Wnt glycoproteins may also be important in regulating *Foxn1* expression. Wnts are expressed by the thymic stroma and by lymphoid cells, although Wnt receptors are exclusively expressed by TECs [196]. The earliest reported expression of Wnt family members during thymus development is at E10.5, immediately prior to strong *Foxn1* expression, when the epithelium of the 3PP and adjacent cells express *Wnt4* [196]. Given this expression pattern and the finding that TEC lines that overexpress Wnt4 display elevated levels of *Foxn1* [196], it is possible that Wnt4 cooperates with Bmp4 to regulate *Foxn1*. *Wnt1*, *Wnt4* and $Wnt1^{-/-};Wnt4^{-/-}$ mice all exhibit hypoplastic thymi characterized by reduced T cell numbers but normal thymocyte developmental progression. As no histological analysis of the thymi in these *Wnt1* mutants has been presented, it is not possible to evaluate whether the primary effect is on the TE or on thymocytes [91,209]. However analysis of $Wnt4^{-/-}$ mice suggests that Wnt4 controls thymus size by modulating expansion of both TECs and immature thymocytes. By E15.5, $Wnt4^{-/-}$ thymi contain fewer TECs than wild-type, and at E18.5, histological analysis reveals smaller medullary areas, reduced expression of medullary markers and disorganization of the CMJ. In addition, loss of *Wnt4* results in a disproportionate decrease in the earliest thymic progenitors (ETPs) and DN2 thymocytes. This effect is likely to be direct, as OP9-DL1 cells modified to overexpress Wnt4 increase the expansion of ETPs and DN2s relative to OP9-DL1 cells [210]. As with Bmp family members, functional redundancy is again a possibility, as other Wnt family members are also expressed in the 3PP and surrounding mesenchyme [196,209].

A role for Wnt signaling in TEC is also suggested by deletion of the Wnt signaling repressor *Adenomatous Polyposis Coli* (*Apc*) in K14-expressing cells. $K14\text{-}Cre;Apc^{CKO/CKO}$ mice have a hypoplastic thymus characterized by variable disruption of epithelial organization, exclusion of lymphocytes, and the presence of enlarged structures expressing keratinocyte markers such as involucrin [211]. Similarly, expression of a stabilized form of beta catenin specifically in TECs resulted in thymus hypoplasia, restricted immigration of thymocytes, and a block in thymocyte development at the DN1 stage. The mutant TE contained a central core of cells that initially appeared committed to a thymus fate, but later downregulated expression of Foxn1, K8 and K5, and expressed involucrin [212]. Collectively, these data suggest that over-stimulation of canonical Wnt signaling in TEC perturbs thymus development and function, possibly by affecting TEPC and/or causing a fate shift in their differentiating progeny.

Recent evidence suggests that Wnt signaling is also required for maintenance of the postnatal thymus. Deletion of the Wnt antagonist Kremen resulted in the development of large epithelial-free zones in the TEC network [213]. Tetracyclin-regulated overexpression of the canonical Wnt inhibitor Dickkopf related protein 1 (encoded by *Dkk1*) in cortical and medullary TECs of postnatal mice resulted in dramatic thymus degeneration [214]. A decrease in the number of $K5^{+}K8^{+}$ cells at the CMJ and reduced proliferation in the MHC Class II^{lo} compartment was observed in this model, perhaps implicating Wnt in the regulation of progenitor TEC. Consistent with this idea, these phenotypes were reversed by withdrawal of Doxycyclin, and restoration of thymus size correlated with recovery of the $K5^{+}K8^{+}$ TEC population [214].

FOXN1 AND REGULATION OF TEC DIFFERENTIATION

The data discussed in the sections 'Origin of thymic epithelial cells' and 'Thymic epithelial progenitor cells (TEPC)' collectively establish that the thymus domain within the 3PP contains a common TEPC, able to generate all subtypes of cortical and medullary TECs. They further provide evidence for the existence of cortical and medullary thymic epithelial sub-lineage-restricted TEPC, which presumably arise downstream of the common TEPC in the fetal TE lineage hierarchy. Thus, in addition to TE lineage specification and expansion of the thymus primordium, mechanisms that support development of these sub-lineage restricted progenitors and their subsequent differentiation into c- and mTEC are an important component of thymus organogenesis. Of these, the mechanisms underpinning differentiation and expansion of mTEC are currently the best understood, and are discussed in detail in the section 'Medullary development and expansion' below; little information exists regarding mechanisms specifically regulating cTEC development.

In addition to sub-lineage specific mechanisms, Foxn1 regulates differentiation of TEC in both sub-lineages in the fetal and adult thymus. As discussed in the section 'Thymic epithelial progenitor cells (TEPC)', in Foxn1 null mice, TEC undergo developmental arrest at the founder/fetal progenitor cell stage of development [44,148], and chimera studies have demonstrated the cell-autonomous requirement for Foxn1 for development of all mature TEC subtypes [215]. The early thymic rudiment in *nude* mice also shows reduced proliferation [216]. Thus, while TEPCs form independently of Foxn1, their proliferation and differentiation into mature c- and mTEC is Foxn1-dependent. However, analysis of the *Foxn1*$^{-/-}$ fetal thymus revealed the presence of K5^{hi}Cldn4^{hi} regions corresponding to presumptive progenitor mTEC suggesting that, during organogenesis, Foxn1 is not required for divergence of the mTEC lineage from the common TEPC [44].

Foxn1 is expressed by most if not all differentiating and mature TECs throughout fetal development and, in the postnatal thymus, it is also expressed in most if not all TECs throughout the lifespan [147](CCB and NRM unpublished). The role of Foxn1 beyond the fetal TEPC stage is incompletely understood. However, our studies have indicated that Foxn1 regulates crosstalk between developing TEC and thymocytes, which is required for expansion and maturation of the medullary compartment [217,218]. Furthermore, we have recently shown that Foxn1 is required in a dosage-dependent manner to regulate multiple stages of differentiation in both the c- and mTEC sub-lineages in the fetal and adult thymus. In both sub-lineages, it is required throughout differentiation, from exit from the earliest TEPC state to terminal differentiation of both cTEC and Aire^{+} mTEC [44].

Importantly, these studies demonstrate that a sub-functional thymus can be generated at sub-optimal levels of Foxn1 expression or function, indicating that full thymus function is generated only in a relatively narrow *Foxn1* dosage range [44,218–221]. This conclusion is also supported by analysis of Foxn1 function in the postnatal thymus, which shows it is required in a dosage-sensitive manner to maintain thymus homeostasis [222], and that complete loss of either Foxn1 expression or Foxn1-expressing TEC postnatally results in loss of thymus function [223–225]. Collectively, these data highlight the importance of understanding the intrinsic and extrinsic mechanisms that regulate Foxn1 expression, and the downstream mechanisms regulated by Foxn1, *in vivo*.

As discussed above, little information exists regarding the transcriptional regulation of *Foxn1* expression. However, direct or indirect transcriptional targets of Foxn1 have now been identified in a number of studies [22,44,219,222,223,226,227], although to date no putative Foxn1 targets in TEC have been verified by chromatin immunoprecipitation. However, since the set of Foxn1-regulated genes includes factors with diverse functions in thymus development and the TEC-mediated support of T cell development, Foxn1 can be considered a 'master regulator' of TEC differentiation, rather than a regulator of a specific aspect of thymus development.

In addition to its expression in TEC, Foxn1 is also expressed in skin and hair follicle keratinocytes, where it regulates normal development. In these cutaneous epithelial lineage cells, Foxn1 is believed to regulate the balance between proliferation and differentiation [228–230]. However, while some parallels may exist between Foxn1 function in skin and thymus, expression of Fgf2, a known direct target of Foxn1 in cutaneous epithelium [230], is not expressed in TEC [44], suggesting Foxn1 may regulate different target genes in the cutaneous and thymic epithelial lineages.

MEDULLARY DEVELOPMENT AND EXPANSION

The development of mature mTECs depends on activation of the non-canonical NF-κB-signaling pathway that culminates in RelB activation. mTEC development is severely compromised in RelB deficient mice [231,232]. In addition, a range of medullary defects occurs in mice that are deficient in various components upstream of RelB in the alternative NF-κB-activation pathway. Mice with a naturally occurring mutation in NF-κB-inducing kinase (*NIK*), and *Ikkα* knockout mice, display abnormal thymic architecture with small, hypocellular medullary regions associated with reduced *Aire* and TRA expression [233–235]. A similar phenotype is found in *TRAF6* knockout mice, indicating that the classical NF-kB-signaling pathway also plays a role in formation of functional medullary regions [236]. Interestingly, each of these mutant strains develops autoimmune manifestations consistent with a breakdown in the establishment of central tolerance due to a deficiency of Aire$^+$ mTECs.

Signals from positively selected thymocytes play an important role in expanding the medullary region in adult thymi, a mechanism often referred to as lympho-epithelial crosstalk. Small medullary regions containing sparse mTEC subsets are found when thymocyte development is blocked at the DP stage, as occurs in *TCRα* deficient mice or in mice lacking both MHC Class I and MHC Class II molecules [237,238]. Despite the paucity of mTECs, the medullary regions these mice nevertheless express *Aire* and TRAs, indicating that mTEC expansion is affected to a greater extent than differentiation. Positively selected DP thymocytes differentiate into SP thymocytes that migrate into the medulla, and these SP cells express various tumor necrosis factor superfamily (TNFSF) ligands, including receptor activator of NF-kB ligand (RANKL), CD40 ligand (CD40L), lymphotoxin-α and lymphotoxin-β [239–242].

Binding of these ligands to their receptors on mTEC activates NF-κB signaling, to promote mTEC proliferation and differentiation: ligand engagement of the TNFSF receptors activates NIK, which phosphorylates homodimers of the downstream kinase, Ikkα. Activated Ikkα in turn phosphorylates the C-terminal region of NF-κB2 (p100) leading to ubiquitin-dependent degradation and release of the N-terminal polypeptide, p52. The formation of RelB/p52 heterodimers permits shuttling of RelB from the cytoplasm into the nucleus where it functions as a transcriptional regulator [243]. Thus, *RANK* or *RANKL* deficient mice have small, poorly developed medullary regions in which mTEC cellularity is decreased and *Aire* expression is reduced [241,244]. Targeted disruption of the *LTβR* gene also results in disorganized medullary regions that contain reduced numbers of mTECs [242]. A recent study indicates that signaling via the LTβR is required to upregulate RANK expression on fetal TECs, implying cooperation between these two TNFSF receptors in mTEC development [245].

Interestingly, medullary region development is initiated in the fetal thymus prior to the appearance of SP thymocytes. This is at least partly due to the presence of lymphoid tissue inducer (LTi) cells that express RANKL, but not CD40L. RANKL signaling induces the generation of Aire$^+$ mTECs before fetal thymocytes of the αβ TCR lineage have matured to the SP stage [244,246]. The early fetal thymus also contains thymocytes expressing invariant Vγ5Vδ1 TCRs. These cells, which are present prior to the development of αβ lineage thymocytes, also express RANKL and play a role in development of Aire$^+$ mTECs [247].

SUMMARY

Thymus organogenesis is a complex process in which a dynamically regulated, three dimensional organ forms from the endoderm of the 3PP. The TE, a critical regulator of thymopoiesis, is comprised of many subtypes of TEC, all of which arise from a common progenitor. Importantly, T cell development depends on interactions with both cortical and medullary TEC subtypes, which mediate different aspects of T cell differentiation and repertoire selection. Therefore, development of functional *in vitro* or transplantable thymic organoids requires the ability to recapitulate all of these functions, with appropriate spatial and temporal constraints. Recent studies have begun to clarify the lineage relationships of the different TEC subtypes, and to unravel the molecular networks that govern thymus and parathyroid organogenesis. However, with respect to the goal of developing *in vitro* or transplantable thymi for therapeutic purposes, several significant questions remain unresolved:

- What factor or factors specify thymus and parathyroid lineages within the 3PP?
- How does the network of transcription factors and signaling molecules that are expressed in the endoderm and surrounding mesenchyme/mesoderm control subsequent thymus and parathyroid development, including the maintenance, proliferation and differentiation of thymic epithelial and parathyroid progenitor cells?
- How is *Foxn1* regulated in fetal and adult TEC?

In addition, further identification of Foxn1 targets in TEC, and the transcriptional networks in which Foxn1 operates to control development and function of c- and mTEC, is an important goal. The potential of factors downstream of Foxn1 to facilitate engineering of hematopoietic progenitor cell niches *ex vivo* has already been demonstrated by transgenic expression of varying combinations of *Ccl25*, *Cxcl12*, *Scf* and *Dll4* in *Foxn1*-deficient TE [248]. It is likely that knowledge of both additional Foxn1-regulated genes and proteins required in conjunction with Foxn1 to regulate specific functions in TEC may prove useful for manipulating or engineering thymus function(s) *in vitro* or *in vivo*.

Finally, important questions remain regarding homeostatic maintenance of the mature thymus, which are relevant to strategies for thymus regeneration or replacement. Of particular importance are the identification of both the stem/progenitor cell types responsible for replenishing TEC in the adult thymus, and of the mechanisms that operate on TEC to induce thymic involution. Progress in each of these issues is likely to facilitate rational design of strategies for therapeutic reconstitution of the adaptive immune system. The development of such strategies should significantly impact the health of the aging population, and other immunocompromised individuals.

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PART 11

Gastrointestinal System

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CHAPTER 44

Stem Cells in the Gastrointestinal Tract

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INTRODUCTION

There has been a tremendous increase in interest in stem cell biology and its potential applications in recent years. Although this has been galvanized by the exploitation of research in pluripotent stem cells, it is interesting to note that what might be called the 'intestinal stem cell community', albeit small, has been working productively for some 40 to 50 years. With stem cells now claiming considerable attention, in retrospect, many of the basic tenets that govern our understanding of organ-specific stem cells have come from studies of the gastrointestinal tract and the hematopoietic system.

In the gastrointestinal tract, there is a large body of evidence that multipotent stem cells are found in specific zones, or niches, within gastric glands and intestinal crypts, composed of and maintained by myofibroblasts in the adjacent lamina propria. In this chapter, we review evidence that these multipotent stem cells generate all gastrointestinal epithelial cell lineages through committed precursor cells housed in the proliferative compartments of intestinal crypts and gastric glands, a concept that has had a long and difficult gestation [1]. Notwithstanding their obvious significance, the gastrointestinal stem cells remain elusive and unidentified, mainly because of a lack of accepted morphological and functional markers at the single cell level. We also explore concepts of stem cell number, location, and fate, and we touch on the ability of gastrointestinal stem cells to regenerate cell lineages of whole intestinal crypts and villi after damage. The luminal gut shows regional specializations of function — the stomach primarily for absorption and the intestinal mucosa for both absorption and secretion. This is reflected by variation in the adult cell lineages native to each tissue, and it is thereby consistent that stem cell fate within each tissue is also different. We are beginning to understand the mechanisms that govern such variation. Controversial recent findings regarding stem cell plasticity in the gastrointestinal tract are examined in this chapter.

Because of their longevity, putatively the same as that of the organism itself, stem cells are often viewed as the target cells for carcinogens and the cells of origin for spontaneous tumors. Recent thought on the location of stem cells in the colon has sparked debate concerning the possible pathways of morphological progression of transformed stem cells. This includes a *top-down* proliferation of mutated stem cells located within intercryptal zones on the mucosal surface,

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downward into the adjacent crypts. Contrasting to this is a *bottom-up* theory of the upward proliferation of mutated stem cells in the crypt base to produce dysplastic crypts that replicate and expand by crypt fission. This brings other facets of gut biology into sharp focus — the mechanisms of crypt reproduction; the clonal architecture of normal and dysplastic gastric glands, intestinal crypts, and their derivative tumors; and the role that stem cells take in these events. Here, we propose that the stem cells accumulate the multiple genetic events leading to tumorigenesis, and we explore the manner by which such mutated clones spread in gastrointestinal epithelia. Many of these concepts are being explored at the level of molecular regulatory pathways, including the signaling pathways of Wnt and transforming growth factor β (TGFβ).

This is quite a brief, since several of the most common tumors originate in the gastrointestinal tract and with increasing incidence in Barrett's oesophagus. Our concepts of stem cell biology impinge considerably on our understanding of how these tumors arise.

GASTROINTESTINAL MUCOSA CONTAINS MULTIPLE LINEAGES

In the small intestine, the epithelial lining forms numerous crypts and larger, finger-shaped projections called villi. In the colon there are many crypts, which vary in size throughout the colon; the shortest is in the ascending colon. Overall, four main epithelial cell lineages exist in the intestinal epithelium. These are the columnar cells, the mucin-secreting cells, the endocrine cells, and Paneth cells in the small intestine. Other less common cell lineages are also present, such as the caveolated cells and membranous or microfold cells. Columnar cells, with apical microvilli, are the most abundant epithelial cells, termed enterocytes in the small intestine and colonocytes in the large intestine. 'Goblet' cells containing mucin granules — and thus producing swollen, goblet-shaped cells — are found throughout the colonic epithelium, secreting mucus into the intestinal lumen. Endocrine, 'neuroendocrine', or 'enteroendocrine' cells form an abundant cell population distributed throughout the intestinal epithelium; these cells secrete peptide hormones in an endocrine or paracrine manner from their contained dense core of neurosecretory granules. Paneth cells are located almost exclusively at the crypt base of the small intestine and ascending colon, contain large apical secretory granules, and express several proteins — including lysozyme, tumor necrosis factor, and the antibacterial cryptins (small molecular-weight peptides related to defensins).

In the stomach, the epithelial lining forms long, tubular glands divided into foveolus, isthmus, neck, and base regions. Gastric foveolar or surface mucus cells are located on the mucosal surface and in the foveola. They contain tightly packed mucous granules in the supranuclear cytoplasm and do not possess a theca. The mucus neck cells are situated within the neck and isthmus of the gastric glands and contain apical secretory mucin granules. The peptic-chief or zymogenic cells are located in the base of the glands in the fundic and body regions; they secrete pepsinogen from oval zymogenic granules. The parietal or oxyntic, acid-secreting cells are located in the body of the stomach in the base of the glands. These cells have many surface infoldings, or canaliculi, which form a network reaching almost to the base of the gland. Endocrine cell families include the enterochromaffin-like cells in the fundus or body that produce histamine; the gastrin-producing cells are a major component of the antral mucosa.

The intestinal crypts and gastric glands are enclosed within a fenestrated sheath of intestinal subepithelial myofibroblasts (ISEMFs). These cells exist as a syncytium that extends throughout the lamina propria and merges with the pericytes of the blood vessels. The ISEMFs play a vital role in epithelial–mesenchymal interactions. ISEMFs secrete hepatocyte growth factor (HGF), TGFβ, [2] and keratinocyte growth factor (KGF) [3], with the receptors for these growth factors are located on the epithelial cells. Thus, the ISEMFs are essential for the regulation of epithelial cell differentiation through the secretion of these and possibly other growth factors [3]. Platelet-derived growth factor-A (PDGF-A), expressed in the intestinal epithelium, acts by paracrine signaling through its mesenchymal receptor, PDGFR-α, to regulate

epithelial–mesenchymal interactions during development. Studies of mice with targeted deletions in the PDGF-A or PDGFR-α genes have shown defects in normal proliferation and differentiation of PDGFR-α positive mesenchymal cells [4]. Typically, ISEMFs are α-smooth muscle actin-positive (αSMA+) and desmin− [3], but some myofibroblasts also express myosin-heavy chains [5]. ISEMFs undergo proliferation despite this MyoD expression – unlike the skeletal muscle myoblast, which decycles once MyoD is expressed. It has been proposed that these cells form a renewing population, migrating upwards as they accompany the epithelial escalator [6]. Although they appear to proliferate and migrate, they move relatively slowly and then move off into the lamina propria to become polyploid [7].

A second myofibroblast population in the intestine is the interstitial cells of Cajal. These cells are located close to neurones in the muscular layers; they act as pacemakers for gastrointestinal smooth muscle activity, propagate electrical events, and modulate neurotransmission [8]. They are said to be αSMA+ and desmin+ and to immunostain for *c-kit* and CD45 [3].

EPITHELIAL CELL LINEAGES ORIGINATE FROM A COMMON PRECURSOR CELL

Little is known of the location and fate of the stem cells within the gastrointestinal tract because of the lack of distinctive and accepted stem cells markers, although they are usually said to appear undifferentiated and can be identified operationally by their ability to repopulate crypts and glands after damage.

The unitarian hypothesis states that all the differentiated cell lineages within the gastrointestinal epithelium emanate from a common stem cell origin [9]. Although widely propounded, until very recently little definitive evidence existed to underpin this hypothesis [10]. Moreover, Pearse and Takor famously proposed that gastrointestinal endocrine cells derive from migrating neuroendocrine stem cells in the neural crest [11], a concept that still has its adherents [12]. Although studies of quail neural crest cells transplanted into chick embryos [13], or experiments where the neural crest is eradicated [14], show gut endocrine cells to be of endodermal origin, Pearse [15] subsequently suggested that the endodermis is colonized by 'neuroendocrine-programmed stem cells' from the primitive epiblast, which generates gut endocrine cells. This hypothesis was not ruled out by chick-quail chimera experiments; therefore, other models must be used to ascertain the gut endocrine cell origins, such as the chimeric mouse studies described later.

Several lines of evidence suggest that stem cells reside in the base of the crypts of Lieberkuhn in the small intestine, just superior to the Paneth cells (approximately the fourth or fifth cell position in mice). In the large intestine, they are presumed to be located in the mid-crypt of the ascending colon and in the crypt base of the descending colon [1]. However, within the gastric glands, migration of cells is bidirectional from the neck-isthmus region to form the simple mucous epithelium of the foveolus or pit, and cells migrate downward to form parietal cells and chief cells. Therefore, the stem cells are believed to be within the neck-isthmus region of the gastric gland [16]. The unitarian hypothesis is now supported by a considerable body of research.

SINGLE INTESTINAL STEM CELLS REGENERATE WHOLE CRYPTS CONTAINING ALL EPITHELIAL LINEAGES

The ability of intestinal stem cells to regenerate epithelial cell populations of entire intestinal crypts and villi following cytotoxic treatment has been demonstrated using the crypt microcolony assay [17]. Four days after irradiation, sterilized crypts undergo apoptosis and disappear, but they can be identified by remaining radio-resistant Paneth cells at the crypt base. At higher radiation dose levels, only single cells survive in each crypt, since a unit increase in radiation leads to unit reduction in crypt survival. Survival of one or more clonogenic cells in a crypt after radiation ensures crypt persistence, and there is regeneration of all epithelial cell populations of that crypt

and, in the small intestine, of the overlying villi. Therefore, following cytotoxic damage, a single surviving stem cell can produce all cell types of the intestinal epithelium to reproduce a crypt [18].

MOUSE AGGREGATION CHIMERAS SHOW THAT INTESTINAL CRYPTS ARE CLONAL POPULATIONS

Mouse embryo aggregation chimeras are readily made, wherein the two populations can be readily distinguished. The lectin *Dolichos biflorus* agglutinin (DBA) binds to sites on the B6-derived but not on the Swiss Webster (SWR)-derived cells in C57BL/6J Lac (B6)×SWR mouse embryo aggregation chimeras, and it can be used to distinguish the two parental strains in gut epithelium. The intestinal crypts in each chimera studied were either positive or negative for DBA, and there were no mixed crypts in the tens of thousands studied [19] (Fig. 44.1A). Therefore, each crypt forms a clonal population. This is the case for Paneth, mucous, and columnar cells, although it was not possible to detect the markers in endocrine cells because of their inability to bind the lectin on their surface [20]. In neonatal C57BL/6J Lac (B6)×SWR chimeras, there were mixed (i.e., polyclonal) crypts for the first two weeks after birth,

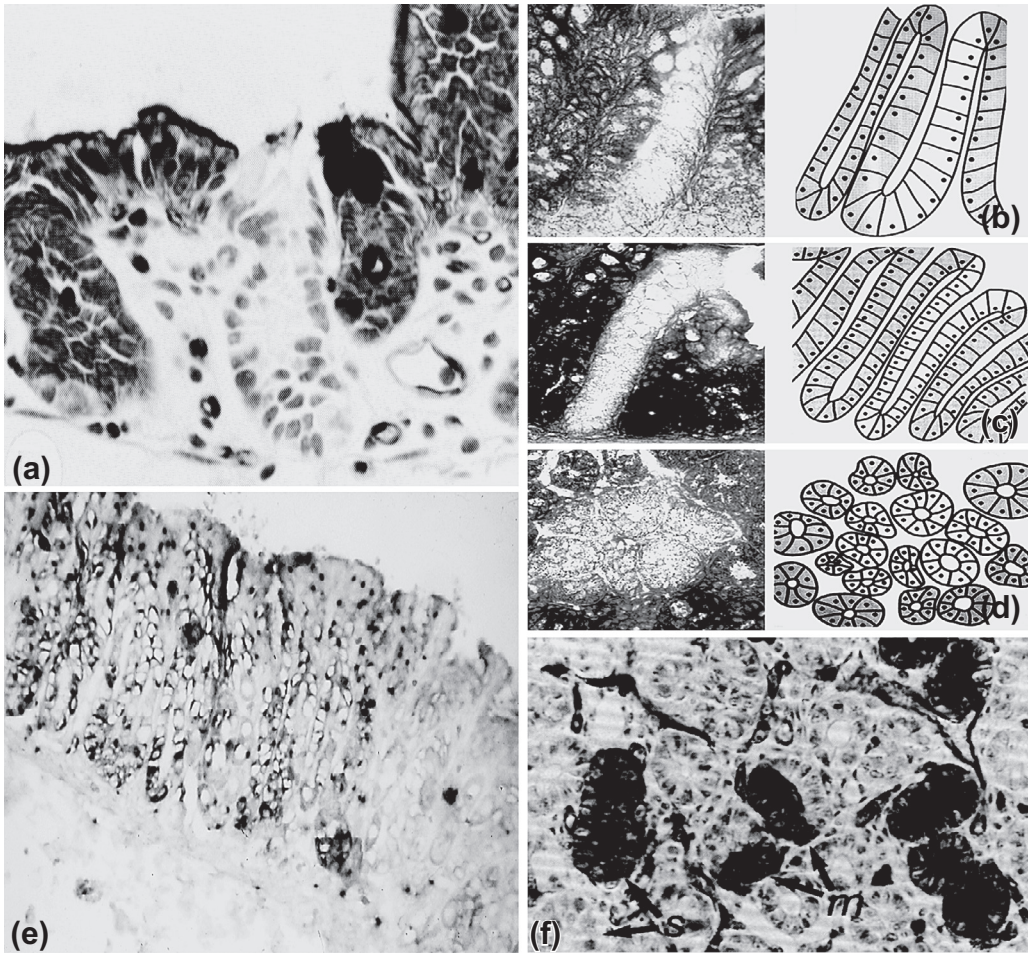

FIGURE 44.1

Gastrointestinal clonality studies. Mouse embryo aggregation chimeras, XX—XY chimeric mice, and X-inactivation mice. (a) DBA staining in the small intestine of ENU-treated (12 weeks) C57BL/6J-SWR F1 chimeras showing entire negative and positive (black) crypts. (b—d) G6PD histochemistry in frozen sections of colonic mucosa in an ENU-treated C3H mouse: (b) partially negative crypt, (c) completely negative crypt, and (d) cross section of an eight-crypt patch at 21 weeks. (e) Y-spot pattern in the gastric mucosa and underlying tissues of an XX—XY chimera. (f) Cross section through crypts in neonatal duodenum of B6-SWR chimera stained with DBA (B6 = black staining, SWR = unstained). A balanced contribution to mixed crypts (m), and monoclonal crypts (s) is seen. *Panel a reproduced with permission from Winton et al., [102] panel b with permission from Park et al., [22] panel e courtesy of E.M. Thompson, and panel f with permission from Schmidt et al. [21] (Please see CD-ROM for color version of this figure.)*

suggesting that multiple stem cells exist during development [21] (Fig. 44.1B through 44.1D). However all crypts ultimately become derived from a single stem cell between birth and postnatal day 14, so-called monoclonal conversion. This apparent cleansing or 'purification' of crypts could be caused by the stochastic loss of one stem cell lineage or by the segregation of lineages because of an extremely active replication of crypts by fission, which occurs at this developmental period[22,23]. To exclude the possibility that crypts from distinct strains segregate differentially during organogenesis, Griffiths et al. [24] confirmed the findings in mice bearing an X-linked defective gene for glucose-6-phosphate dehydrogenase (*G6PD*) (Fig. 44.1e and later sections of this chapter).

In the stomach, the situation is similar although more complex. Epithelial cell lineages in the antral gastric mucosa of the mouse stomach, including the endocrine cells, derive from a common stem cell. Identification of the Y-chromosome by *in situ* hybridisation in XX—XY chimeric mice showed that gastric glands were also clonal populations [21] (Fig. 44.1f). These findings were confirmed by Tatematsu et al. [25] in 1994 in CH3×BALB/c chimeric mice, where each gastric gland was composed of either CH3 or BALB/c cells; there were no mixed glands. Thus, we might advance the general hypothesis that gastric glands in the mouse, in addition to the intestinal crypts, are clonally derived. Additionally, by combining immunohistochemistry for gastrin, an endocrine cell marker, with *in situ* hybridization to detect the Y-chromosome, the male regions of the gastric glands were shown to be almost exclusively Y-chromosome positive with gastrin-positive endocrine cells, whereas the female areas in the chimeric stomach were gastrin positive and Y-chromosome negative [21] (Fig. 44.1d). These results finally negate the Pearse concept that gut endocrine cells originate from a separate stem cell pool.

Nomura et al. [26] used X-inactivation mosaic mice expressing a *lacZ* reporter gene to study clonality of gastric glands in the fundic and pyloric regions of the developing mouse stomach. As in the intestine, most glands are initially polyclonal, with three or four stem cells per gland, but they become monoclonal during the first six weeks of life — again either by purification of the glands, where division of one stem cell eventually overrides all other stem cells, or by gland fission. A population of approximately 5—10% of mixed, polyclonal glands persists into adulthood. The significance of these mixed glands with an increased stem cell number is not known, but it is possible that they do not undergo fission or have reduced fission rates; perhaps they even have an increased stem cell number during development and therefore maintain a higher number of stem cells after crypt fission.

SOMATIC MUTATIONS IN STEM CELLS REVEAL STEM CELL HIERARCHY AND CLONAL SUCCESSION

Somatic mutations at certain loci allow us to study stem cell hierarchy and clonal succession within the gastrointestinal tract. Mutations in the *DLb-1* gene on chromosome 11 are one good example of this; C57BL/6J×SWR F1 chimeric mice show heterozygous expression of a binding site on intestinal epithelial cells for the DBA lectin. This binding site can be abolished when the *Dlb-1* locus becomes mutated either spontaneously or by the chemical mutagen ethyl nitrosourea (ENU). After ENU treatment, crypts emerge that initially are partially and then are entirely negative for DBA staining [27]. Perhaps the simplest explanation for this phenomenon is that a mutation occurs at the *Dlb-1* locus in a stem cell within the small intestinal crypt. This mutated cell could expand stochastically to produce a clone of cells that cannot bind DBA and remain unstained [27] (Fig. 44.1ee). If this is the case, then a single stem cell can generate all the epithelial lineages within an intestinal crypt of the small intestine.

A 'knock-in' strategy at the *Dlb-1* locus can also be used to explain the preceding findings. If SWR mice do not express a DBA-binding site on their intestinal epithelial cells but can be induced to bind DBA by ENU treatment, wholly DBA+ or DBA− intestinal crypts would result. From the use of this model, Bjerknes and Cheng [23,28] proposed that 'committed epithelial

progenitor' cells exist in mouse intestinal crypts by visualizing the morphology, location, and longevity of mutant clones in crypts and villi of the mouse small intestine. These transitory committed progenitor cells — the columnar cell progenitors (C_o) and the mucus cell progenitors (M_o) — evolve from pluripotential stem cells and then differentiate further into adult intestinal epithelial cell types.

Not much is known about the mechanisms that regulate the proliferation of these progenitor cells, but administration of proglucagon-derived, glucagon-like peptide 2 (GLP-2) to SWR mice was found to induce intestinal epithelial growth and repair specifically by stimulating the columnar cell progenitors, resulting in increased crypt and villus size in the normal small intestine [28]. The receptor for GLP-2 (GLP2R) was recently shown to be located on enteric neurons [28] and not on the gut enteroendocrine cells and in the brain as previously thought [29]. GLP-2 activation of enteric neurons produces a rapid induction in *c-fos* expression, which signals growth of columnar epithelial cell progenitors and stem cells that generate adult columnar cell types. There is no stimulatory effect on the mucus cell lineage; instead, it is stimulated by KGF. Thus, the committed progenitor cells are involved in regeneration of damaged epithelia, possibly through a neural regulatory pathway (Fig. 44.2b).

There remains the possibility that cells from different parental strains of chimeric animals segregate independently during development to produce *monophenotypic* crypts and not *monoclonal* crypts. Mosaic expression of the electrophoretic isoenzymes PGK-1A and PGK-1B were analyzed in colonic crypts of mice heterozygous for the X-linked alleles *Pgk-1*^{a} and *Pgk-1*^{b} [19]. In agreement with the results from the chimeric studies, no mixed crypts were seen, thus eliminating the possibility that these crypts are monophenotypic and verifying that they are derived from a single progenitor cell. In a further model, mice that heterozygously express a defective *G6PD* gene have a crypt-restricted pattern of *G6PD* expression, thus confirming that the intestinal crypts are derived from a single stem cell [24]. Moreover, mice treated with the colon carcinogen dimethylhydrazine (DMH) or ENU also develop crypts that initially are partially and later are wholly negative for *G6PD*. The partially negative crypts could conceivably result from the

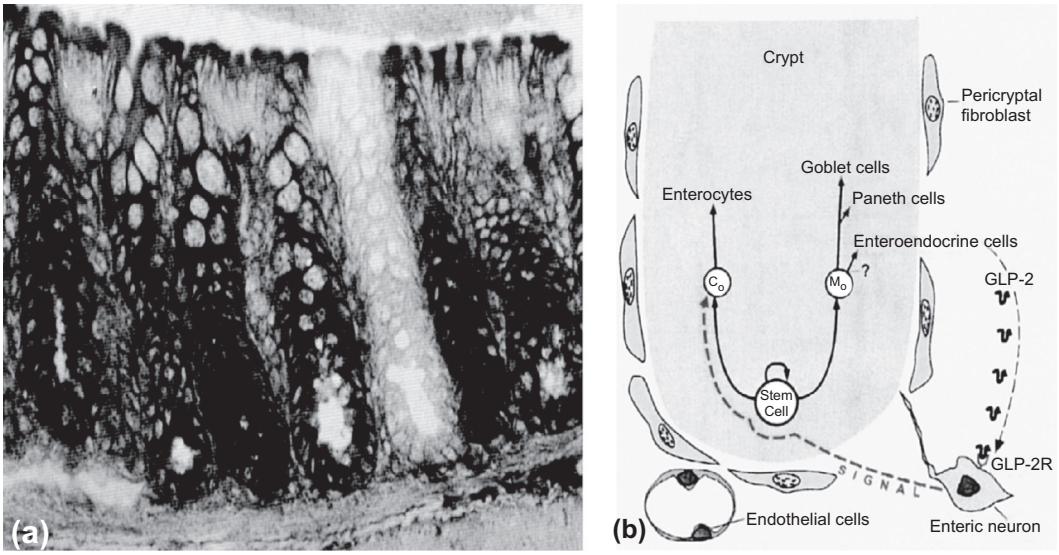

FIGURE 44.2

Stem cell niche hypothesis. (a) G6PD-stained frozen section of colonic mucosa from a CH3 mouse 14 days after a single dose of DMH, showing a single, wholly mutated crypt in which all cells have been replaced by a mutant phenotype. (b) Diagrammatic representation of the stem cell niche. An active, multipotent stem cell produces a daughter cell (C_o) generates enterocytic lineages and another that generates goblet, Paneth, and enteroendocrine cells (although it is not known whether all derive from M_o). GLP-2, produced by a subset of enteroendocrine cells, stimulates proliferation of the C_o daughter through an interaction with enteric nervous system neurons that express the GLP-2 receptor (GLP-2R). *Panel a reproduced with permission from Williams et al., [35] and panel b reproduced with permission from Mills et al. [165] (Please see CD-ROM for color version of this figure.)*

mutation of a cell in the dividing transit population of the crypt that lacks stem cell properties. This is supported by the observation that these partially negative crypts are transient and decrease in frequency parallel to an increase in wholly negative crypts. Conversely, such partially negative crypts could become wholly negative by stochastic expansion of a mutant stem cell. Wholly negative crypts would then be a clonal population derived from this mutant stem cell.

After administration of a mutagen, in both the Dlb-1 and the G6PD models, the time taken for the decrease in partially mutated crypts and the emergence of entirely negative crypts to reach a plateau is approximately four weeks in the small intestine and up to 12 weeks in the large intestine (Fig. 44.2a). This difference was initially thought to be because of cell cycle time differences between the two sites. However, a favored explanation can be found in the *stem cell niche hypothesis*. This hypothesis suggests that multiple stem cells occupy a crypt with random cell loss after stem cell division. It was originally formulated as the stem cell zone hypothesis by Bjerknes and Cheng (see Wright [1] for review and Bjerknes et al.[30–34]). The numbers of stem cells may be greater in the small intestine than in the large intestine, causing the difference in time taken for phenotypic changes following mutagen treatment as the mutant stem cell expands stochastically [35] (Fig. 44.2a). An alternative hypothesis might lie in crypt

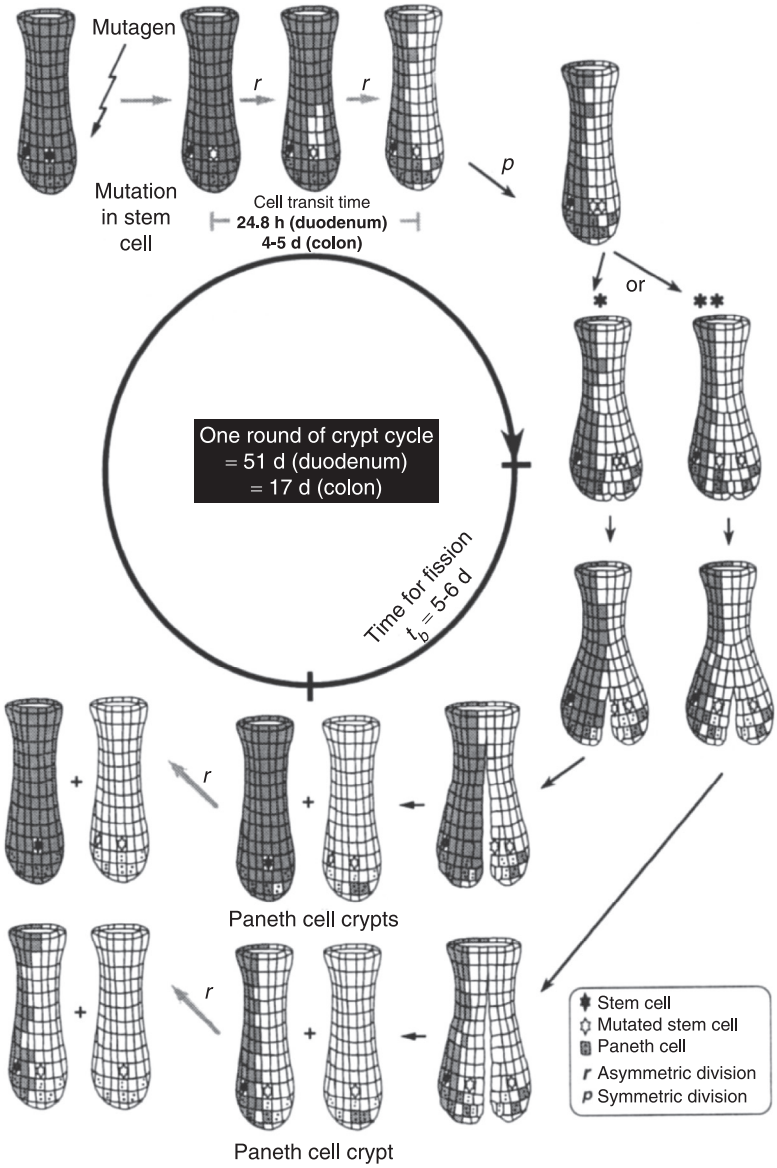

FIGURE 44.3
Crypt cycle. Emergence of transformed crypts after a single stem cell mutation. Crypt division is triggered by symmetric stem cell divisions (p), indicated by light-colored arrows, whereas dark arrows show asymmetric stem cell divisions (r). This is independent of the crypt cycle and enables individual crypts to maintain a constant number of stem cells and to continuously replace differentiated progeny over a crypt cell turnover time, on average. *Reproduced with permission from Park et al. [22] (Please see CD-ROM for color version of this figure.)*

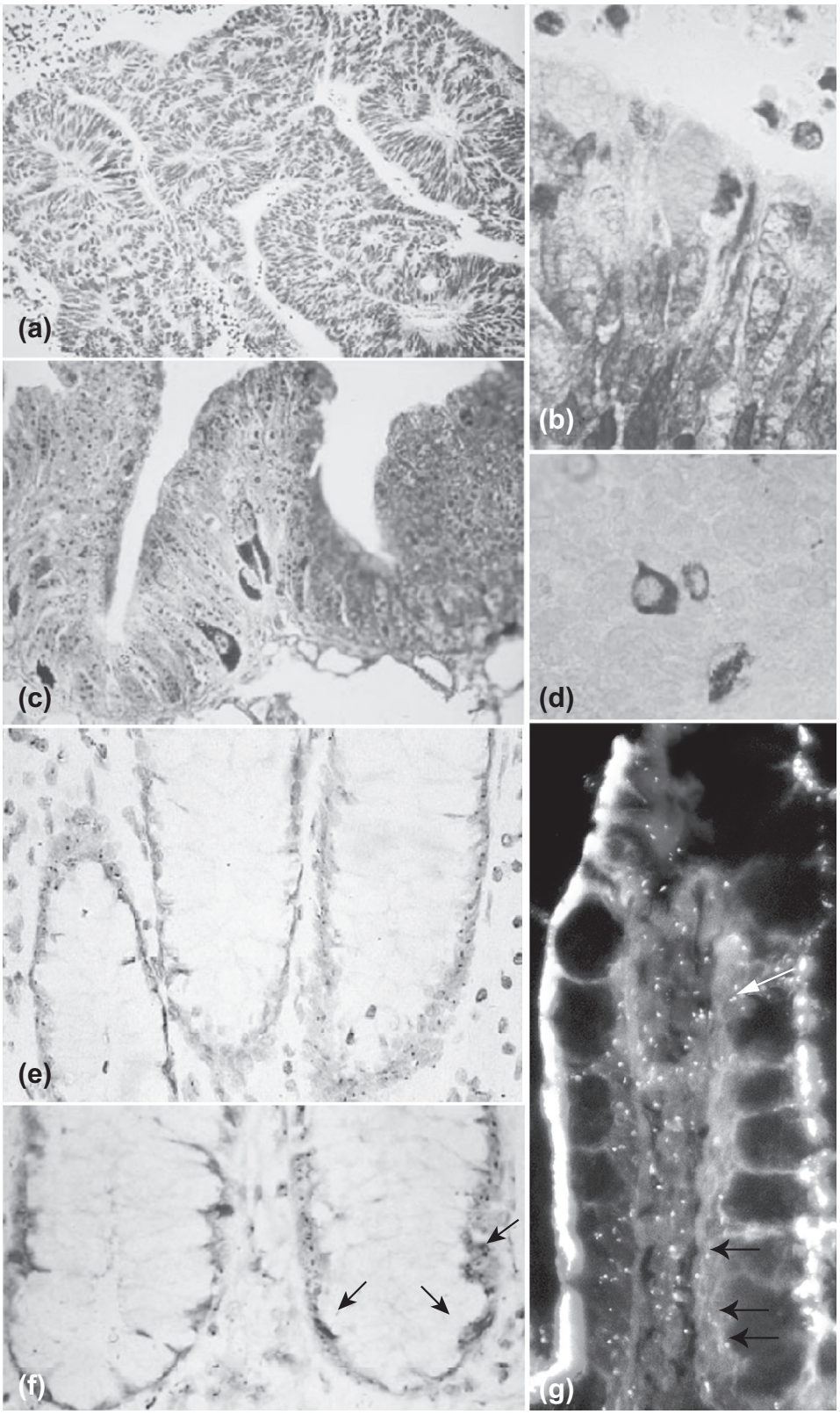

FIGURE 44.4

Monoclonal origin of human clonic crypts. (a) A tumor growing in a nude mouse derived from a single cell cloned human colorectal carcinoma cell line, stained (b) with Alcian blue to show goblet cells, and with (c) Grimelius and (d) an antibody against chromogranin A to demonstrate endocrine cells. (e) Monoclonal origin of human colonic crypts: normal colonic mucosa in an XO—XY mosaic individual stained by *in situ* hybridization for a Y-chromosome-specific probe showing an XO crypt (central) surrounded by two XY crypts. (f) Endocrine cells, highlighted by their chromogranin A content, also show clonality with the

fission: the rate of fission at the time of mutagen administration was higher in the colon than in the small intestine. During crypt fission, when crypts divide longitudinally, selective segregation of the two cell populations could occur, 'cleansing' the partially mutated crypts by segregating the mutated and non-mutated cells and by duplicating the wholly negative crypts to create monoclonal crypts [22] (Fig. 44.3). First proposed as a concept by Park et al. [22], this phenomenon was illustrated by Bjerknes and Cheng [23].

HUMAN INTESTINAL CRYPTS CONTAIN MULTIPLE EPITHELIAL CELL LINEAGES DERIVED FROM A SINGLE STEM CELL

The investigation of gastrointestinal stem cells in humans has proved difficult. The human colorectal carcinoma cell line HRA19 was derived from a primary adenocarcinoma of the rectum. A colony of single morphology was cloned and grown as a monolayer *in vitro* [36]. When engrafted subcutaneously into nude mice, these clones produce tumors histologically identical to the original tumor and contain columnar, goblet, and neuroendocrine cells (Figs. 44.4a through 44.4d). These malignant epithelial cells are therefore multipotent and can produce all differentiated cell types in the human colorectal epithelium [10]. However, these results cannot definitively be applied to normal human gastrointestinal epithelia.

In most of the human population, the colonic goblet cells secrete O-acetylated mucin. However, approximately 9% of the human Caucasian population have a homozygous genetic mutation in the enzyme O-acetyl transferase ($OAT^{-/-}$) and in goblet cells secreting this non-O-acetylated sialic acid; these are then positive when stained with mild periodic acid-Schiff (mPAS) stain[37,38]. In heterozygotes, which comprise approximately 42% of the population (OAT^{-}/OAT^{+}), O-acetylation proceeds, and crypts thereby stain negative for mPAS. Loss of the remaining active *OAT* gene converts the genotype to OAT^{-}/OAT^{-}, resulting in occasional, apparently randomly located, positive mPAS-stained crypts with uniform staining of goblet cells from base to luminal surface, an effect that increases with age [39]. This could be because of a somatic mutation or nondisjunction in a single crypt stem cell and subsequent colonization of the crypt by the mutated stem cell. The frequency of these events is racially determined [40] and increases after irradiation [41]. This has been interpreted as indicating an increased rate of stem cell mutation. Interestingly, there is no increase in the rate of apparent stem cell mutation in hereditary nonpolyposis carcinoma patients [42] or in the background mucosa of left- and right-sided carcinomas [43]. However, just as in the mouse stem cell mutation models, when patients are followed by the mPAS method over the period of time after irradiation, initially there is partial crypt staining, and then whole crypts appear where the goblet cells are mPAS+ [44]. The *clonal stabilization time* (defined as the period required for the emergence of most of such wholly stained crypts) in humans is approximately one year, a process we referred to previously as monoclonal crypt conversion.

These results have implications for the origins of goblet cell lineages in the gut: they indicate that they arise from crypt stem cells; however, they say little about the other cell lineages.

Perhaps the best evidence for the clonality of human intestinal crypts, and the stem cell derivation of all contained epithelial cell lineages, comes from studies of the colon of a rare XO—XY patient who received a prophylactic colectomy for familial adenomatous polyposis (FAP) [45,46]. Nonisotopic *in situ* hybridization (NISH) using Y-chromosome-specific probes showed the patient's normal intestinal crypts to be composed almost entirely of either Y-chromosome positive or Y-chromosome negative cells (Fig. 44.4e), with about 20% of crypts being XO. Immunostaining for neuroendocrine specific markers and Y-chromosome NISH

other crypt cell lineages in the same patient. (g) Villi, receiving cells from more than one crypt of different clonal derivation, show a polyclonal pattern in this XO—XY patient. Apart from the occasional Y-chromosome positive inflammatory cell (white arrow), most cells on the right of this villus are XO (black arrows); on the left side, the cells are XY. *Panels a—d courtesy of S. Kirkland, panels e and f courtesy of M. Novelli, and panel g courtesy of R. Poulsom. (Please see CD-ROM for color version of this figure.)*

used in combination showed that crypt neuroendocrine cells shared the genotype of other crypt cells (Fig. 44.4f). In the small intestine, the villus epithelium was a mixture of XO and XY cells, in keeping with the belief that the villi derive from stem cells of more than one crypt (Fig. 44.4g). Of the 12,614 crypts examined, only four crypts were composed of XO and XY cells, which could be explained by nondisjunction with a loss of the Y-chromosome in a crypt stem cell. Importantly there were no mixed crypts at patch boundaries. These observations agree with previous findings using chimeric mice; i.e., that intestinal crypt epithelial cells, including neuroendocrine cells, are monoclonal and derive from a single multipotent stem cell. Consequently, the hypothesis that enteroendocrine cells and other differentiated cell types within the colorectal epithelium share a common cell of origin (the unitarian hypothesis) appears to apply to both mice and humans. These observations have recently been confirmed in Sardinian women heterozygous for a defective *G6PD* gene [47].

It has been proposed that insight into stem cell organization can be gained from the study of the methylation pattern of nonexpressed genes in the colon. In the normal human colon, methylation patterns are somatically inherited endogenous sequences that randomly change and increase in occurrence with aging. Investigation of methylation patterns is a possible alternative to histological markers for investigating crypt histories, and should allow fate mapping. Examination of methylation tags of three neutral loci in cells from a normal human colon showed variation in sequences between crypts and in mosaic methylation patterns within single crypts. Multiple unique sites were present in morphologically identical crypts; for example, one patient had no identical methylation sequences of one gene within any of the crypts studied, even though all sequences were related [48]. This indicates that some normal human colonic crypts are quasi-clonal with multiple stem cells per crypt. Differences in methylation tags can highlight relationships among cells in a crypt where less closely related cells show greater sequence alterations and where closely related cells have similar methylation patterns. Sequence differences suggest that crypts are maintained by stem cells, which are randomly lost and replaced in a stochastic manner, eventually leading to a 'bottleneck' effect in which all cells within a crypt are closely related to a single stem cell descendant. This reduction to the most recent common crypt progenitor is predicted to occur several times during life, superficially resembling the clonal succession of tumor progression [49].

In situ analyses of glandular clonality in the human stomach have been more problematic. Nomura et al. [50] used X-chromosome-linked inactivation to study fundic and pyloric glands in human female stomachs. Studies using polymorphisms on X-linked genes, such as the androgen receptor (HUMARA) to distinguish between the two X-chromosomes, revealed that, although pyloric glands appear homotypic and thus monoclonal, about half of the fundic glands studied were heterotypic for the HUMARA locus and were consequently polyclonal. This finding suggests that a more complex situation occurs in humans than the studies of gastric gland clonality in chimeric mice indicate. However, we have seen that some glands in the mouse remain polyclonal throughout life [26].

BONE MARROW STEM CELLS CONTRIBUTE TO GUT REPOPULATION AFTER DAMAGE

The hematopoietic bone marrow stem cell is of mesodermal origin, and its functionality and cell surface markers have been well characterized [51]. When transplanted into lethally irradiated animals and humans, as in clinical bone marrow transplantation, it has been long considered that the hematopoietic stem cell colonizes host tissues to form only new erythroid, granulocyte-macrophage, megakaryocyte and lymphoid lineages [52]. Although earlier studies suggest that vascular endothelium could derive from transplanted donor marrow, more recent studies not only have confirmed these earlier proposals concerning endothelial cells [53,54] but also have indicated that adult bone marrow stem cells possess a considerable degree of plasticity and can differentiate into different cell types, including hepatocytes [55–59], biliary epithelial cells [56],

skeletal muscle fibers [60], cardiomyocytes [61], central nervous system cells [62], and renal tubular epithelial cells [63]. These pathways can be bidirectional, as muscle [64] and neuronal stem cells [65] can also apparently form bone marrow. Furthermore, it appears that selection pressure induced by target organ damage can intensify the efficacy of this process as bone marrow stem cells differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells in mice, with ischemic cell death following myocardial infarction and coronary artery occlusion[61,66]. Bone marrow stem cells have also been shown to differentiate into pancreatic β-cells [67], and possibly more persuasively, fully differentiated cells can transdifferentiate into other adult cell types without undergoing cell division; for example, exocrine pancreatic cells can differentiate into hepatocytes *in vitro* [68]. Furthermore, isolated potential hepatic stem cells from fetal mouse livers, which differentiate into hepatocytes and cholangiocytes when transplanted into recipient animals, can form pancreatic ductal acinar cells and intestinal epithelial cells when transplanted directly into the pancreas or duodenal wall [69]. Thus, the conventional view that bone marrow stem cells generate cell types of a single lineage (i.e., all formed elements in the peripheral blood) has been rectified in favor of the findings that adult bone marrow stem cells are highly plastic and can differentiate into many cell types within various organs.

These observations have raised the possibility of regeneration of a failing organ by transplanting an individual's own bone marrow stem cells to colonize and repopulate the diseased tissue, thus avoiding the allograft reaction. In apparent proof of principle, fumarylacetoacetate hydrolase (FAH)-deficient mice, which resemble type 1 tyrosinaemia in humans, are rescued from liver failure by transplantation of purified hematopoietic stem cells that become morphologically normal hepatocytes, express the FAH enzyme, and therefore are functionally normal [59].

There are now several reports that bone marrow cells can repopulate both epithelial and mesenchymal lineages in the gut. Brittan et al. [70] analyzed the colons and small intestines of female mice that had received a bone marrow transplant from male mice donors and gastrointestinal biopsies from female patients with graft versus host disease following bone marrow transplant from male donors. Bone marrow cells frequently engraft into the mouse small intestine and colon and differentiate to form ISEMFs within the lamina propria. *In situ* hybridization confirmed the presence of Y chromosomes in these cells; their positive immunostaining for αSMA and negativity for desmin, the mouse macrophage marker F4/80, and the hematopoietic precursor marker CD34 determined their phenotype as pericryptal myofibroblasts in the lamina propria derived from transplanted bone marrow. This engraftment and transdifferentiation occurred as early as one week after bone marrow transplantation; almost 60% of ISEMFs were bone marrow-derived six weeks after the transplantation, indicating that transplanted bone marrow cells are capable of a sustained turnover of the ISEMF cells in the lamina propria (Fig. 44.5). Y-chromosome-positive ISEMFs were also seen in the human intestinal biopsy material [70]. Lethally irradiated female mice given a male bone marrow transplant and a subsequent foreign body peritoneal implant formed granulation tissue capsules containing myofibroblast cells derived from the hematopoietic stem cells of the transplanted bone marrow [71]. This suggests that myofibroblasts may generally derive from bone marrow cells.

There are a growing number of reports that bone marrow cells can repopulate gastrointestinal epithelial cells in animals and man. Krause et al. [72] found bone marrow-derived epithelial cells in the lung, gastrointestinal tract, and skin 11 months after transplantation of a single hematopoietic bone marrow stem cell in the mouse. In the gastrointestinal tract, engrafted cells were present as columnar epithelial cells in the oesophageal lining, a small intestinal villus, colonic crypts, and gastric foveola. No apparent engraftment into the pericryptal myofibroblast sheath was reported, and as only a single hematopoietic stem cell was transplanted, it is possible that the ISEMFs derive from mesenchymal stem cells within transplanted whole bone marrow. It is, however, generally believed that stromal cell populations do not survive following bone marrow transplantation — although if an empty niche exists, as after irradiation in the gut, engraftment might occur.

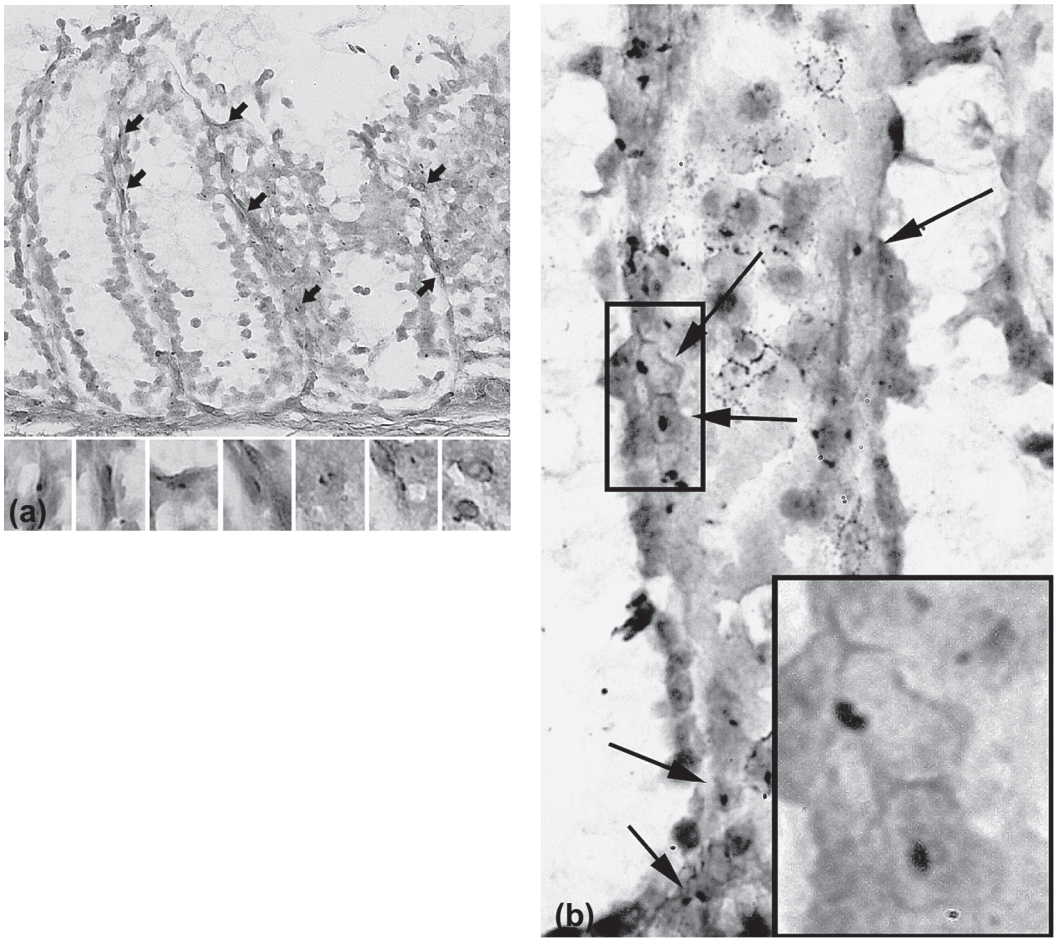

FIGURE 44.5

Bone marrow derived ISEMFs in the lamina propria of the mouse colon. Female mouse colon following a male whole bone marrow transplant. Bone marrow derived ISEMFs are present as Y-chromosome-positive cells, immunoreactive for α-SMA (a) two weeks after transplant (arrows and high-power inserts) and as columns reaching from crypt base to tip (b) six weeks after transplant (arrows and high-power inserts). The Y-chromosome is seen as a black punctate density, and cytoplasmic staining indicates immunoreactivity for α-SMA. *Courtesy of M. Brittan. (Please see CD-ROM for color version of this figure.)*

In biopsies from female patients who had undergone sex-mismatched hematopoietic bone marrow transplantation, *in situ* hybridization for a Y-chromosome-specific probe with immunohistochemical staining for cytokeratins demonstrated mucosal cells of donor origin in the gastric cardia [73]. Moreover, Okamoto et al. [74] reported four long-term bone marrow transplant survivors with multiple engraftment of oesophageal, gastric, small intestinal, and colonic epithelial cells by donor bone marrow cells up to eight years after transplantation, emphasizing the long-term nature of this transdifferentiation.

It would be impossible to finish even such a short section on this topic without mentioning the mechanisms and significance of such phenomena. At first considered to be caused by trans-differentiation or lack of lineage fidelity — commonly seen in invertebrates, during gastrulation, or during organogenesis — it is becoming clear that such changes are neither simple nor readily reproducible. Some labs have been unable to reproduce earlier findings [75,76], and there are claims that adult tissues are contaminated with bone marrow precursors [64]. Finally, the fusion of a transplanted bone marrow cell with an indigenous adult cell has been proposed as the mechanism by which bone marrow stem cells acquire the phenotype of target cell lineages. Initially, cell fusion was seen as a rare event, occurring only *in vitro* and under circumstances of extreme selection [77]. In the FAH model described previously, cell fusion was recently shown to be common, and it cannot be ruled out as the main mechanism by which bone marrow stem

cells transform to functional hepatocytes [78,79]. However, the genetic mechanisms whereby gene expression is switched off in the recipient cells — followed by clonal expansion to repopulate large areas of the liver, for example — are as yet unclear. We should also recall that several tissues in the mouse are polyploid, such as the liver and the acinar cells of the exocrine pancreas [80]. Other studies in which mixed-sex bone marrow transplants have been used to show plasticity have not reported evidence of cell fusion in animals or man. For example, bone marrow-engrafted cells in the human stomach, intestine, buccal mucosa, and pancreatic islet cells showed a normal complement of X and Y chromosomes [67,73,74,81]. Whatever the mechanism, it is clear that the most important criterion for altered lineage commitment, that of function [82], has only been fulfilled in a few models, such as the FAH model and possibly post-infarction cardiomyocyte engraftment. The future in this field will prove interesting.

In inflammatory bowel diseases, such as Crohn's disease, intestinal myofibroblasts are activated to proliferate and synthesize an extracellular matrix, and excessive collagen deposition causes fibrosis and post-inflammatory scarring in the lamina propria and muscularis layers of the gut wall. Intestinal inflammation is believed to be mediated by luminal bacteria and bacterial wall polymers [83], and tumor necrosis factor (TNF) plays a key role in the pathogenesis of intestinal inflammatory disease, since mice with targeted deletion of the AU-rich elements in TNF develop chronic ileitis resembling a Crohn's disease-like phenotype [84] (Figs. 44.6a through 44.6d). Moreover, a single dose of anti-TNF antibody to Crohn's disease patients can dramatically

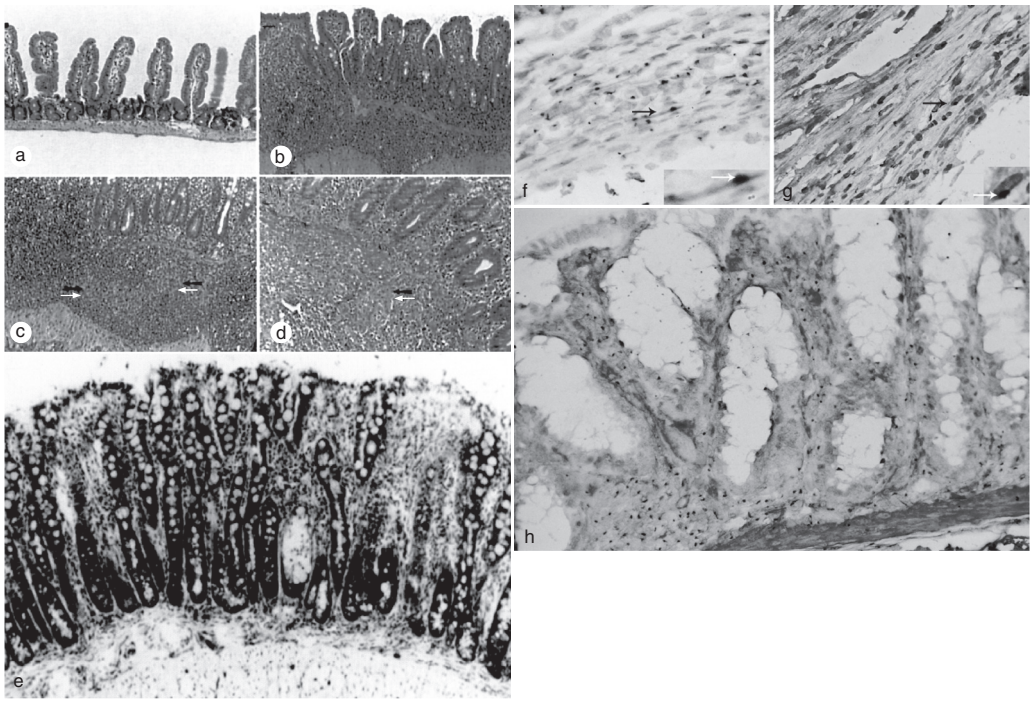

FIGURE 44.6

Murine models of fibrosis. Inflammatory bowel disease in TNFαARE and SAMP/Yit mouse ilea resembling human Crohn's disease. (a) Normal ileal morphology in an 8-week control mouse. (b) TNFαARE homozygous knockout at 7 weeks with blunt, distorted villi and increased inflammatory infiltrate. (c) TNFαARE heterozygote at 16 weeks with villus blunting and chronic inflammation. An ill-defined noncaseating granuloma is in the submucosa (arrows). (d) Ileocaecal region from a human patient with Crohn's disease demonstrating similar location and composition of granuloma compared with mouse model (arrow). (e) SAMP/Yit mouse at 20 weeks showing severe mucosal inflammation, crypt hyperplasia, elongation, and villous atrophy. (f) A fibrotic reaction in the serosal tissues of a paracetemol-treated male-female radiation chimeric mouse, showing numerous spindle-shaped cells with Y-chromosomes revealing their bone marrow origin, which are also (g) vimentin-positive, confirming their fibroblast lineage. (h) Massive expansion of myofibroblasts of bone marrow origin in a male-female chimeric mouse with colitis induced by TNBS. *Panel d reproduced with permission from Kontoyiannis et al., [84] panel e with permission from Matsumoto et al., [166] panels f—g courtesy of N. Direkze, and panel h courtesy of M. Brittan. (Please see CD-ROM for color version of this figure.)*

alleviate inflammation [85]. Other cytokines, including interleukin-10 [86] and TGFβ [87] are implicated in the development of fibrosis in inflammatory bowel disease.

It has previously been concluded that fibrotic reactions, which occur in inflammatory bowel disease, and other diseases are caused by the local proliferation of myofibroblasts and fibroblasts [88]. Our recent data showing that transplanted bone marrow contributes to the intestinal subepithelial myofibroblast population indicates that extraintestinal cells may have a role in fibrosis. In this respect, we have shown that cells with a fibroblast-fibrocyte phenotype can derive from transplanted bone marrow and contribute to fibrotic reactions in and around the intestinal wall [89] (Fig. 44.6f). The concept that cells of the lamina propria, other than the lymphoid and myeloid lineages, exist in equilibrium with bone marrow precursors is an interesting one and could provide an opportunity for therapeutic delivery of cytokines to the intestine to prevent the development of fibrosis, or even to treat it. Further recent data [90] show that many of the myofibroblasts and fibroblasts recruited to, or expanding in, the lamina propria and submucosa of mice with induced trinitrobenzene sulfonic acid (TNBS) colitis are bone marrow derived (Fig. 44.6g).

GASTROINTESTINAL STEM CELLS OCCUPY A NICHE MAINTAINED BY ISEMFs IN THE LAMINA PROPRIA

Stem cells within many tissues are thought to reside within a niche formed by a group of surrounding cells and their extracellular matrices, which provide an optimal microenvironment for the stem cells to function. The identification of a niche within any tissue involves knowledge of the location of the stem cells; as we have seen, this has proved problematical in the gastrointestinal tract. According to Spradling et al. [91], to prove that a niche is present, the stem cells must be removed and subsequently replaced while the niche persists, providing support to the remaining exogenous cells. Although this has been accomplished in *Drosophila* [92], such manipulations have not yet been possible in mammals. In this context, the survival of a single epithelial cell following cytotoxic damage to intestinal crypts in the microcolony assay is interesting, as many of the intestinal subepithelial myofibroblasts are also lost after irradiation [93], although sufficient numbers may remain or be replaced by local proliferation or migration from the bone marrow to provide a supportive niche for the surviving stem cell or cells. The ISEMFs surround the base of the crypt and the neck-isthmus of the gastric gland, a commonly proposed location for the intestinal and gastric stem cell niches, respectively. It is proposed that ISEMFs influence epithelial cell proliferation and regeneration through epithelial-mesenchymal crosstalk, and that they ultimately determine epithelial cell fate [3].

There has been a long quest for markers of stem cells in the intestine. The neural RNA-binding protein marker Musashi-1 (Msi-1) is a mammalian homolog of a *Drosophila* protein evidently required for asymmetrical division of sensory neural precursors. In the mouse, Msi-1 is expressed in neural stem cells [94,95] and has been proposed as the first intestinal stem cell marker because of its expression in developing intestinal crypts and specifically within the stem cell region of adult small intestinal crypts. This is further substantiated by its expanded expression throughout the entire clonogenic region in the small intestine after irradiation [94,95].

The regulatory mechanisms of stem cell division within the niche to produce, on average, one stem cell and one cell committed to differentiation is as yet unknown, although there is no shortage of potential models [96]. In the stem cell zone hypothesis, the bottom few cell positions of the small intestinal crypt are occupied by a mixture of cell types: Paneth, goblet, and endocrine. The migration vector is toward the bottom of the crypt [30–33]. Above cell position 5, cells migrate upward, although only the cells that divide in the stem cell zone beneath are stem cells. Other models envisage the stem cells occupying a ring immediately above the Paneth cells [97], although there is little experimental basis for such an assertion,

since 'undifferentiated' cells of similar appearance are seen among the Paneth cells in thin sections[31,32]. Moreover, there is no difference in the expression of Msi-1 and Hes1 — a transcriptional factor regulated by the Notch signaling pathway also required for neural stem cell renewal and neuronal lineage commitment — in undifferentiated cells located in either the stem cell zone or immediately above the Paneth cells [98]. This suggests that both populations may have the same potential as putative stem cells.

The number of stem cells in a crypt or a gland is presently unknown. Initially, all proliferating cells were believed to be stem cells [99]. Although clonal regeneration experiments using the microcolony assay indicated that intestinal crypts contained a multiplicity of stem cells, it was clear that this was less than the proliferative cellularity. Proposed stem cell numbers have varied from a single stem cell [100] to 16 or more [101]. Others have proposed that the number of stem cells per crypt varies throughout the crypt cycle, with the attainment of a *threshold number* of stem cells per crypt being the signal for fission to occur [102]. There is little experimental evidence to support these proposals. Although all cells in a crypt are initially derived from a single cell, as shown by the chimeric and X-inactivation experiments discussed previously, mutagenesis studies such as those shown in Fig. 44.1e and Fig. 44.2a argue strongly for more than one stem cell per crypt, with stochastic clonal expansion of a mutant clone. A three stem cell colonic crypt has been suggested by Williams et al. [35] on this basis.

In organisms such as *Drosophila* and *Caenorhabditis elegans* stem cell divisions are known to be asymmetric. We have no such firm concept in the mammalian gut, although there is some evidence to support the proposal [103,104]. By labeling DNA template strands in intestinal stem cells with tritiated thymidine during development or tissue regeneration, and by labeling newly synthesized daughter strands with bromodeoxyuridine, segregation of the two markers can be studied. The template DNA strand labelled with tritiated thymidine is retained, but the newly synthesized strands labeled with bromodeoxyuridine become lost after the second division of the stem cell. This indicates not only that asymmetric stem cell divisions occur but also that by discarding the newly synthesized DNA, which is prone to mutation, into the daughter cell destined to differentiate, a mechanism of stem cell genome protection is afforded [104].

When a stem cell divides, the possible outcomes are that two stem cells (P) are produced, that two daughter cells destined to differentiate (Q) cells are produced, or that there could be an asymmetric division resulting in one P and one Q cell. These are sometimes called p, q, and r divisions [102] or p and q divisions [107] (Fig. 44.7). If $p = 1$ and $q = 0$, then regardless of the number of stem cells per crypt, the cells are immortal and there will be no drift in the niche with time. Such a situation is called 'deterministic.' [48,105] However, if $p < 1$ and $Q > 0$ (i.e.,

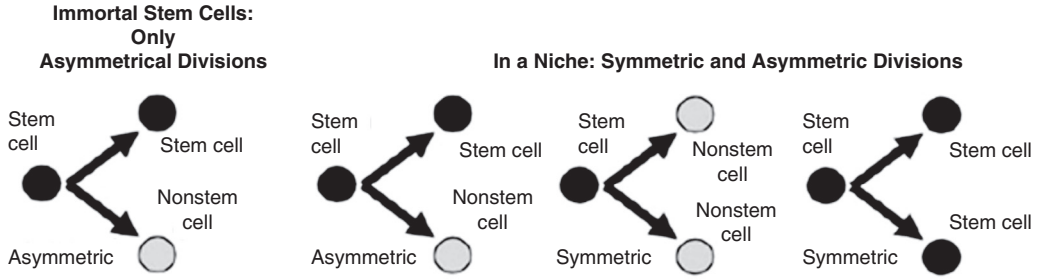

FIGURE 44.7

How stem cells divide in a niche. Classically, stem cells always replace themselves by asymmetrical division; therefore, their lineages never become extinct. However, although the stem cell number remains constant in a niche, both asymmetrical and symmetrical divisions occur. Symmetrical division can lead to one stem cell lineage becoming extinct as both daughters leave the niche, but to maintain a constant stem cell number in the niche, this loss is balanced by stem cell expansion, where both daughters remain in the niche as stem cells. Such stem cell loss with ensuing replacement can lead to the extinction of all lineages bar one, or clonal succession. *Reproduced with permission from Kim et al. [159] (Please see CD-ROM for color version of this figure.)*

a stochastic model), there will be eventual extinction of some stem cell lines and a drift toward a common stem cell from which all other cells derive. We previously described the variation in methylation patterns or 'tags' that occur in human colonic crypts [48,105] and explained that crypts apparently show several unique tags. The variance of these unique tags was compared with those expected using a variety of models, including no drift with aging (the deterministic model), drift with immortal stem cells with divergence (the numbers of unique tags are proportional to the stem cell number), drift with one stem cell per crypt, and a stem cell niche with more recent divergence (with loss of stem cells occurring proportional to the time since divergence). Multiple unique tags were found in some crypts, and the number of unique tags increased with the number of markers counted, which favors random tag drift and multiple stem cells per crypt. The variances were consistent with drift in immortal stem cells, where N (the number of stem cells) $= 2$, but favored a model where $0.75 < P < 0.95$ and $N < 512$. Thus, the data supported a stochastic model with multiple stem cells per crypt. However, as in many such attempts, there are several major assumptions necessary, such as a constant stem cell number. It is clear that variation in both P and N occur in this model. However, this analysis is consistent with the results of Campbell et al. [44], who deduced that the time taken for monoclonal conversion, or the 'clonal stabilization time', of $OAT^{+/-}$ individuals to convert to $OAT^{-/-}$ cells following irradiation was found to be about one year. Assuming 64 stem cells per niche and $P = 0.95$, the mean time for conversion should be some 220 days. The same assumptions suggest a bottleneck, where all stem cells are related to the most recent common ancestral cell, occurring every 8.2 years [104].

MULTIPLE MOLECULES REGULATE GASTROINTESTINAL DEVELOPMENT, PROLIFERATION, AND DIFFERENTIATION

Although the molecular mechanisms by which pluripotent stem cells of the gastrointestinal tract produce differentiated cell types are not clearly understood, an increasing number of genes and growth factors have been identified that regulate development, proliferation, and differentiation, as well as development of tumors. These are expressed by intestinal mesenchymal and epithelial cells and include members of the fibroblast growth factor family [106], epidermal growth factor family, TGFβ (reviewed by Dignass et al. [107]), insulin-like growth factors 1 and 2 [108], HGF-scatter factor [109], Sonic and Indian hedgehog [110], and PDGF-α [5], among others.

WNT/β-CATENIN SIGNALING PATHWAY CONTROLS INTESTINAL STEM CELL FUNCTION

The Wnt family of signaling proteins is critical during embryonic development and organogenesis in many species. There are 16 known mammalian *Wnt* genes, which bind to receptors of the frizzled (*Fz*) family, eight of which have been identified in mammals. The multifunctional protein β-catenin normally interacts with a glycogen synthase kinase 3-β (GSK3-β), axin, and adenomatous polyposis coli (APC) tumor suppressor protein complex [111]. Subsequent serine phosphorylation of cytosolic β-catenin by GSK3-β leads to its ubiquitination and to its proteasomal degradation, thereby maintaining low levels of cytosolic and nuclear β-catenin. Wnt ligand binding to its *Fz* receptor activates the cytoplasmic phosphoprotein *dishevelled*, which in turn initiates a signaling cascade resulting in increased cytosolic levels of β-catenin. β-Catenin then translocates to the cell nucleus, where it forms a transcriptional activator by combining with members of the T cell factor/lymphocyte enhancer factor (Tcf/LEF) DNA-binding protein family. This activates specific genes, resulting in the proliferation of target cells, for example, in embryonic development (Fig. 44.8). In addition to its role in normal embryonic development, the Wnt/β-catenin pathway plays a key role in malignant transformation [112,113]. Mutations of the *APC* tumor suppression gene are present in up to 80% of human sporadic colorectal tumors [114,115]. This mutation prevents normalβ-catenin

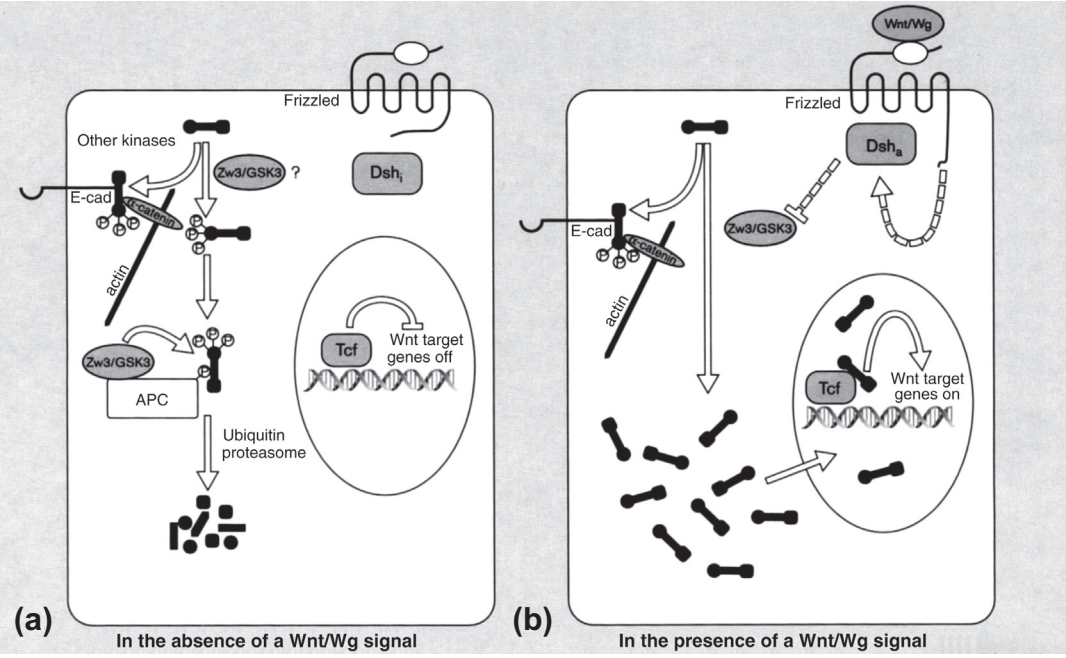

FIGURE 44.8

Wnt signaling pathway. (a) In the absence of Wnt signaling, *dishevelled* is inactive (Dshi), and Drosophila zeste-white 3 or its mammalian homolog glycogen synthase kinase 3 (Zw3/GSK3) is active. β-Catenin (black dumbbell), through association with the APC—Zw3/GSK3 complex, undergoes phosphorylation and. degradation by the ubiquitin-proteasome pathway. Meanwhile, T cell factor (Tcf) is bound to its DNA-binding site in the nucleus, where it represses the expression of genes such as Siamois in Xenopus. (b) In the presence of a Wnt signal, dishevelled is activated (Dsha), leading to inactivation of Zw3/GSK3 by an unknown mechanism. β-Catenin fails to be phosphorylated and is no longer targeted into the ubiquitin-proteasome pathway; instead, it accumulates in the cytoplasm and enters the nucleus by an unknown pathway, where it interacts with Tcf to alleviate repression of the downstream genes and provide a transcriptional activation domain. *Reproduced with permission from Willert et al. [113] (Please see CD-ROM for color version of this figure.)*

turnover by the GSK3β/axin/APC complex. This results in increased nuclear *β-catenin/Tcf/LEF* gene transcription and a subsequent increase in β-catenin-induced Tcf/LEF transcription [116,117]. One of the main functions of *APC* appears to be the destabilization of β-catenin. Free β-catenin is one of the earliest events, or perhaps even the initiating event, in tumorigenesis in the murine small intestine and in the human colon [118]. Many genes, including *c-myc*, *cyclin D1*, *CD44*, *c-Jun*, *Fra-1*, and urokinase-type plasminogen receptor have been identified as targets of the β-catenin/Tcf/LEF nuclear complex, although the precise mechanisms that lead to carcinogenesis are not entirely understood [118—122].

The Tcf/LEF family of transcription factors has four members; Tcf-1, LEF1, Tcf-3, and Tcf-4. Tcf-4 is expressed in high levels in the developing intestine from embryonic day (E) 13.5 and in the epithelium of adult small intestine, colon, and colon carcinomas. When there is loss of function of APC or mutations in β-catenin, increased β-catenin/Tcf-4 complexes are formed that lead to uncontrolled transcription of target genes [123,124]. Mice with targeted disruption of the *Tcf-4* gene have no proliferating cells within their small intestinal crypts and lack a functional stem cell compartment [124] (Fig. 44.9a through Fig. 44.9f). This suggests that *Tcf-4* is responsible for establishing stem cell populations within intestinal crypts; this in turn is thought to be activated by a *Wnt* signal from the underlying mesenchymal cells in the stem cell niche. Chimeric ROSA26 mice expressing a fusion protein containing the high mobility group box domain of Lef-1 linked to the transactivation domain of β-catenin (B6Rosa26<>129/Sv(Lef-1/β-cat) display increased intestinal epithelial apoptosis. This occurs specifically in 129/Sv cells throughout crypt morphogenesis, unrelated to enhanced cell proliferation. On completion of crypt formation and in adult mice, there is complete loss of all 129/Sv cells. Stem cell selection appears to be biased toward the unmanipulated ROSA26 cells in these

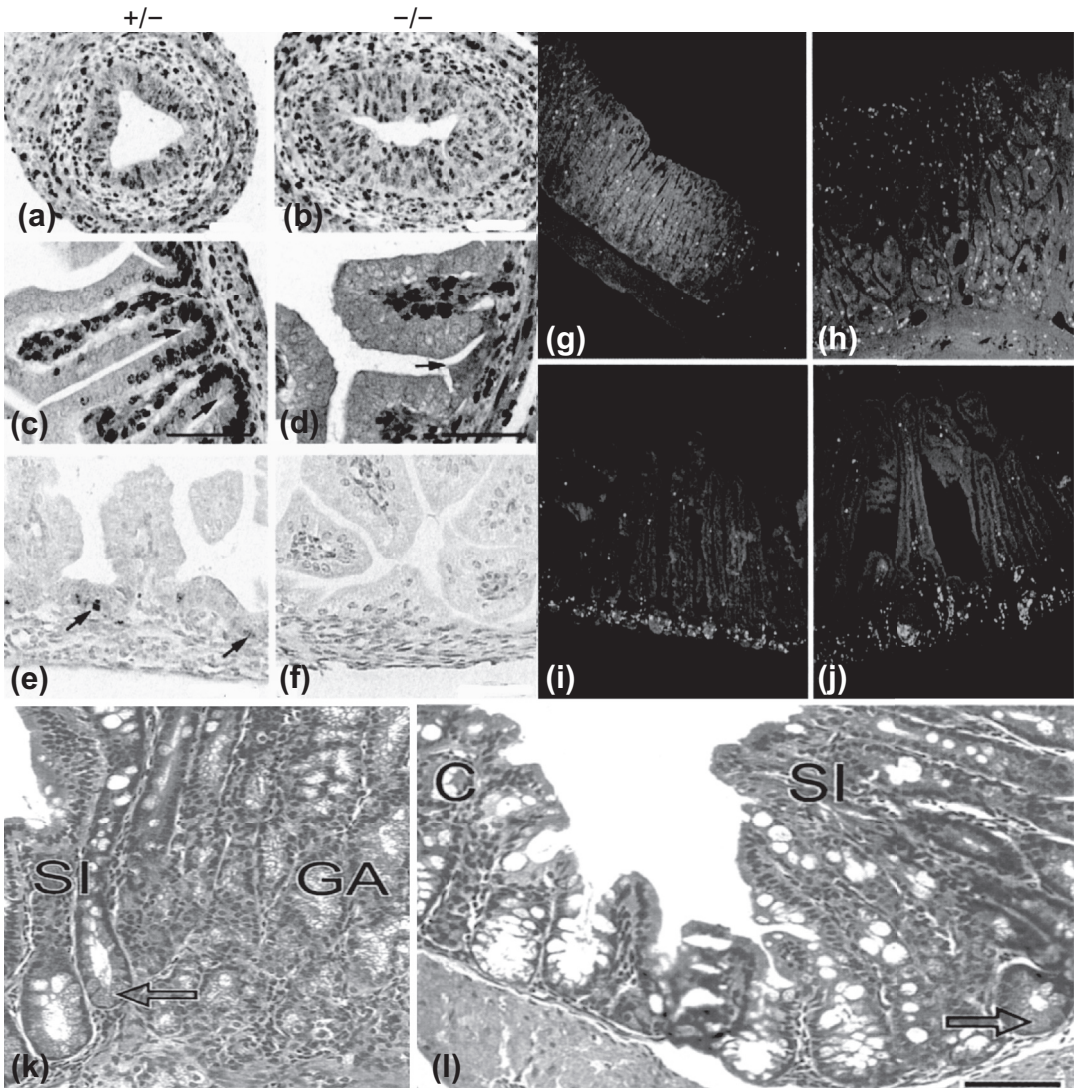

FIGURE 44.9

Tcf-4 knockout mice, Foxl1 knockout mice, and Cdx-2 knockout mice. (a–f) Small intestine from (a) E14.5 Tcf712+/– heterozygous, (b) Tcf712–/– homozygous, (c) E16.5 Tcf712+/–, and (d) Tcf712–/– embryos stained with Ki-67 antibody. (e) E17 heterozygous and (f) homozygous bromodeoxyuridine-labeled embryos stained with antibromodeoxyuridine. Numerous proliferating cells are present (a and b) throughout the epithelium at E14.5 in heterozygotes and homozygotes, but are restricted (c) to intervillous regions in E16.5 heterozygotes and absent (d) in E16.5 homozygotes. bromodeoxyuridine-labeled cells are present in intervillous regions of the (e) Tcf712+/– embryos (arrows), but are absent from (f) Tcf712–/– embryos. (g–j) Horizontal bars indicate 0.1 mm. Epithelial proliferation is increased in Foxl1 mutant mice shown by injection of bromodeoxyuridine 1.5 hours before sacrifice (g and h) in the stomach and (i and j) jejunum of (g and i) wild-type and Foxl1-null mice in increased levels (h and j) shown by immunofluorescence, where labelled nuclei represent proliferating cells. (k–l) The lateral edge of a colonic polyp in Cdx-2+/– mouse encompassing the gastric antrum (GA), small intestine (SI), and colon (C). Bar indicates 5.55 mm. (k) Goblet cells in the villi and Paneth cells (arrow) in the crypts adjacent to the branched mucus-secreting cells of the gastric antrum. (i) Transition from SI to C shows stunted villi with goblet cells and Paneth cells (arrow) in the crypts between villi. *Panels a–f reproduced with permission from Korinek et al., [124] panels g–j with permission from Perreault et al., [132] and panels k–l with permission from Beck et al. [127] (Please see CD-ROM for color version of this figure.)*

chimeras, suggesting that 'adequate threshold' levels of β-catenin during development permit sustained proliferation and selection of cells, establishing a stem cell hierarchy. Increased β-catenin expression appears to induce an apoptotic response, and thus the stem cell niche is unaffected by increased Lef-1/β-catenin during intestinal crypt development [125].

TRANSCRIPTION FACTORS DEFINE REGIONAL GUT SPECIFICATION AND INTESTINAL STEM CELL FATE

Hox genes define regional gut specification

The mammalian homeobox genes *Cdx-1* and *Cdx-2* display specific regional expression in developing and mature colon and small intestine. During embryogenesis, *Cdx-1* localizes to the proliferating cells of the crypts and maintains this expression during adulthood. The Tcf-4 knockout mouse does not express *Cdx-1* in the small intestinal epithelium, thus the Wnt/β-catenin complex appears to induce *Cdx-1* transcription in association with Tcf-4 during the development of intestinal crypts [126]. Mice heterozygous for a *Cdx-2* mutation develop colonic polyps composed of squamous, body, and antral gastric mucosa with small intestinal tissue (Fig. 44.9). Proliferation of *Cdx-2*colonic cells with low *Cdx-2* levels can produce clones of cells phenotypically similar to epithelial cells of the stomach or small intestine [127]. This could indicate a possible homeotic shift in stem cell phenotype. Region-specific genes such as *Cdx-1*, *Cdx-2*, and *Tcf-4* appear to define the morphological features of differential regions of the intestinal epithelium and regulate the proliferation and differentiation of the stem cells.

Forkhead family is essential for intestinal proliferation

The winged helix-forkhead family of transcription factors is essential for proper development of the ectodermal and endodermal regions of the gut. There are nine murine forkhead family members, which generate the forkhead box (Fox) proteins [128], three homologs of the rat hepatic nuclear factor 3 gene (*HNF3*α, -β, and -γ), and six genes referred to as forkhead homologs (*fkh-1* through *fkh-6*) [129]. *Fkh-6* is expressed in gastrointestinal mesenchymal cells [130] now reclassified as *Foxl1* [128]. *Foxl1* knockout mice have a dramatically altered gastrointestinal epithelium with branched and elongated glands in the stomach, elongated villi, hyperproliferative crypts, and goblet cell hyperplasia because of increased epithelial cell proliferation (Fig. 44.9g through 44.9j) [131]. They show upregulated levels of heparin sulfate proteoglycans (HSPGs), which increase *Wnt*-binding efficacy to the *Fz* receptors on gastrointestinal epithelial cells. This results in overactivation of the Wnt/β-catenin pathway and increased nuclear β-catenin [132]. The resultant β-catenin/Tcf/LEF complex activates target genes such as *cyclin D1* and *c-myc*, which increase epithelial cell proliferation. Therefore, *Foxl1* regulates the Wnt/β-catenin pathway in association with an increase in HSPGs, demonstrating epithelial cell regulation by mesenchymal factors during embryogenesis in the gastrointestinal tract. As *c-myc* is a known proto-oncogene, a mutation of *Foxl1* and the resultant increase in epithelial cell proliferation through inappropriate *c-myc* activation may lead to the development of colorectal cancers [122].

E2F transcription family is essential for development of the crypt proliferative zone

The E2F family of transcription factors regulates cell proliferation, allowing transit from the G_1 to the S phase. E2F4 is expressed in the proliferative regions of embryonic intestine and in the adult small intestine and colon. In E2F4-knockout mice, intestinal crypts fail to develop, and the lamina propria appears thickened. E2F4 is thus essential for the development of the proliferative compartment of the intestinal epithelium, although the molecular pathways that influence E2F4 during development are unknown [133].

MULTIPLE MOLECULES DEFINE STEM CELL FATE AND CELL POSITION IN THE VILLUS-CRYPT AXIS

The colonic crypt and the villus-crypt axis offer a system in which the fate of stem cell progeny is defined. Their position within this axis can readily be determined. The goblet cell number normally remains relatively constant, and Paneth cells derive positional information and use it

to remain in the crypt base. In mice, deletion of the *Math1* gene, a basic helix-loop-helix transcription factor and downstream component of the *Notch* signaling pathway, depletes goblet, Paneth, and enteroendocrine cell lineages in the small intestine. This indicates that Math1 is essential for stem cell commitment to one of three epithelial adult cell types. Math1 progenitors merely become enterocytes [134]. High levels of Notch switch on the Hes1 transcriptional repressor. This in turn blocks expression of *Math1* so that cells remain progenitors and ultimately become enterocytes. Conversely, low *Notch* expression increases levels of its ligand Delta, which induces *Math1* expression by blocking Hes1, causing cells to become goblet cells, Paneth cells, or enteroendocrine cells [135]. Hes1-null mice have elevated *Math1* expression, with increased enteroendocrine and goblet cells and fewer enterocytes. This supports the evidence that *Math1* regulates the determination of cell fate through a Notch-Delta signaling pathway [136] (Fig. 44.10).

Recent studies show that β-catenin and Tcf inversely control the expression of the EphB2/EphB3 receptors and their ligand ephrin-B1 in colorectal cancer and along the crypt-villus axis [137]. When the *EphB2* and *EphB3* genes are disrupted, cell positioning within the crypt is also disrupted. For example Paneth cells do not migrate downward to their normal position at the bottom of the crypt but scatter along crypt and villus. This indicates that β-catenin and Tcf contribute to the sorting of cell populations through the EphB/ephrin-B system.

In the future, it is clear that functional genomics will have an increasing role to play in the study and identification of intestinal stem cells. Stappenbeck et al. [138] studied a consolidated population of stem cells isolated by laser-capture microdissection from germ-free

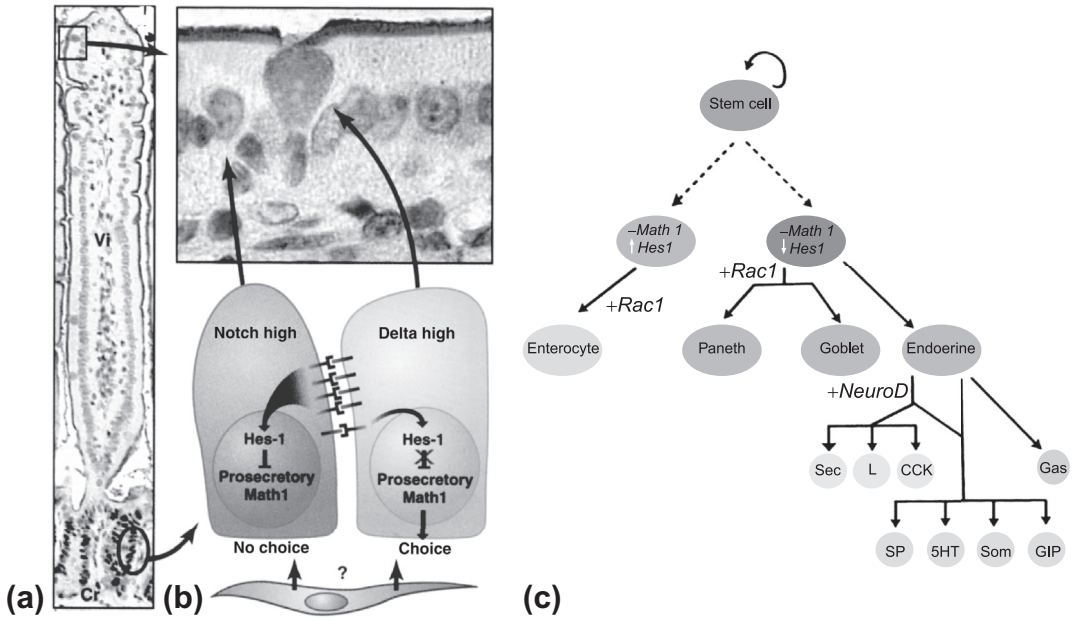

FIGURE 44.10

Math1 signaling pathway. (a) Low power section of adult murine small intestine. Precursor cells are stained for cyclin proliferating cell nuclear antigen, enterocytes express intestinal alkaline phosphatase, and goblet cells secrete mucins. Inset shows high-power image of small intestinal enterocytes and goblet cells. (b) Math1, a component of the Notch signaling pathway, influences intestinal epithelial cell fate decisions. In crypt progenitor stem cells that express high levels of Notch, the Hes1 transcription factor is switched on, and the expression of Math1 and of other 'prosecretory' genes is blocked. The result is that the precursor cells become enterocytes. In cells expressing low amounts of Notch, levels of Delta are high, production of Hes1 is blocked, and Math1 expression is induced. Production of the Math1 helix-loop-helix transcription factor allows precursor cells to make a choice: whether to become goblet cells, Paneth cells, or enteroendocrine cells (c) Math1 is essential for secretory cells. Whether Math1-expressing cells descend directly from stem cells or an intermediate progenitor remains unknown. (Vi: villus, Cr: crypt, Sec: secretin, L: glucagons—peptide YY, CCK: cholecystokinin, SP: substance P, 5HT: serotonin, Som: somatostatin, GIP: gastric inhibitory peptide, and Gas: gastrin). *Panels a and b reproduced with permission from van Den Brink et al., [135] and panel c reproduced with permission from Yang et al. [134] (Please see CD-ROM for color version of this figure.)*

transgenic mice lacking Paneth cells. There were no less than 163 transcripts enriched in these stem cells compared with normal crypt base epithelium, which contains a predominance of Paneth cells. The profile showed prominent representation of genes involved in *c-myc* signaling, as well as in the processing, localization, and translation of mRNAs. Similar studies in the mouse stomach showed that growth factor response pathways are prominent in gastric stem cells, examples including insulin-like growth factor [139]. A considerable fraction of stem cell transcripts encode products required for mRNA processing and cytoplasmic localization. These include numerous homologs of *Drosophila* genes needed for axis formation during oogenesis.

GASTROINTESTINAL NEOPLASMS ORIGINATE IN STEM CELL POPULATIONS

We can use the development of colorectal carcinoma as a paradigm. The concept of the adenoma-carcinoma sequence, whereby adenomas develop into carcinomas, is now widely accepted [140], and most colorectal carcinomas are believed to originate in adenomas. The initial genetic change in the development of most colorectal adenomas is thought to be at the *APC* locus, and the molecular events associated with these stages are clear: a second hit in the *APC* gene is sufficient to give microadenoma development, at least in FAP [141]. There are basically two models for adenoma morphogenesis, both of which closely involve basic concepts of stem cell biology in the colon: in the first, recently formulated, mutant cells appear in the *intracryptal zone* between crypt orifices, and as the clone expands, the cells migrate laterally and downward, displacing the normal epithelium of adjacent crypts [142]. A modification of this proposal is that a mutant cell in the crypt base, classically the site of the stem cell compartment [1], migrates to the crypt apex where it expands. These proposals are based on findings in some early non-FAP adenomas, where dysplastic cells were seen exclusively at the orifices and on the luminal surface of colonic crypts [142]; measurement of loss of heterozygosity (LOH) for *APC* and nucleotide sequence analysis of the mutation cluster region of the *APC* gene carried out on microdissected, well-orientated histological sections of these adenomas showed that half the sample had LOH in the upper portion of the crypts, most with truncating *APC* mutations. Only these superficial cells showed prominent proliferative activity, with nuclear localization of β-catenin, indicating an *APC* mutation only in these apical cells (Fig. 44.11c). Earlier morphological studies have drawn attention to the same appearances [143]. This top-down morphogenesis has wide implications for concepts of stem cell biology in the gut. It is clear that most evidence indicates that crypt stem cells are found at the origin of the cell flux, near the crypt base [23]. These proposals, however, either re-establish the stem cell compartment in the intracryptal zone or make the intracryptal zone a favored locus where stem cells, having acquired a second hit, clonally expand.

An alternative hypothesis proposes that the earliest lesion is the *unicryptal* or *monocryptal adenoma*, where the dysplastic epithelium occupies an entire single crypt. These lesions are common in FAP, and although they are rare in non-FAP patients, they have been described [144]. Here, a stem cell acquires the second hit, and then it expands, stochastically or more likely because of a selective advantage, to colonize the whole crypt. Such monocryptal lesions thus should be clonal [46]. Similar crypt-restricted expansion of mutated stem cells has been well documented in mice after ENU treatment [22] and in humans heterozygous for the *OAT* gene, where after LOH, initially half then the whole crypt is colonized by the progeny of the mutant stem cell [44]. Interestingly, OAT+/OAT$^-$ individuals with FAP show increased rates of stem cell mutation with clustering of mutated crypts. Thus, in sharp contrast the mutated clone expands not by lateral migration but by *crypt fission* in which the crypt divides, usually symmetrically at the base (Fig. 44.12a) or by budding (Fig. 44.11b). Several studies have shown that fission of adenomatous crypts is the main mode of adenoma progression — predominantly in FAP, where such events are readily evaluated [145,146], but also in sporadic

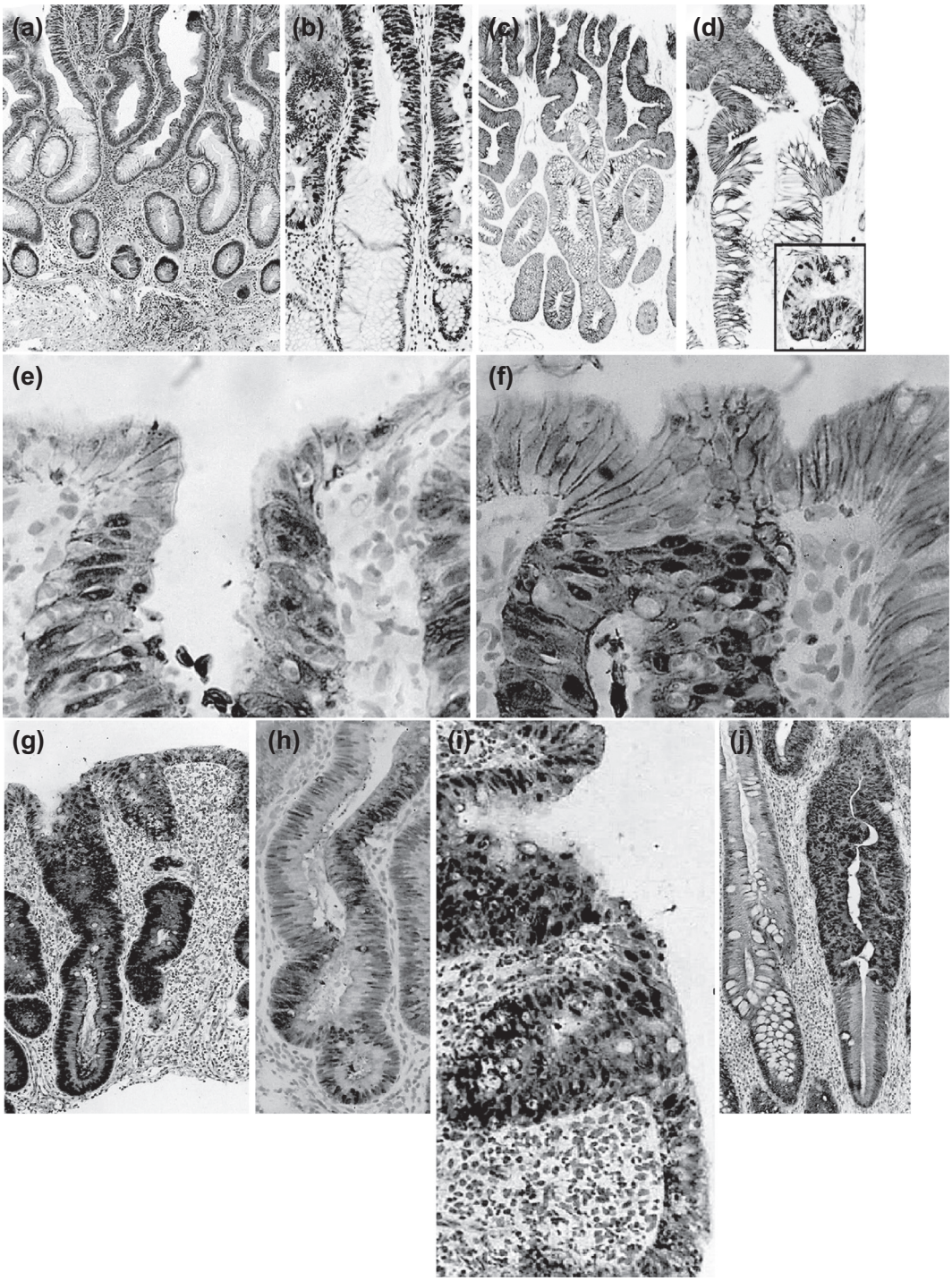

FIGURE 44.11

Contrasting theories for the morphogenesis of adenomas and the part played by stem cells. (a) Hematoxylin- and eosin-stained sections of a small tubular adenoma. Dysplastic epithelium is superficial within the crypts, with histologically normal underlying epithelium. (b) Abrupt transition between dysplastic and normal-appearing epithelial cells at the midpoint of this crypt. Proliferative activity assessed with the Ki-67 antibody, distributed throughout the dysplastic epithelium at the top of the crypts. (c) Nuclear β-catenin is highly expressed and distributed throughout the dysplastic epithelium at the top of the crypts but not in the crypt bases. (d) β-Catenin in the nuclei of adenomatous crypts from a tiny tubular adenoma. (e) Nuclear β-catenin extends to the bottom of crypts in early adenomas, including the very bases of the crypts. (f) β-Catenin staining in nuclei of budding crypts. (g) Junction between early adenomatous crypts, showing a sharp junction on the surface with accumulation of nuclear β-catenin giving way sharply to membranous staining in the normal surface cells. (h) High-power serial sections, demonstrating the sharp junction between nuclear staining in the adenomatous cells and membranous staining in normal surface epithelial cells. (i) Surface continuity between crypts showing nuclear β-catenin staining. (j) Crypts from a larger adenoma stained for β-catenin, showing invasion of adjacent crypt territories in a top-down fashion. *Panels a—d reproduced with permission from Shih et al., [142] and panels e—j reproduced with permission from Preston et al. [152] (Please see CD-ROM for color version of this figure.)*

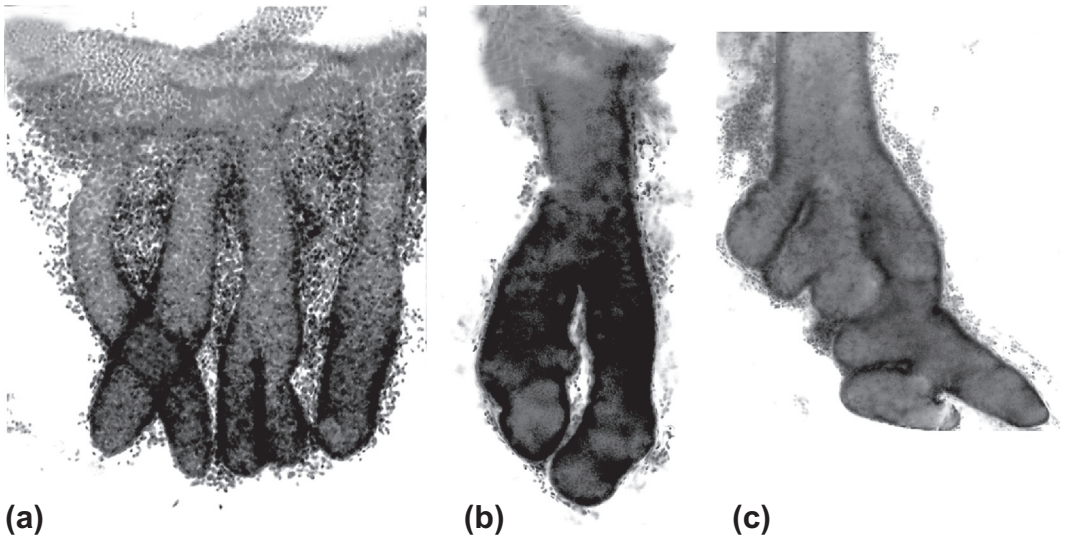

FIGURE 44.12

Microdissected crypts from normal colonic mucosae and adenomas. (a) Symmetrical fission of normal colonic crypts. (b) Isolated adenomatous crypt showing frequent crypt fission with atypical and asymmetrical branching. (c) Another crypt from an adenoma with bizarre shape, asymmetrical branching, and multiple budding. *Reproduced with permission from Preston et al. [152] (Please see CD-ROM for color version of this figure.)*

adenomas [147]. The nonadenomatous mucosa in FAP, with only one *APC* mutation, shows a large increase in the incidence of crypts in fission [145]. Aberrant crypt foci, thought to be precursors of adenomas, grow by crypt fission [148,149], as do hyperplastic polyps [150]. This concept does not exclude the possibility that the clone later expands by lateral migration and downward spread into adjacent crypts, but with the initial lesion the monocryptal adenoma, this model of morphogenesis is conceptually very different.

Recent work supporting the bottom-up spread of colorectal adenomas looked at a number of small (< 3 mm) tubular adenomas. Here, nuclear accumulation of β-catenin (Fig. 44.11g) was seen, indicating loss of function of one of the genes in the Wnt pathway, most likely *APC*, with subsequent translocation of β-catenin to the nucleus. Serial sections (Figs. 44.11e and 44.11f) showed that the β-catenin nuclear staining extended to the bottom of the crypts and was present in crypts in the process of crypt fission (Fig. 44.11h). β-Catenin expression was particularly marked in the nuclei of buds. At the surface, there was a sharp cut-off between the adenomatous cells in the crypt that showed nuclear β-catenin and those surface cells that did not (Figs. 44.11e and 44.11f). The adjacent crypts were filled with dysplastic cells containing nuclear β-catenin, which were not confined to the upper portions of the crypts. In larger adenomas, there was unequivocal evidence of surface cells growing down and replacing the epithelium of normal-looking crypts (Fig. 44.11j). Crypt fission was rare in normal and noninvolved mucosa and usually began with basal bifurcation at the base of the gland (Fig. 44.12a), whereas in adenomas, fission was commonly asymmetrical (Fig. 44.12b) with budding from the superficial and mid-crypt (Fig. 44.12b). Multiple fission events were frequently observed in adenomas (Fig. 44.12c). The *crypt fission index* (the proportion of crypts in fission) in adenomas was significantly greater than that in noninvolved mucosa [150] (Fig. 44.12d).

It is usually stated that adenomas do not display a stem cell architecture, but recent observations [151,152] show that adenomatous crypts in early sporadic adenomas show superficial similarities to normal crypts in the distribution of their proliferative activity. Observations on possibly older adenomas have suggested that maximum proliferative activity is found towards the top of the crypts, indicating that that migration kinetics are reversed, with cells flowing toward the bottom of the crypt [153,154]. These observations are corroborated by the report of

increased apoptosis at the bottom of adenomatous crypts [155]. Such a distribution could support a top-down mechanism. But there is evidence from examining the methylation histories of cells in adenomas for a discrete stem cell architecture [156]; moreover, although crypt mitotic scores are significantly greater in adenomas than in noninvolved mucosa and normal controls, the zonal distribution of mitoses in adenomatous crypts mitoses is evenly distributed throughout the crypt, which does not suggest a concentration of dysplastic cells in the tops of adenomatous crypts.

Examination of the adenomas of an XO-XY individual with FAP showed none exceeded 2.5 mm in diameter [46].The monocryptal adenomas showed either the XO or the XY genotype and hence are clonal proliferations, as would be expected from the observation that crypts are clonal (Fig. 44.13a). Many microadenomas showed the XY genotype, and none of these early lesions showed mixture of XO and XY nuclei occupying the same crypt (Fig. 44.13b). It was shown that 76% of the microadenomas were polyclonal. There were also sharp boundaries at the surface between adjacent adenomatous crypt territories [152] (Fig. 44.3e).

What are the implications of these considerations upon our concepts of the single (stem) cell origin or clonality in colorectal adenomas? We have seen that crypts are clonal units; thus, these lesions would be polyclonal because of the mixture of clonal crypts and clonal adenoma — though in this instance, they have different clonal derivation. A study of both sporadic and FAP adenomas, using X-linked restriction fragment length polymorphisms, showed that such lesions were apparently monoclonal in origin [45]. On the other hand, the X-linked patch in the colon is large — and can be in excess of 450 crypts in diameter [47] (see Fig. 44.14). So, unless an adenoma grows over a patch boundary and involves crypts on either side of that boundary, X-inactivation analysis will always show that such lesions are monoclonal.

On the 'bottom-up' proposals, expansion of a clonal monocryptal adenoma by crypt fission would inevitably lead to a monoclonal microadenoma and thus adenoma. However, as

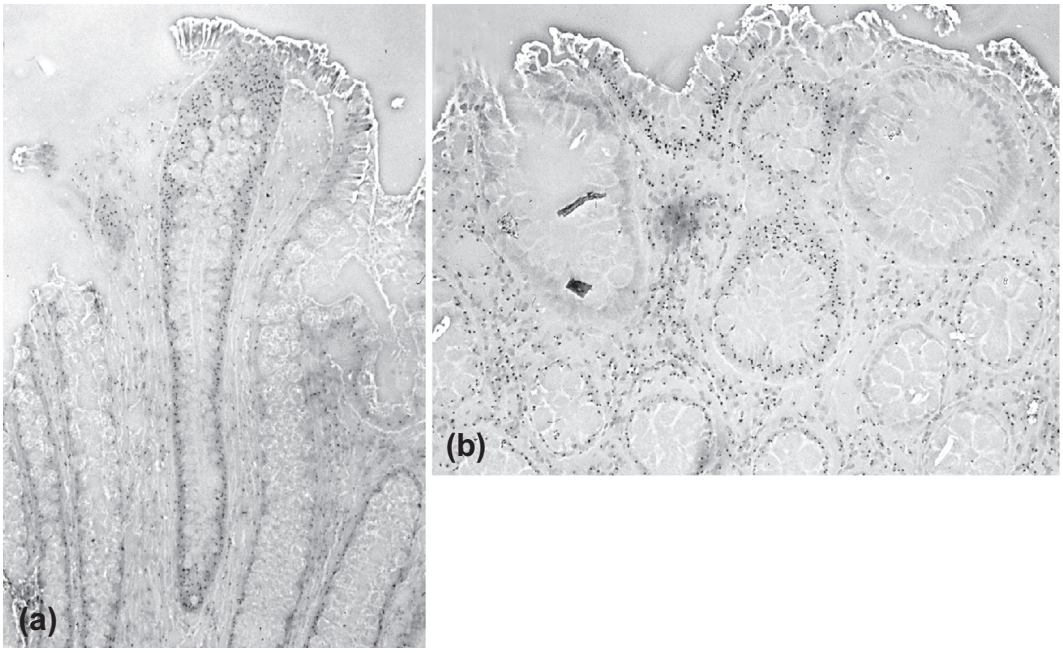

FIGURE 44.13
Adenomas from an XO—XY mosaic individual with FAP, stained by *in situ* hybridisation for a Y-chromosome—specific probe. (a) Clonal monocryptal adenoma. (b) Polyclonal adenoma, with a mixture of XO and XY crypts. Note that there is a sharp margin between the territories of XO and XY adenomatous crypts at the surface with no evidence of invasion. *Reproduced with permission from Preston et al. [152] (Please see CD-ROM for color version of this figure.)*

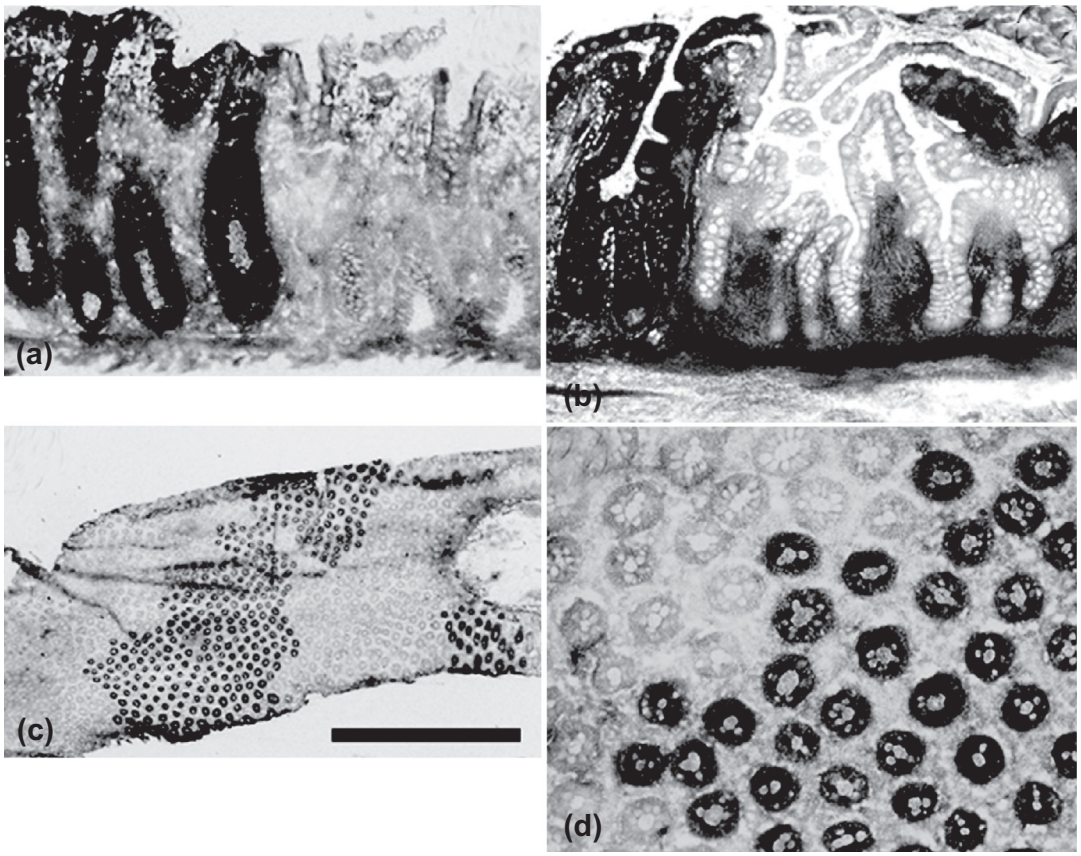

FIGURE 44.14
Visualization of X-inactivation patches directly by enzyme histochemistry in surgical resections from Sardinian females heterozygous for the G6PD Mediterranean mutation (563 C 3 T), previously shown to have reduced G6PD enzyme activity. Heterozygosity for the G6PD Mediterranean mutation was confirmed by polymerase chain reaction analysis of genomic DNA followed by MBOII restriction endonuclease. (a) G6PD staining in longitudinal sections of colonic crypts. In individual crypts, all epithelial cells show a similar staining pattern. (b) Longitudinal sections of crypts and villi in small intestinal mucosa stained for G6PD activity. Epithelial cells in individual crypts show a similar staining pattern, but the villous epithelium shows areas of positive and negative staining, confirming its polyclonal derivation. (c) G6PD staining in colonic patches in low power. (d) High-power view of large patches of crypts with irregular patch borders. Bar indicates 2 mm. *Reproduced with permission from Novelli et al. [47] (Please see CD-ROM for color version of this figure.)*

mentioned before, studies on the XO-XY individual, who has a mean patch size of 1.48 crypts, indicated that some 76% of adenomas were polyclonal [46], supported by observations in Rosa26-Min chimeric mice [157]. This could be explained by the transformation of noninvolved crypts by transformed stem cells.

So what is happening to the stem cells in the development of these early, monocryptal lesions? Bjerknes et al. [158] showed that adenomatous crypts in FAP contained two stem cell lines, $APC^{-/+}$ and $APC^{-/-}$, and interpreted this to mean that the $APC^{-/-}$ cells were expanding stochastically within the same crypt. There is good evidence that such monoclonal conversion occurs as a stochastic process [48,158]. It has previously been shown that a quantitative analysis of age-related methylation suggests that crypts are maintained by niches containing multiple stem cells [48]. Random stem cell loss with replacement suggests that all niche lineages except one will become extinct. It is fairly clear that clonal succession is related to tumor progression [48]. Although we have mentioned previously that Shibata has argued for clonal succession occurring in a stem cell niche without mutation, genetic alterations aiding clonal expansion of a mutant clone might confer a growth advantage [159]. Several mouse genotypes exist in which crypt morphology — and therefore implicitly stem cell proliferative behavior — is normal, such as $APC^{+/-}$, $TP53^{+/-}$, $Trp53^{-/-}$, $Apc^{+/-}$, $Trp53^{-/-}$, $MLH1^{-}/0$, $Apc^{+/-}$, $Mlh1^{-/-}$, and $Tgfbr2^{+/-}$. However, more subtle alterations, such as an increased incidence of crypt

fission that may be dependent upon stem cell behavior, might well be missed (see Wasan et al. [145], for example). These are special cases, so consider the situation in a normal individual, where stem cells are wild-type ($APC^{+/+}$) and one APC allele is lost in a single stem cell. This $APC^{+/-}$ stem cell, unless it possesses a growth advantage, could be lost by the stochastic ejection from the niche — indeed, it could be argued that this will happen in the overriding majority of instances [159], but the $APC^{+/-}$ cell could survive and the niche will be populated by progeny of this $APC^{+/-}$ cell, which will resemble an FAP crypt. A further hit in this crypt will lead to the formation of the monocryptal adenoma on the model proposed previously [152].

Others have also concluded that migrating crypt epithelial cells in the upper part of the crypt are the primary targets for transformation by *APC* mutation [160], and this has received some experimental support [161]. At the bottom of the crypt, progenitor cells accumulate nuclear β-catenin and express *Tcf* target genes as a result of *Wnt* stimulation from surrounding basal pericryptal myofibroblasts. In normal crypts, cells that reach the mid-crypt region down-regulate β-catenin/*Tcf*, resulting in cell cycle arrest and differentiation. Cells that bear a β-catenin or an *APC* mutation do not respond to signals controlling β-catenin/*Tcf* activity, and these cells continue to behave as crypt progenitor cells in the surface epithelium, generating microadenomas. Boman et al. [162] used computer modeling to suggest that an expansion in the crypt stem cell population explained the putative proliferative abnormality in FAP, namely an upwards shift in the proliferative compartment toward the top of the crypt [163]. Simulation of labeling index distribution curves from FAP crypts using a single mechanistic design was able to fit the data from both control and FAP crypts, indicating that the proliferative abnormality does not alter the rate of cell cycle proliferation, differentiation, or apoptosis of proliferating crypt cells. Instead, it suggests an expansion in the crypt stem cell population sufficient to explain the observed proliferative abnormality in FAP. Thus, β-catenin signaling in the colonic crypt controls the number of stem cells. The stem cell population is expanded in FAP crypts because of a germ line APC mutation activating Tcf-4. Any increase in the size of the stem cell population might be expected to result in an increase in the rate of crypt fission [164].

Crypt fission is therefore an essential event in the expansion of mutated clones in adenomas. Although the morphology of this process is distinct, the molecular mechanisms that govern it are far from clear. We further conclude that the initial event in the genesis of colorectal adenomas, of both sporadic and FAP adenomas, is the monocryptal adenoma; initial growth occurs through crypt fission, and spread into adjacent crypt territories is a later, secondary event.

SUMMARY

The cells of the gastrointestinal tract undergo constant renewal and respond to damage by regeneration and repopulation. Each region of the gastrointestinal tract is morphologically distinct with its own repertoire of cell types. Although the stem cells are the most important cells of the gastrointestinal tract, responsible for the production of every other cell type in the gastrointestinal mucosa, they have not yet been closely characterized. There is, initially at any rate, a single stem cell in every intestinal crypt or gastric gland that indirectly generates a clone containing further stem cells, transit amplifying and differentiated cells, through the production of committed progenitor cells. This cell also produces new crypts by crypt fission, repairs entire crypts and villi when damaged, and generates gastrointestinal tumors. The stem cell or cells occupy a niche, formed by mesenchymal cells such as the ISEMFs, and extracellular matrix molecules, which regulate epithelial stem cells through mesenchymal-epithelial crosstalk. The molecular events that regulate the development of the gastrointestinal tract and epithelial cell turnover in the normal tissue and in formation of carcinomas are beginning to be identified. It is clear that the Wnt/β-catenin signaling pathway and downstream molecules such as APC, Tcf-4, Fkh-6, Cdx-1, and Cdx-2 are vital for normal gastrointestinal stem cell function. We are beginning to identify molecular pathways that determine further

proliferation of committed progenitor cells into specific epithelial cell lineages: the Notch-Delta signaling pathways involving Hes1 and Math1 transcription factors regulate differentiation of goblet, Paneth, and enteroendocrine cells in the small intestine. Factors secreted and expressed by the mesenchymal cells (KGF, HGF, etc.) that regulate gastrointestinal mucosal development and epithelial proliferation are rapidly becoming identified. Epithelial and intestinal subepithelial myofibroblast lineages apparently derived from bone marrow, presenting the possibility of delivering therapeutic genes to the damaged lamina propria — for example, in diseases that cause fibrosis, such as Crohn's disease — and even repopulating the damaged gut. Finally, we conclude that the colonic stem cell is pivotal in understanding mechanisms of tumorigenesis in the colon. The isolation and characterization of gastrointestinal stem cells is a priority in gut biology.

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CHAPTER 45

Liver Stem Cells

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INTRODUCTION

The hepatobiliary system consists of the liver, the intrahepatic bile ducts (IHBDs) and extrahepatic bile ducts (EHBDs) including the gallbladder. The identification of stem cells in the hepatobiliary system has importance for both basic biology and cell-based therapy. As candidate cell-based therapy, hepatobiliary stem cells could be an alternative to whole liver transplantation for patients suffering from chronic liver disease. However, the presence of stem cells in the adult liver remains a controversial concept, and there is a paucity of data regarding EHBD stem cells. The controversy surrounding the existence of liver stem cells is partially due to the extraordinary proliferative ability of adult hepatocytes, which are terminally differentiated cells that have a well-documented capacity for self-renewal and can accomplish regeneration of the liver after injury. In addition, there is a lack of clear evidence for the presence of a liver stem cell in the uninjured liver. However, there have been many recent reports of the prospective isolation of candidate stem cell populations in the liver and EHBD systems. Here we review recent literature regarding the lineage organization and self-renewal in liver and EHBD systems, as well as the studies evaluating presence of stem cells in both these systems.

DEFINITION OF A TISSUE-DERIVED STEM CELL

The classic definition of a tissue-derived adult stem cell comes from the study of hematopoietic stem cells. These stem cells have been defined as clonogenic cells capable of both self-renewal and colony formation, and differentiation to multiple committed progenitor cells that in turn proliferate and differentiate into functionally specialized mature cells [1]. In the search for hematopoietic stem cells, one which was initially driven by the need for bone marrow transplantation in patients, a hierarchical system organized around primitive stem cells, together with their committed progenitors and more mature cells, was uncovered [2–7]. With the prospective isolation of hematopoietic stem cells [6,7], it was possible to compare the regenerative abilities of these cells to all the other blood cells in mouse models of bone marrow transplantation. To the surprise of many, only the hematopoietic stem cells and not the more committed progenitor cells were able to sustain long-term engraftment *in vivo*. Thus, in the context of bone marrow transplantation, only the stem cells appeared important for the sustained regeneration of the hematopoietic system. This is in part due to the foremost characteristic of a stem cell: its capacity for self-renewal. Cells without long-term self-renewal potential, such as committed hematopoietic progenitor cells, would be capable of generating enough progeny for short-term engraftment, but would not be able to sustain long-term engraftment over many years. Therefore, self-renewal is a key function of stem cells.

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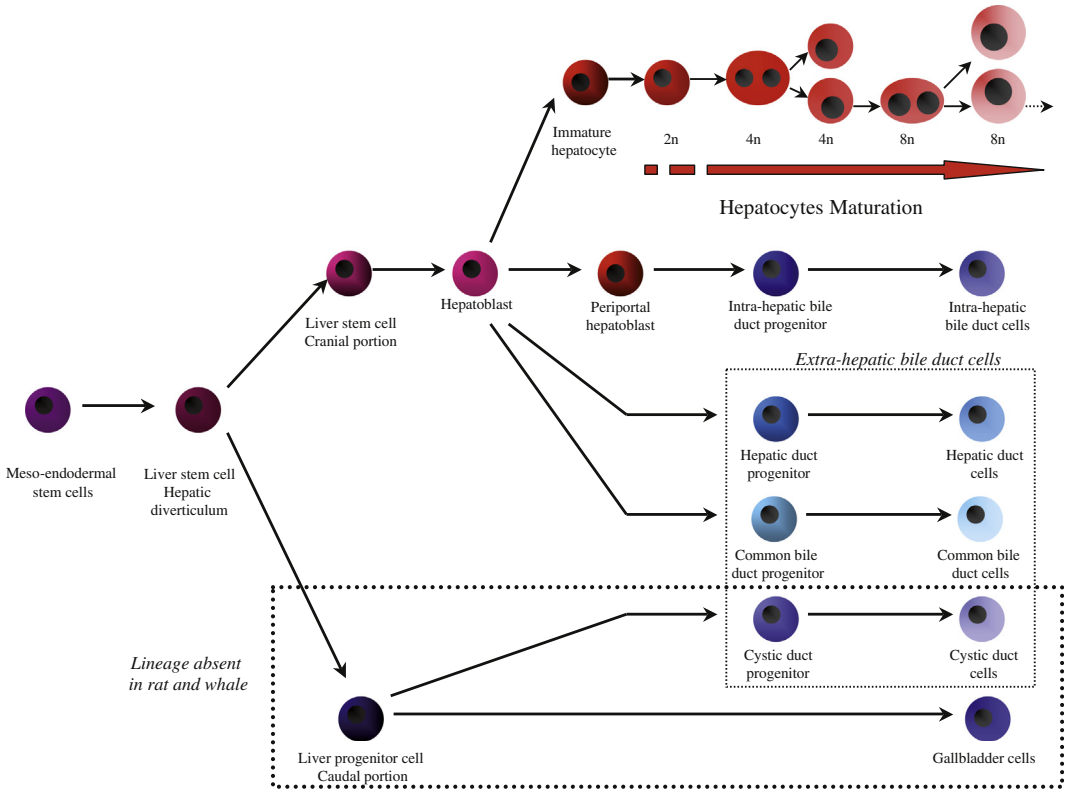

FIGURE 45.1

Model of development of the hepatobiliary system and ventral pancreas. The gallbladder and cystic duct do not develop in the rat and whale. Hepatocyte polyploidization is driven by failed cytokinesis. *Based on review by Shiojiri et al. [32] and Zong and Stanger [113].*

Another property of stem cells is their multipotency, which is their ability to produce progeny of more than one lineage. Again, the best-known example of a multipotent tissue-derived stem cell is the hematopoietic stem cell. Hematopoietic stem cells have the capacity to give rise to many lineages, from short-term multipotent progenitor cells to more differentiated progeny [1]. Finally, clonality, i.e., the ability to self-renew from a single cell is probably the ultimate proof of the presence of stem cells. Clonal self-renewal allows one to demonstrate both self-renewal and mulitpotency at the single cell level. However, for stem cells to maintain their stemness, at least some of the cells must undergo division without differentiation while others will differentiate. Any claim for the isolation and identification of a resident tissue-specific stem cell has to satisfy two minimal requirements: the demonstration that it can be prospectively isolated and is capable of clonal self-renewal, and that it can differentiate to cell lineages of that tissue. Thus, a liver stem cell should be able to generate the two epithelial cells in the liver, namely the hepatocytes and the bile duct cells (Fig. 45.1). Multipotency in the EHBD system is harder to define, as the heterogeneity of epithelial cells has not been completely elucidated yet. In this chapter, we present a compilation of experiments that seek to identify liver stem cells as well as EHBD stem cells.

CELLULAR ORGANIZATION OF THE HEPATOBILIARY SYSTEM

Organization of liver and IHBD system

The liver is one of the largest organs of the body, composed of two epithelial cell types, the hepatocytes and IHBD cells. Both the hepatocytes and IHBD cells belong to the same hepatic lineage and are derived from a common stem cell during development (Fig. 45.1). The basic

functional unit of the liver is the *hepatic lobule*, which is demarcated by the portal triad and central vein. The portal triad is composed of the hepatic artery, portal vein and IHBD cells. Blood enters the liver from the portal vein and hepatic artery, and flows between liver sinusoids, which have a unique fenestrated endothelium, toward the central vein. Hepatocytes organized into hepatic plates are present between individual sinusoids, which allow the filtration of the blood. The junction of the hepatocytes and endothelial cells is their basolateral surface. The apical surface of hepatocytes is formed by bile canaliculi, which are spaces into which bile acids are pumped. Bile canaliculi form channels that drain bile secreted by hepatocytes into the bile ducts of the portal triad. In addition to hepatocytes and IHBD cells, sinusoidal endothelial cells, Stellate cells (formerly called *Ito cells*; resident fibroblasts found in the Space of Disse between hepatocytes and endothelial cells), Kupffer cells (resident macrophages of the liver), and leukocytes are also present in significant numbers in the liver.

There is functional heterogeneity of hepatocytes along the hepatic plate, from the portal triad to the central vein. Differences in heterogeneous biochemical properties as well as patterns of gene expression have been distinguished in periportal hepatocytes, i.e., those close to the portal triad and pericentral hepatocytes close to the central vein. Periportal hepatocytes are considered more primitive. This notion is reinforced by their proximity to the Canals of Hering, which represent the connection between the bile canaliculi and the bile ducts and is in the periportal zone and which have been proposed to be the site of the liver stem cells. Midacinar hepatocytes in the middle of the hepatic lobule and pericentral hepatocytes are considered more mature. However, these zonal differences depend on blood flow and more importantly, can be reversed when the blood flow through the liver is reversed [8].

Hepatocytes also exhibit heterogeneity in their ploidy. This is especially unique as hepatocytes are highly differentiated cells capable of multiple synthetic and metabolic functions. Hepatocytes can be diploid or polyploid, mononucleated or binucleated (Fig. 45.2). Newborn human or rat hepatocytes are exclusively diploid ($2n$) but will subsequently generate $4n$, $8n$, and eventually $16n$ mono- and binucleated hepatocytes. Aneuploidy is also frequently present in both human and mouse hepatocytes [9,10]. The mechanism for this polyploidization has been shown to be aborted cytokinesis, with mononucleated hepatocytes forming binucleated hepatocytes [11]. In rodents, studies of the transplanted hepatocytes separated by flow cytometry using the DNA profiles of diploid, tetraploid and octoploid liver cells, showed that all fractions could engraft, proliferate and regenerate liver tissues [12]. These data supported the conclusion that multiple hepatocyte ploidy classes can serve as progenitors for regenerating hepatocyte foci in damaged livers.

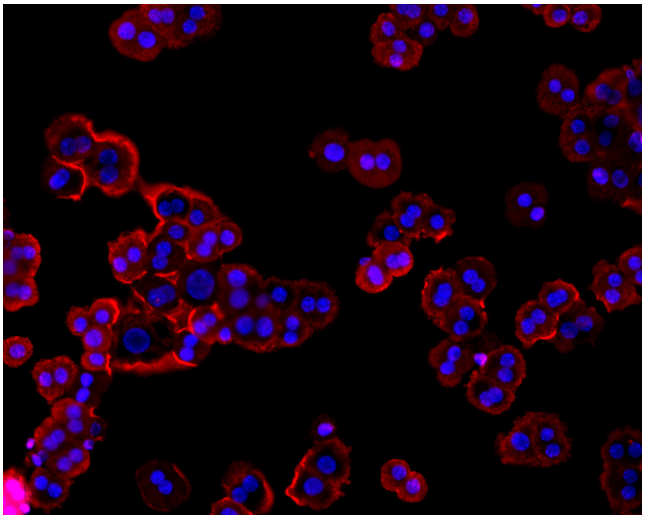

FIGURE 45.2
Cytospsin of mouse adult liver cells.
Hepatocytes are a heterogeneous population of cells: small and large, mono- and binucleated. Cells were stained with propidium iodide and counterstained with Hoechst.

Functional heterogeneity is also observed with IHBD cells. The bile ducts begin with the Canals of Hering within the hepatic lobule, and progressively increase in diameter into a system of interlobular, septal, area, segmental and hepatic ducts that eventually join with the EHBD system [13,14]. IHBD cells that line the Canals of Hering are small, cuboidal cells with a high nuclear to cytoplasmic ratio [15,16]. By contrast, larger bile ducts are lined with columnar epithelial cells that have low nuclear to cytoplasmic ratios, apical Golgi apparatus and relatively abundant rough endoplasmic reticulum. Beyond morphology, small and large IHBD cells differ by gene expression pattern and response to injury such as bile duct ligation (BDL) [17,18]. These data have led to the notion that small IHBD cells may represent committed bile duct progenitor cells that give rise to the large IHBD cells upon injury [16].

Organization of the EHBD system

The EHBD system consists of the common hepatic duct, gallbladder, cystic duct and common bile duct (CBD) [19,20]. Bile is concentrated and stored in the gallbladder, from where it is pumped through the CBD into the duodenum [21,22]. The gallbladder therefore plays an important role in the digestive process. However, this role appears to be dispensable, as cholecystectomy, or removal of the gallbladder, does not have major long-term consequences on the health of the organism. The epithelium of the EHBD system consists for the most part of a single layer of epithelial cells [23]. The CBD and Ampulla of Vater also consist of peribiliary glands (PBGs). PBGs are mucus secreting epithelial cells buried in the bile duct walls and are mostly found in the CBD and the Ampulla of Vater [24–26]. The gallbladder does not contain PBGs [20] but consists of Rokitansky-Aschoff (R-A) sinuses, which are invaginations of the epithelial cells through the muscle layer into the subserosal connective tissue have been identified by histology [27]. However, R-A sinuses are considered to represent early pathological changes in the gallbladder as their presence has been noted in gallbladder cancer [28, 29] and in gallstones [30,31]. Their existence in the uninjured gallbladder is controversial.

DEVELOPMENT OF THE HEPATOBILIARY SYSTEM

The liver, EHBD system and the ventral pancreas develop from the posterior ventral foregut endoderm [32–34]. Specifically, the liver primordium is formed by the closure of the lateral and medial endodermal domains [34], followed by its evagination into the septum transversum, which is the mesenchymal tissue located between the cardiac mesoderm and the ventral endoderm [33]. This induction requires Fibroblast Growth Factor (FGF) from the cardiac mesoderm and Bone Morphogenic Protein (BMP) from the septum transversum. At the same time, the ventral pancreas is induced in the lateral endodermal cells that are caudal to the hepatic endoderm [33]. Both these events occur at roughly embryonic day (ED) nine and three weeks post gestation in the mouse and human respectively.

The liver primordium is divided into cranial and caudal portions. The cranial portion of the liver primordium after its evagination into the septum transversum gives rise to the hepatocytes and the IHBD cells [32,35]. Hematopoietic cells migrate into the developing liver bud by ED 11 in the mouse. At this point, liver cells are referred as hepatoblasts and express liver specific genes like albumin (ALB), alpha-fetoprotein (AFP) and cytokeratin 19 (CK19) (Fig. 45.3). Hepatoblasts eventually give rise to mature hepatocytes and IHBD cells.

Developmental link between the EHBD system and ventral pancreas

The caudal portion of the liver primordium is thought to give rise to the EHBD system, including the gallbladder. However, this notion is based on histological data that show that EHBD and IHBD systems develop together. The exact mechanism of development and the cell-intrinsic factors by which the IHBD and EHBD segregate have heretofore been unclear.

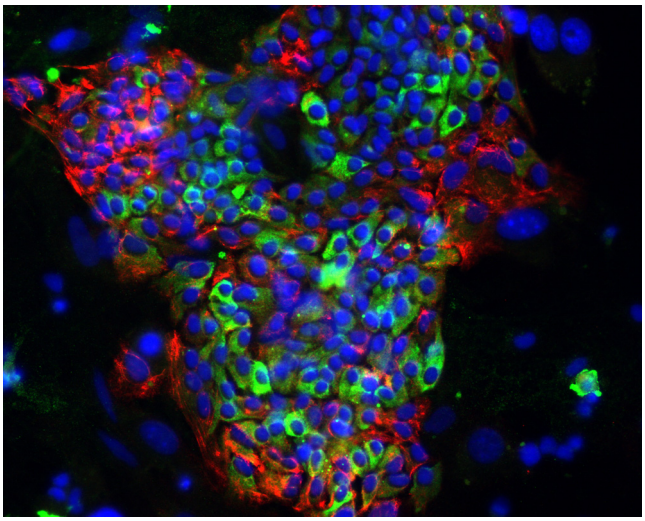

FIGURE 45.3
***In vitro* culture of human fetal liver cells.** Human fetal liver cells were cultured for several weeks on rat feeder cells. Colonies were stained with AFP, a marker for the hepatic lineage and CK19, a marker for bile duct cells. This colony contains a mix of cells positive for CK19 only (bile duct cells), AFP only (hepatoblasts and small hepatocytes) and for both (bipotent progenitor cells).

Sumazaki et al. [36] have noted gallbladder agenesis and ectopic pancreatic tissue in the common bile duct (CBD) in hairy and enhancer of split 1 (*Hes1*) deficient mice. Fukuda et al. [37] corroborated these data in a later study where they observed ectopic pancreatic tissue in the CBD of *Hes1*-knockout mice. In both reports, IHBD system development appeared normal though the connection to the CBD was lacking, suggesting a shared ontogeny between the EHBD system and ventral pancreas and one that is separate from the IHBD system. This shared ontogeny between the EHBD system and ventral pancreas has been much elucidated in a recent study by Spence et al. [38]. The authors used a pancreatic and duodenal homeobox-1 (PDX1)-Cre mouse to demonstrate that hepatocytes and IHBD cells derive from PDX1- cells while EHBD cells and ventral pancreas derive from PDX1+ cells. They found that SRY- (sex determining region)-box (SOX)-17 controlled the specification of the EHBD and pancreatic cells from PDX-1+ cells and this specification required HES-1. SOX-17 loss-of-function at ED 8.5 resulted in gallbladder agenesis and the presence of ectopic pancreatic tissue (PDX-1+ cells) in the EHBD. Conversely SOX17 gain-of-function at ED 9.5 resulted in ectopic ductal tissue (SOX-17+ cells) in the developing pancreas. In both the foregoing conditions, the IHBD system was not affected. The regulation of SOX-17 expression and its interaction with HES-1 and PDX-1 have yet to be completely understood. However, these data definitively demonstrate that during murine development, the IHBD and EHBD systems originate from distinct progenitor cells.

HEPATOCYTES: FUNCTIONAL UNITS OF THE LIVER WITH STEM CELL PROPERTIES

The liver is an amazing organ because it can regenerate

The Greek myth of Prometheus tells of the mortal who stole fire from Zeus and was punished by having his liver devoured by an eagle at dusk every day, only to have it regenerate by the following morning. The myth exaggerates the regenerative potential of the liver and the foregoing phenomenon was recognized much later in medicine. The classic experiment by Higgins and Anderson in 1931 demonstrated experimentally that surgical removal of two-thirds of a rat liver was possible without significant mortality, and more importantly resulted in regeneration of the remaining lobes of the liver within five to seven days by compensatory hyperplasia [39].

Serial surgical resection of the liver (hepatectomy) is therefore possible in experimental animals, and each time results in the extraordinary compensatory growth of the remaining liver lobes. This model of hepatectomy has been extensively studied and, to the surprise of

many, is not dependent on resident stem cells in the liver. In contrast to other regenerative tissues like the bone marrow or the gut, mature cells — mostly hepatocytes — in response to specific stimuli like growth factors enter into cell cycle and drive liver regeneration. Hepatocytes have been shown to be the primary cell responsible for this enormous proliferation and regeneration of the liver. They are the most abundant epithelial cells in the liver (~60%) and represent most of the mass of the organ due to their large size. Hepatocyte proliferation starts in the periportal region and spreads to the pericentral region. In general, the regeneration of the liver mass requires each hepatocyte to undergo an average of 1.4 rounds of replication to restore the liver mass. Other type of cells including IHBD cells, stellate cells, endothelial cells and Kupffer cells are also involved in the process of liver regeneration [40].

HEPATOCYTES TRANSPLANTATION: 'PROOF-OF-CONCEPT' FOR CELL-BASED THERAPY FOR LIVER DISEASE

Our understanding of the potential of cell-based therapy for liver disease has come from studies of transplanted liver cells into animal models. The first such experiment described the transplantation and survival of hepatocytes in the spleen [41]. This work was followed by the demonstration that these ectopic hepatocytes were functional and could proliferate [42–44]. Hepatocyte transplantation has also been demonstrated in experimental animal models with inherited metabolic liver diseases [45,46]. These experiments established that allotransplantation of normal hepatocytes and autotransplantation of genetically modified hepatocytes can correct metabolic defects.

More recently, transgenic and knockout mice have provided excellent models of liver regeneration by cell transplantation. In these models, inherited chronic hepatocellular toxicity induces massive necrosis of the recipient's hepatocytes, and a quasi-clonal proliferation of the healthy donor hepatocytes.

The transgenic mouse containing the urokinase plasminogen activator (uPa) downstream of the albumin promoter was one of the first such genetic models used to study liver regeneration [47]. Albumin-driven uPa expression results in hepatotoxicity, and this confers a selective growth advantage to the transplanted donor hepatocytes, allowing their massive proliferation. The FAH knockout mouse is another genetic model of hepatocellular toxicity with the added advantage that it reproduces a lethal human inherited disease, Type 1 Tyrosinemia [48]. In this model, deletion of the fumarylacetoacetate hydrolase (*Fah*) gene, which encodes a key enzyme in the tyrosine metabolic pathway, results in the buildup of toxic metabolites. $Fah^{-/-}$ mice develop severe liver disease, but can be rescued by blocking the tyrosine pathway upstream of FAH by using the drug 2-(2-nitro-4-trifluoro-methyl-benzyol)-1,3 cyclohexanedione (NTBC). $Fah^{-/-}$ mice on NTBC can develop to adulthood, breed and remain healthy. Removal of NTBC results in liver failure within 6–8 weeks and subsequent death. When $Fah^{-/-}$ mice are transplanted intra-splenically with single cell suspensions of syngeneic hepatocytes from wild-type mice, the $Fah^{+/+}$ cells migrate to the liver, invade the hepatic lobules and divide until > 95% of the liver cell mass is replaced by the donor hepatocytes [49]. Repopulated animals are healthy and as few as 100 donor hepatocytes are sufficient to rescue the animal from tyrosinemia. Moreover, repopulating hepatocytes can be serially transplanted to measure their cell division capacity, which highlights their tremendous proliferative ability [50].

As hepatocytes are such terminally differentiated cells, it was originally thought they only had a limited capacity for proliferation. However, both the uPa transgenic and $Fah^{-/-}$ mouse models demonstrated the opposite. In the uPa transgenic mouse, 12 or more hepatocyte cell divisions were necessary for liver regeneration. Serial transplantations in $Fah^{-/-}$ mice showed that donor hepatocytes were capable of 69 cell doublings. This rather unique capacity of hepatocytes to regenerate the liver has two essential requirements: extensive and continuous liver injury and a strong selective advantage for the donor cells to survive relative to the recipient

cells. However, these conditions are usually not present optimally in situations involving transplantation of human hepatocytes, as will be discussed later.

LIVER STEM CELLS

The presence of a stem cell distinct from IHBD cells and hepatocytes in the uninjured liver is hotly debated. In part this is because during homeostasis in the uninjured liver, new hepatocytes and IHBD cells are thought to be generated by duplication of each respective epithelial cell type [51–54]. However, in a recent study Furuyama et al. [55] using the SOX9 transgenic mouse, which labels most of if not all IHBD cells, it was demonstrated that new hepatocytes derive from IHBD cells. These data were contradicted in another more recent paper that used an adenoviral labeling system in the adult mouse to specifically label hepatocytes. Malato et al. [56] determined that new hepatocytes derive from hepatocytes. Both these studies use state-of-the art tools such as transgenic mice and viral serotypes that afford good target cell specificity and low toxicity, and provide direct evidence for their respective conclusions. Yet the stark difference in these conclusions highlights much of the disagreement surrounding our current knowledge of liver homeostasis, which extends to studies of liver stem cells.

Hepatocytes and IHBD cells as facultative stem cells

TRANSDIFFERENTIATION OF HEPATOCYTES INTO IHBD CELLS

The ability of hepatocytes to proliferate extensively *in vivo* raises the possibility that they could be stem cells. Interestingly, hepatocytes have been reported to potentially transdifferentiate into biliary cells, both *in vitro* [57,58] and *in vivo* [59]. However, neither the prospective isolation of hepatocytes nor their clonal expansions were reported in these studies, making a definitive conclusion regarding their multipotentcy and consequently, stemness difficult. If these studies were confirmed though, it can be postulated that hepatocytes would behave like facultative stem cells, having both self-renewal and lineage commitment capacities. Last, hepatocytes are a heterogeneous population of cells (Fig. 45.2) containing small and large, mono- and binucleated cells. Therefore, it can be argued that only a distinct population of 'primitive hepatocytes' may have stem cell properties.

OVAL CELLS AND DUCTULAR REACTIONS

In rodent injury models, damage to the liver combined with an arrest in the proliferative capacity of hepatocytes leads to the proliferation of 'oval cells'. These cells are not small hepatocytes but a distinct population of liver cells, which differentiates into hepatocytes and restores the hepatic parenchyma [60,61]. A similar population of liver cells called 'ductular hepatocytes' has been reported in patients suffering a variety of liver diseases [62]. These cells have a gene expression associated with both hepatocytes and cholangiocytes, and their presence has been reported in histopathology of human diseases from fulminant hepatitis to primary biliary cirrhosis.

In rodent models, experimental studies suggest that oval cells derive from IHBD cells. Using models of injury that inhibit hepatocyte proliferation, the Canals of Hering have long been identified as a reservoir of what would be later characterized as oval cells [63]. Recent studies have described the Canals of Hering as the structures that penetrate deep inside the hepatic lobules and could be considered as a candidate stem cell niche [64]. Studies in the rat treated with partial hepatectomy followed by the administration of *N*-2 acetyl-aminofluorene (AAF), a protocol used to generate large populations of oval cells, have demonstrated that these cells are located in the Canals of Hering before differentiating into hepatocytes [65]. In a more recent report, Kuwahara et al. [66] use a label retention assay to identify the regenerative cells during liver injury. They find that all the candidate stem cell niches localize to or around the Canals of Hering. However, correlating these data to the uninjured liver and the subsequent identification of stem cells has proved difficult.

Prospective identification of fetal liver stem/progenitor cells

The first clonogenic liver progenitor cells to be prospectively isolated were from fetal rats [67]. Using an *in vitro* colony forming assay and flow cytometry, a population of fetal rat liver cells RT1A^{1}/ OX18^{low}/ ICAM-1^{+} was isolated at ED13. These cells were considered as hepatoblasts and were shown to be bipotent *in vitro* (by expression of hepatocyte and IHBD cell markers). This report was followed by the identification of a similar population of hepatoblasts in mouse fetal liver [68,69]. By combining a different culture system with flow cytometry, a more primitive fetal liver cell population capable of forming large colonies and long-term expansion was isolated from mouse fetal livers [70,71]. In these studies, the c-Met^{+}/CD49f$^{+/low}$/c-kit^{-}/ CD45^{-}/TER119^{-} fetal liver cells were clonally propagated in culture and were shown to continuously produce hepatocytes and IHBD cells, while remaining primitive. These clonally propagated cells were transplanted into mice and shown to morphologically and to some extent, functionally differentiate into hepatocytes and IHBD cells. Furthermore, these mouse fetal liver cells were capable of differentiating into pancreatic acinar cells, gastric as well as intestinal epithelial cells upon transplantation into these organs. Therefore, these cells were regarded as more of a general endodermal stem cell population [71]. More recently rodent studies have identified select markers that significantly enrich stem/progenitor cells. Using colony forming assays and flow cytometry, Kakinuma et al. [72] demonstrated that CD13^{+}/ Dlk^{+}/CD45^{-}/TER119^{-} cells at ED13.5 in mouse livers form the largest colonies *in vitro*, differentiate to produce ALB- and ck19-producing cells and engraft into recipient livers. Similarly, Oertel et al. [73] identified Dlk^{+} cells at ED14 in rat livers as being significantly enriched in liver stem/progenitor cells.

We know very little about the different progenitor populations in the human fetal liver. Recently, several isolations and characterizations of human fetal liver stem/progenitor cells were described. Using EpCAM and magnetic activated cell sorting (MACS), EpCAM^{+} hepatoblasts and hepatic stem cells were isolated from fetal, neonatal and adult liver and cultured in short-term (10 days) and long-term (20 days) assays [74]. EpCAM^{-} liver cells proved to be more than 95% diploid and polyploid hepatocytes. Hepatic stem cells were EpCAM^{+}, NCAM^{+}, CLDN-3^{+}, ck19^{+}, c-kit^{+}, aquaporin 4^{+} Alb^{low} and AFP^{-} while hepatoblasts were EpCAM^{+}, NCAM^{-}, CLDN-3^{-}, ck19^{low}, c-kit^{low}. In a later study, the authors localized the hepatic stem cells to the ductal plates in the human fetal livers and confirmed that they are capable of long-term expansion *in vitro* and are able to form hepatocyte-like cells (ALB^{+}/AFP^{+}/ Cytochrome P450-A7^{+}) and IHBD cells (ck19^{+}) [75]. In addition, transplantation into NOD-SCID mice showed that the hepatic stem cells could generate hepatocytes *in vivo*.

In another report, a multipotent progenitor population (hFLMPC) was reported to be capable of differentiating into liver and mesenchymal lineages [76]. Human fetal liver cells were maintained in culture on feeder cells and colonies were clonally isolated by serial dilution. These progenitor cells have the immunophenotype CD34^{+}, CD90^{+}, c-kit^{+}, EpCAM^{+}, c-met^{+}, SSEA-4^{+}, CK18^{+}, CK19^{+}, Albumin^{-}, alpha-fetoprotein^{-}, CD44h^{+} and vimentin^{+}. Placed in appropriate media, hFLMPC differentiated into hepatocytes and bile ducts as well as into fat, bone, cartilage, and endothelial cells. Similar to the findings of Schmelzer et al. [75], hFLMPCs in this study engrafted and formed hepatocytes *in vivo* when transplanted into animal models of liver disease. Dan et al. noted that hFLMPCs do not express hepatic markers like albumin, alpha-fetoprotein or primitive hepatic transcription factors (HNF1a, HNF3b, and HNF4a) in their undifferentiated state, but express mesenchymal markers. These results suggest that hFLMPCs are mesenchymal-epithelial transitional cells that could have a mesendodermal origin. In all, there is significant discrepancy between the foregoing reports regarding methods of *in vitro* culture, clarity of cell surface markers to identify stem cells, clonal expansion assays and *in vivo* transplantation experiments. For all these reasons, further analysis and characterization are necessary to determine the true origin of human fetal liver progenitor cells.

Prospective identification of adult liver stem/progenitor cells

Most studies of the identification of adult liver stem/progenitor cells in rodent systems focus on diseased liver conditions in which hepatocyte proliferation is blocked and oval cells emerge (see the section 'Oval cells and ductular reactions') [77–82]. As a result there are few reports of the presence of stem cells in the uninjured rodent and human livers. Suzuki et al. [78] and Qiu et al. [79] used CD133 and CD24 expression respectively, to isolate candidate stem cells from uninjured adult mouse livers. In both reports, the isolated cell populations expanded *in vitro* and appeared to differentiate into hepatocytes. Suzuki et al. [78] demonstrated that $CD133^+$ cells were capable of clonal self-renewal and gave rise to hepatocytes and IHBD cells *in vitro*. In both the foregoing reports, the authors determined that the candidate stem cell populations were capable of engraftment and differentiation into hepatocytes in $Fah^{-/-}$ mice. Suzuki et al. also observed that the frequency of $CD133^+$ cells increased upon DDC treatment.

Dorrell et al. [81] have used specific antibodies developed in-house [83] along with CD133 expression to identify a subpopulation of non-hepatocyte small liver cells capable of *in vitro* expansion and hepatocyte differentiation. They demonstrate that these candidate liver stem cell populations could engraft and differentiate into hepatocytes in $Fah^{-/-}$ mice. In addition, they determined that these stem cell populations were the most enriched for SOX9 expression, and were enriched upon DDC treatment.

While interesting, these studies suffer from the same general problems. The specific markers used usually co-stain with IHBD cells in the liver, confusing the interpretation of the results. Expansion of the stem cells was not completely homogeneous but contained contaminating mesenchymal cells, making the discernment of a resident epithelial stem cell difficult. Finally, the hepatocyte differentiation observed with these cells was inconclusive and occurred at very low frequencies. This could be for various reasons, beginning with the lack of a definitive *in vitro* hepatocyte differentiation protocol. However, it could also be because these candidate stem cells are not capable of *de novo* hepatocyte differentiation. In all, the identification of a liver stem cell is inconclusive and consequently, there are no specific markers for its isolation.

STEM CELLS IN THE EHBD SYSTEM

There are few reports of the identification of stem cells in the EHBD system, and specifically in the gallbladder. Irie et al. [84] observed c-kit^+ cells in the mouse CBD and gallbladder following BDL that expressed albumin and cytochrome P450 enzymes. These cells engrafted into livers of mice injured with carbon tetrachloride (CCl_4) and one-third partial hepatectomy, suggesting that they were capable of hepatocyte differentiation. However, this study does not mention the presence of c-kit^+ cells in the uninjured EHBD, which makes evaluating the origin of these cells difficult.

Recently, there have been reports indicating that PBGs in the EHBD system are a reservoir for multipotent stem cells that are capable of differentiation into hepatocytes, bile duct cells and pancreatic islets [20,85]. In a recent report, Cardinale et al. [20] demonstrate that the PBGs express the transcription factors SOX-9, SOX-17 and PDX-1 as well as EpCAM and CD133. They found that cells from the PBGs could expand *in vitro* long-term and when placed in the appropriate culture conditions or animal models, were capable of differentiating into hepatocytes, bile duct cells and glucose-responsive pancreatic islets *in vitro* and *in vivo* respectively [20]. However, these reports while interesting, fall short of definitively identifying the initial cell population isolated from the primary tissue and make no mention of separation of the PBGs from the luminal epithelium of the CBD. Furthermore, the gallbladder does not contain PBGs [20].

Our group has recently identified an $EpCAM^+/CD49f^{hi}/CD29^+/CD133^+/CD45^-/TER119^-$ epithelial subpopulation from adult mouse gallbladders that expanded *in vitro*, formed large colonies and exhibited single cell clonal self-renewal and differentiation to gallbladder-like

structures *in vitro* and *in vivo* [86]. The gallbladder stem cells also expressed the bile duct transcription factors SOX9 and SOX17. However, we did not observe any hepatocyte markers in these cells and were not able to obtain engraftment of the gallbladder stem cells in the livers of $Fah^{-/-}$ mice. Interestingly, we found that the transcriptional profiles of gallbladder stem cells and candidate IHBD stem cells were distinct from each other [86] corroborating the evidence that they descend from separate progenitors [38]. Functional annotation of differentially expressed genes suggested that lipid and drug metabolism are different in these two cell types, and consequently that their functions might be different as well. To our knowledge, this is the first direct comparison of the expression profiles of IHBD and gallbladder cells.

THERAPEUTIC APPROACHES FOR USING STEM CELLS IN THE HEPATOBILIARY SYSTEM

Treating orthotopic liver transplantation (OLT): searching for alternatives to hepatocyte transplantation

Liver disease affects a large part of western society and is among the top causes of death in the US [87]. Roughly 10% of all Americans suffer from liver disease. In addition, 170 million people worldwide and 4 million in the US are infected with Hepatitis C [88], which is a major cause of liver failure [89]. Currently the only widespread treatment for chronic liver disease is allogeneic orthotopic liver transplantation (OLT). However, OLT is hindered by the severe lack of donated livers. There are over 15,000 people on the waiting list for liver transplant and only 5,000 available donors [90]. Furthermore, this procedure requires the use of lifelong immunosuppressive drugs and is complicated by graft loss. In addition, OLT is an expensive procedure that not everyone can afford. As organ transplantation is unavailable for a large fraction of patients with acute and chronic liver disease, hepatocyte transplantation has become an increasingly attractive prospect. This foregoing need, along with the success of hepatocyte transplantation in animal models has resulted in several groups evaluating the efficacy hepatocyte-based cell therapy in a variety of human diseases [91–93]. Hepatocyte transplantation is less expensive and invasive than OLT, and unlike the whole organ, hepatocytes can be cryopreserved and stored.

Patients have undergone hepatocyte transplantation for inborn inheritable metabolic diseases. These surgeries have been performed in individual cases and in small, uncontrolled groups of patients [93,94]. Although the beneficial outcome in some of these liver cell transplantations has been very encouraging [95,96], the limited clinical experience with hepatocyte transplantation is still a problem. In addition, the single biggest obstacle to the widespread use of hepatocyte transplantation is the limited availability of donor liver tissue. Most hepatocytes used in clinical transplantation settings are obtained from donors that are deemed unsuitable for OLT and are subsequently of poor quality and viability. And it has been difficult to argue that an already limited donor pool should be shared between what is still an experimental cell transplantation protocol and the more established whole organ transplantation one. This problem of availability could be solved by alternative sources of hepatocytes, such as liver or EHBD stem/progenitor cells, pluripotent cell-derived hepatocytes or the transdifferentiation of other somatic cells such as fibroblasts into hepatocytes. The latter two options are discussed below.

Pluripotent cells as a candidate cell source of hepatocytes

Adult hepatocytes are scarce and refractory to *in vitro* expansion. For these reasons, embryonic stem cells (ESCs) and more recently induced pluripotent stem cells (iPSCs) are being evaluated as alternative cell sources for hepatocyte generation. iPSCs are better favored as their generation circumvents the ethical considerations that plague ESC generation and because of the possibility of autologous hepatocyte transplantation. A complete account of hepatocyte generation from pluripotent cells is beyond the scope of this chapter (see review in reference [97]).

We will instead focus on the seminal papers here and briefly discuss progress in and obstacles to this field.

Rambhatla et al. were the first to show that human ESCs could generate hepatocyte-like cells [98]. Since then various groups have reported the same result, specifically enhancing culture conditions to obtain a more homogenous and mature hepatocyte population [99–101]. Using similar protocols, a few groups have shown differentiation of iPSCs into hepatocyte-like cells [102–104]. However, in all these studies, the end products of differentiation are a heterogeneous population of cells including hepatocyte-like cells. Moreover, the hepatocyte-like cells are considered immature rather than terminally differentiated hepatocytes. Finally, only a few studies have evaluated these cells *in vivo*, and those that have, have observed teratoma formation, suggesting contamination of pluripotent cells in the donor cells. In all, there is currently no definitive protocol for the differentiation of pluripotent cells into terminally differentiated hepatocytes.

Non-hepatic somatic cells as a cell source of hepatocytes

Mouse gallbladder epithelial cells have been shown to differentiate into hepatocyte-like cells *in vitro* [105]. In this study, the authors cultured mouse gallbladder cells in transwell inserts above a layer of myofibroblast feeder cells, a technique that supports expansion of epithelial cells [106]. Following *in vitro* expansion, gallbladder epithelial cells were grown between collagen and matrigel in the presence of epidermal growth factor (EGF), hepatocyte growth factor (HGF) and dexamethasone (Dex). In these conditions, the authors noted the up-regulation of the hepatocyte markers HNF4α and ALB. The functionality of these hepatocyte-like cells was determined by bile acid synthesis, uptake of low-density lipoprotein and benzodiazepine metabolism [105]. In a later study, the same group observed engraftment of freshly isolated and *in vitro* expanded gallbladder epithelial cells in the livers of non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice [107]. However, this engraftment was possible only with tremendous injury to the recipient liver. Even then reported engraftment was only 1.94% ± 2.18%, with the majority of donor cells engrafting in the IHBD system [107]. Therefore, the *de novo* differentiation of gallbladder cells into hepatocytes does not appear to be very frequent or robust. In all, these data establish the proof-of-concept for the differentiation of gallbladder cells into hepatocytes. In addition, the gallbladder is considered to be a non-vital organ and is routinely removed during surgery, with 500,000–600,000 surgeries every year [108]. This sheer volume of available tissue combined with the shared ontogeny of the liver and gallbladder presents exciting opportunities for candidate cell-based therapy.

Two more recent studies have detailed the reprogramming of mouse fibroblasts into hepatocyte-like cells using specific transcription factors [109,110]. In both reports, the authors began by over-expressing transcription factors that play a role in liver development, in mouse fibroblasts and eliminated those that did not give rise to a hepatic phenotype [33,111,112]. In the first study, Huang et al. [109] observed that over-expression of GATA4, HNF1α and Forkhead box A3 (FOXA3) in *p19*^{Arf}-null (p19^{Arf} is a cell cycle inhibitor) adult mouse fibroblasts, converted them into induced hepatocyte-like (iHep) cells. iHep cells exhibited hepatic morphology and expressed hepatic genes. Moreover, they were able to significantly (~80%) engraft and rescue five out of twelve *Fah*$^{-/-}$ mice.

In the second study, Sekiya et al. [110] observed that over-expression of combinations of two transcription factors – HNF4α and either FOXA1, FOXA2 or FOXA3 – in embryonic and adult mouse fibroblasts resulted in iHep cells. Similar to the previous study, the authors found that the iHep cells resembled hepatocytes in morphology and gene expression patterns and rescued significant (40%) numbers of transplanted *Fah*$^{-/-}$ mice. Both these studies confirm that somatic cells can be successfully reprogrammed into functional hepatocytes capable of engraftment *in vivo*. Furthermore, they suggest that reprogramming is more effective with specific transcription factors than defined culture conditions.

CONCLUSION

Major progress has been made in the identification of liver stem/progenitor cells. However more needs to be learned regarding the phenotypes and plasticity of these cells. So far only a few laboratories have prospectively isolated fetal liver stem cells, and as yet there is no consensus about their phenotypes. And in the adult liver, the presence of a stem/progenitor cell is still disputed. Finally, there is a clear paucity of data regarding EHBD including gallbladder stem/progenitor cells.

Transplantation of hepatocytes in small-animal models has demonstrated their ability to proliferate *in vivo*. Clinical trials for hepatocyte cell-based therapy to treat end-stage liver disease are subsequently underway (reviewed in reference [93]). But much remains to be discovered. Adult and fetal hepatocytes and fetal and adult liver stem/progenitor cells need to be compared to determine the most optimal cell for engraftment. This is especially important given the shortage of adult donor livers, and hepatocytes. Significant effort is necessary to demonstrate the reprogramming of fibroblasts and pluripotent cells into hepatocytes and the efficacy of this approach for cell-based therapy. Cell expansion will also be an issue for a viable therapeutic procedure. Finally, transplanted cells must have a clear selective advantage to engraft and repopulate the diseased liver. Starting points are the metabolic disorders that provide the necessary environment for efficient cell transplantation. However in acute and chronic liver disease where such an environment does not exist, a procedure similar to myeloablation would be necessary. In all, there is much more to learn about hepatobiliary stem cells. However, given the prevalence of liver disease, progress is necessary.

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CHAPTER 46

Hepatic Tissue Engineering

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LIVER FAILURE AND CURRENT TREATMENTS

Liver disease afflicts over 600 million people worldwide, 30 million of whom are Americans. Liver disease leads to the death of over 40,000 individuals in the United States every year. Liver failure can be generally separated into two major categories: fulminant hepatic failure, also referred to as acute liver failure, and chronic hepatic failure resulting from chronic end-stage liver disorders. The term fulminant hepatic failure is utilized for cases in which hepatic encephalopathy and impaired synthetic function (i.e., coagulopathy) develops within 26 weeks of the initial onset of jaundice. Hepatic encephalopathy is a neuropsychiatric condition, which can be divided into four stages ranging from minor effects such as mild confusion and sleep disorder, to deep coma. Although fulminant hepatic failure is relatively rare, with approximately 2,000 cases in the United States per year, it exhibits a high mortality rate of approximately 28% [1]. The major identified causes of fulminant hepatic failure include acetaminophen overdose, idiosyncratic drug reactions, and viral hepatitis A and B [1]. Of note, a recent multicenter etiology study showed that 17% of fulminant hepatic failure cases remained of indeterminate origin [2]. In addition to hepatic encephalopathy, other clinical manifestations of fulminant hepatic failure include bacterial and fungal infection, coagulopathy, as well as metabolic, cardiorespiratory, and hemodynamic abnormalities. Though spontaneous recovery has been observed due to the regenerative capacity of the liver, this type of recovery is difficult to predict, and rarely occurs in various etiologies, such as idiosyncratic drug toxicity and hepatitis B [1]. Liver transplantation is currently the only therapy shown to directly alter mortality, and therefore, is the standard of care in most clinical settings. As a result, all fulminant hepatic failure patients that meet the criteria for orthotopic liver transplant are immediately listed as United Network for Organ Sharing Status 1 (highest priority) upon presentation. Factors that preclude this designation are irreversible brain damage, unresponsive cerebral edema, uncontrollable sepsis, malignancy, and multisystem organ failure. Despite the effectiveness of liver transplant in improving short-term survival of fulminant hepatic failure patients, the utility of this approach remains limited due to the scarcity of donor organs.

Liver failure due to chronic liver diseases, while exhibiting a longer time-course of disease pathogenesis, is much more common than fulminant failure, with chronic liver disease

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and cirrhosis being the twelfth leading cause of death (1.3% of total deaths) in the United States in 2010 [3]. The most common causes of chronic liver disease are hepatitis B virus, hepatitis C virus, and alcohol-induced and non-alcoholic fatty liver disease (NAFLD) [4]. Other etiologies largely include metabolic and autoimmune etiliogies: primary sclerosing cholangitis, primary biliary cirrhosis, α_1-antitrypsin deficiency, autoimmune hepatitis, hereditary hemochromatosis, Wilson's disease, and liver cancer. The prevalence of hepatitis C infection in the United States population has been estimated at 1.8%, or nearly 4 million individuals [5]. Notably, cirrhosis initiated by hepatitis C infection is the most frequent cause for liver transplantation, accounting for 40–50% of both individuals who have undergone transplant and those on the waiting list [6]. In addition, the long-term inflammation precipitated by chronic hepatitis B and C infection can also promote the development of hepatocellular carcinoma. As a result, substantial efforts are focused on understanding hepatitis B and C viral pathogenesis and the development of approaches for the improved control of the viruses before and after transplantation. Taken together, so-called fatty liver diseases also comprise a major proportion of chronic liver disease patients [4]. In particular, NAFLD is an increasingly prevalent condition in the United States; present in approximately 20% of adults, of which a subset (2–3% of adults) exhibit non-alcoholic steatohepatitis (NASH), defined by the presence of characteristic injury and necroinflammatory changes in addition to excessive fat accumulation. NAFLD pathogenesis has been shown to be associated with risk factors and conditions such as obesity, type 2 diabetes mellitus, hyperlipidemia, hyperinsulinemia, and insulin resistance. Collectively, chronic liver disorders can progress towards the eventual development of cirrhosis and sequelae of decompensated cirrhosis: ascites, portal hypertension, variceal bleeding, and hepatic encephalopathy. Unfortunately, these complications represent decompensation of liver function and evidence of liver dysfunction. Medical management can minimize the impact on patient quality of life but transplantation is the only currently effective therapy. As a means to more accurately determine organ allocation to patients on the liver transplant waiting list the MELD (mathematical model for end-stage liver disease) system was implemented in 2002, which assigns a priority score based on three prognostic indicators, bilirubin level, creatine level, and INR (a measure of blood clotting time) [6]. Although overall improvements in liver allocation have been achieved following the introduction of the MELD system, regional variations in MELD scores exist, and the ability of this model to accurately predict outcomes across the entire score distribution and for distinct pathologies is less clear.

Unlike other major causes of mortality, liver disease death rates are rising rather than declining. Hepatitis B and hepatitis C virus infect 370 million and 130 million people, respectively. Current hepatitis B vaccination campaigns may help to decrease hepatitis B virus (HBV) associated morbidity although the rapid increase in HIV coinfected patients has represented a challenge for clinical management. The rising rates of obesity have also resulted in the rapid rise of NAFLD worldwide with recent epidemiologic studies suggesting a prevalence rate from 6% to 35% with a median of 20%. Up to 20% of patients with NAFLD may progress to cirrhosis, raising the possibility that liver disease may become one of the most common causes of mortality worldwide. Moreover, patients with cirrhosis or hepatitis B are at greater risk of developing hepatocellular carcinoma. Consequently, hepatocellular carcinoma is the third and sixth most common cause of cancer death among men and women, respectively.

Given the steady rise in patients with liver disease, the need for liver transplantation has continued to increase. However, the number of available donor livers has not changed significantly in five years. As a result several different approaches have been undertaken to address this growing of donor organs. Several surgical options have been pursued including the use of non-heart-beating donors or split liver transplants from cadaveric or living donors [7]. Split liver transplants depend on the significant regeneration capacity of the mammalian liver. This regenerative process has been extensively examined via experiments in rodent models, which demonstrate that partial hepatectomy or chemical injury induces the

proliferation of the existing mature cell populations within the liver including hepatocytes, bile duct epithelial cells, and others, resulting in the replacement of lost liver mass. However, liver regeneration is difficult to predict clinically, and although partial liver transplants have demonstrated some effectiveness, biliary and vascular complications are major concerns in these procedures [7]. In addition, the surgical risk to the living donors (which has included several donor deaths) has raised significant ethical questions. Furthermore, in spite of these surgical advances and improvements in organ allocation, the increasing divergence between the number of patients awaiting transplantation and the number of available organs suggests that it is unlikely that liver transplantation procedures alone will expand the supply of donor livers to meet the increasing demand. Alternative approaches are therefore needed and are actively being pursued. These include several non-biological extracorporeal support systems that will be discussed in more detail later in this chapter such as plasma exchange, plasmapheresis, hemodialysis, molecular adsorbents recirculation system, or hemoperfusion over charcoal or various resins. These systems have shown limited success likely due to the narrow range of functions inherent to each of these devices relative to the complex array of functions performed by the healthy liver, which include detoxification, synthetic, and metabolic processes. Recapitulation of a substantial range of liver functions will be required to provide sufficient liver support, and it is unclear which known liver functions (e.g., gluconeogenesis, serum protein production, coagulation factor production, toxin-mediating encephalopathy) should be prioritized to improve clinical outcomes. Given this complexity, extracorporeal support systems that do not incorporate a hepatocyte component may not be able to ameliorate and augment liver disease outcomes as illustrated by the failure of several non-biological extracorporeal support systems. In contrast, limited hepatocyte transplantation trials have been shown in some cases to ameliorate and improve liver function. These cellular therapies encompass approaches at providing temporary support such as extracorporeal bioartificial liver (BAL) devices as well as more permanent adjunct interventions such as cell transplantation, transgenic xenografts, and implantable hepatocellular constructs (Fig. 46.1). Collectively, the development of these types of cell-based therapies for liver disease is a major aim of liver tissue engineering, and fundamental advances and current status of these approaches are reviewed in this chapter.

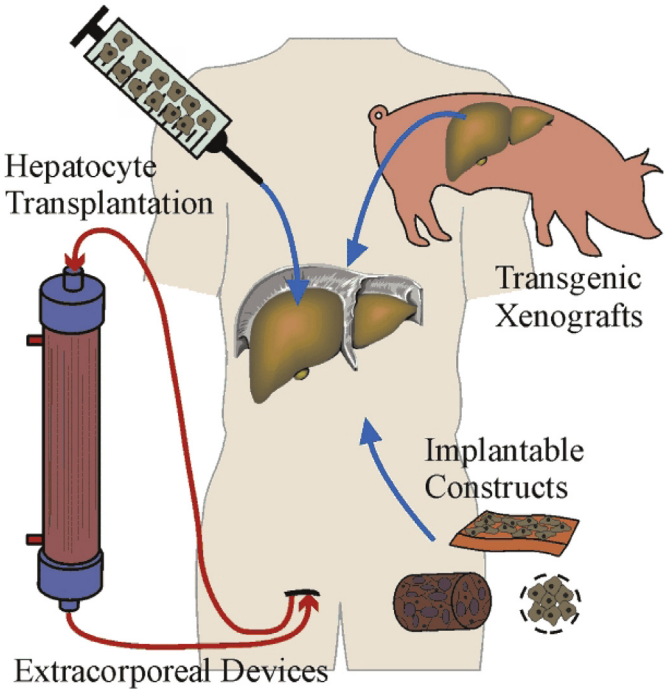

FIGURE 46.1
Cell-based therapies for liver disease. Extracorporeal devices perfuse patient's blood or plasma through bioreactors containing hepatocytes. Hepatocytes are transplanted directly or implanted on scaffolds. Transgenic animals are being raised in order to reduce complement-mediated damage of the endothelium [114].

CELL SOURCES FOR LIVER CELL-BASED THERAPIES

Cell-based therapies hold great promise, but their clinical use has been hindered by the inability of hepatocytes isolated from the *in vivo* hepatic microenvironment to maintain hepatocyte-specific phenotype and function *in vitro*. Due to the paucity of human liver tissue as a cell source, alternative cell sources have been explored (Table 46.1) with inherent strengths and drawbacks. However, the criteria to characterize these alternative cell sources as a hepatocyte or hepatocyte-like cells have not been standardized and vary greatly among different studies. No single test has been demonstrated to be sufficient to determine whether a particular cell type truly recapitulates hepatocyte function; as a result, several tests must be carried out to query various domains of hepatocyte function, including bile production, detoxification, metabolic, and synthetic functions (Table 46.2).

Cell lines

Immortalized hepatocyte cell lines such as HepG2 (human hepatoblastoma) [8], the HepG2 derived line C3A [9], HepLiu (SV40 immortalized) [10], or immortalized fetal human hepatocytes [11], have been utilized as readily available surrogates for hepatic tissue. However, these cell lines lack the full functional capacity of primary adult hepatocytes, and for clinical applications there is a risk that oncogenic factors or transformed cells could be transmitted to the patient. Thus, the generation of conditionally immortalized lines and the incorporation of inducible suicide genes have been considered as potential precautionary measures.

Primary cells

The use of primary hepatocyte-based systems could potentially eliminate the issues faced by immortalized lines, and provide the appropriate repertoire of liver functions. Primary porcine hepatocytes have been utilized in a range of BAL device configurations with some encouraging results. However, the utility of xenogeneic porcine cells for human liver therapies is restricted by immunogenicity and the potential for xenozoonotic transmission of infectious agents such as porcine endogenous retrovirus (PERV). Recognizing these concerns, recent efforts have led to the development of PERV-free pigs as well as genetically modified pigs that are transgenic for human proteins, thereby decreasing their immunogenicity.

Primary human hepatocytes are the ideal cell type for cell-based therapies, and the development of primary hepatocyte-based approaches is the focus of substantial ongoing research. However, progress has been hindered by the limited supply of primary human hepatocytes and the difficulty of maintaining hepatocyte function *in vitro*. Discussed in more detail later, hepatocytes exhibit a loss of liver-specific functions under many conditions *in vitro* and despite their significant proliferative capacity during regenerative responses *in vivo*, mature hepatocyte proliferation in culture is limited [12]. A variety of techniques have been developed

TABLE 46.1 Cell sources for liver therapies

Cell source	Critical issues
Primary hepatocytes Human adult and fetal, xenogeneic	Sourcing, expansion, phenotypic instability, immunogenicity, safety (xenozoonotic)
Immortalized hepatocyte lines Tumor-derived, SV40, telomerase, spontaneously immortalized	Range of functions, genomic instability, safety (tumorigenicity)
Stem Cells Embryonic, liver progenitors (hepatoblasts, oval cells), other lineages (HSC, MAPC), Induced pluripotent stem cells, direct reprogramming to hepatocytes	Sourcing, differentiation efficiency, phenotypic instability, immunogenicity, safety (tumorigenicity)

TABLE 46.2 Hepatic functions

Functional classification	Examples
Synthetic	Albumin Secretion Alpha-1-antitrypsin Secretion Coagulation Factor Production (II, IX, X) Lipoprotein and apoprotein synthesis Ceruloplasmin production Ferritin production Complement production
Metabolic	Ureagenesis and metabolism Bilirubin Metabolism Steroid Metabolism Gluconeogenesis/Glycogen Production Lipid metabolism
Detoxification pluripotent stem cells, direct reprogramming to hepatocytes	Metabolize, detoxify, and inactivate exogenous and endogenous compounds via cytochrome P450 enzymes, methyltransferases, sulfotransferases, acetyltransferases, UDP-glucuronosyltransferases, and Glutathione S-transferases
Bile Production	

to enable the cryopreservation of human hepatocytes [13]. This enables the large number of hepatocytes that are prepared from a single liver to be stored and thawed with reproducible cellular function. This option has opened the door to a variety of *in vitro* pharmacologic and infectious disease studies [14–16].

Due to the limitations in mature hepatocyte expansion *in vitro*, alternative cell sources are being pursued. These include various stem cell and progenitor populations, which can self-renew *in vitro* and exhibit multipotency or pluripotency and thereby serve as a possible source of hepatocytes, as well as other non-parenchymal liver cells.

Fetal and adult progenitor cells

A variety of fetal and adult progenitor cell types have been explored. Current investigations are focused on determining the differentiation potential and lineage relationships of these populations. Fetal hepatoblasts are liver precursor cells present during development that exhibit a bipotential differentiation capacity, defined by the capability to generate both hepatocytes and bile duct epithelial cells. Sourcing problems associated with fetal cells have led researchers to search for resident cells that have progenitor properties in the adult. Towards this end, a few groups have argued that rare resident cells (which may represent embryonic remnants) exhibit properties consistent with the hypothesized adult hepatic stem cells and share phenotypic markers and functional properties with fetal hepatoblasts. In adult animal livers suffering certain types of severe and chronic injury, such oval cells can mediate liver repair through a program similar to hepatic development. Whether or not these processes occur in normal human liver injury processes remains controversial. In either case, there is little evidence that cells other than adult hepatocytes participate in the daily turnover of healthy, undamaged rodent or human liver and therefore the existence and role of hepatic stem cells or progenitors in healthy rodent and human liver homeostasis remains controversial.

Along similar lines, Weiss and colleagues have demonstrated the development of bipotential mouse embryonic liver (BMEL) cell lines derived from mouse E14 embryos that exhibit characteristics comparable to fetal hepatoblasts and oval cells [17]. These BMEL cells are proliferative, can be induced to be hepatocyte-like or bile duct epithelial-like *in vitro*, and can

home to the liver to undergo bipotential differentiation *in vivo* within a regenerative environment. More recently, biopotential human embryonic liver cells have been isolated and, similar to mouse BMEL cells, are proliferative and capable of bipotential differentiation [18].

Pluripotent stem cells

Human embryonic stem cells (hES) first isolated by James Thomson in 1998 offer the ability to generate large numbers of pluripotent cells that have the potential to form all three germ layers and differentiated cells including hepatocytes. Subsequently, Yamanaka and colleagues demonstrated that fully differentiated adult cells, such as fibroblasts or skin cells, could be reprogrammed to a undifferentiated, pluripotent state similar to embryonic stem cells through the forced expression of reprogramming factors Oct3/4 and Sox2 along with either Klf4 or Nanog and Lin28. These reprogrammed cells are termed induced pluripotent stem (iPS) cells and highly resemble hES cells, sharing many characteristics such as self-renewal capabilities *in vitro* and pluripotent differentiation potential *in vitro* and *in vivo* (though epigenetic differences between hES and iPS have been identified). Since iPS cells are sourced from adult somatic cells unlike embryonic stem cells, ethical considerations associated with hES are eliminated. Moreover, iPS cells permit the generation of patient-specific cell populations, potentially enabling therapies to be developed according to the characteristics of an individual patient or to study a variety of metabolic disorders and genetic variations that only manifest in an adult.

Inspired by the understanding of how a totipotent stem cell proceeds to become a hepatocyte during normal development, multiple protocols have been described that enable hES cell differentiation into hepatocyte-like cells. Duncan and colleagues, as well as other researchers, demonstrated that through iPS reprogramming and a subsequent multistep differentiation protocol, skin fibroblasts can give rise to hepatocyte-like cells, which not only exhibit a variety of hepatocyte-specific functions *in vitro*, but can also be induced to generate intact fetal livers in mice *in vivo* via tetraploid complementation [19]. Pluripotent stem cell-derived hepatocytes exhibit both the phenotypic and functional characteristics associated with hepatocytes, though the resulting differentiated cells exhibit a more fetal state of maturation compared to adult human hepatocytes [20]. In addition, as with adult primary hepatocytes, it is not clear whether stem cell-derived 'hepatocyte-like cells' will maintain their phenotype *in vitro*. To date, only one example of *in vivo* engraftment of pluripotent stem cell-derived hepatocyte-like cells has been reported; this group utilized a unique and specific injury mouse model, and the authors noted little to no human-specific hepatic functions after engraftment [21]. Future studies will need to verify that pluripotent stem cell-derived hepatocyte-like cells can engraft *in vivo*, and will also need to address safety concerns, such as the potential for pluripotent cell-derived teratoma formation, and the oncogenic risks associated with integrating vectors used to generate some iPS lines. Human embryonic stem (ES) cells have recently been approved by the FDA (Food and Drug Administration) for safety trials to commence such studies.

Over the past few years a variety of lineages have been generated via the direct reprogramming of one adult cell type into another, without an undifferentiated pluripotent intermediate. Similar to the use of master transcriptional regulators in the reprogramming to iPS cells, the expression of a key sets of genes have been used to directly generate skeletal muscle tissue, cells that highly resemble β-cells, and neurons [22]. More recently, mouse fibroblasts have been directly reprogrammed to generate hepatocytes [23,24]. These findings raise future possibilities for deriving human hepatocytes directly from another adult cell type, although it is unclear how reprogrammed hepatocyte-like cells may behave compared to primary cells, or whether this approach will also be successful using human cells.

Ultimately, understanding the mechanisms governing the fates of stem and progenitor cell populations can empower the development of cell-based therapies. However, many challenges remain, including the ability to program differentiation completely, beyond a fetal hepatocyte stage. Furthermore, regardless of the cell source, phenotypic stabilization of

hepatocytes *ex vivo* remains a primary issue. Microenvironmental signals including soluble mediators, cell-extracellular matrix interactions, and cell-cell interactions have been implicated in the regulation of hepatocyte function. Accordingly, the development of robust *in vitro* liver models is an essential stepping-stone towards a thorough understanding of hepatocyte biology and improved effectiveness of cell-based therapies for liver disease and failure.

IN VITRO HEPATIC CULTURE MODELS

An extensive range of liver model systems have been developed, some of which include: perfused whole organs and wedge biopsies; precision cut liver slices; isolated primary hepatocytes in suspension or cultured upon extracellular matrix; immortalized liver cell lines; isolated organelles, membranes or enzymes; and recombinant systems expressing specific drug metabolism enzymes [25]. While perfused whole organs, wedge biopsies, and liver slices maintain many aspects of the normal *in vivo* microenvironment and architecture, they typically suffer from short-term viability (< 24 hours) and limited nutrient/oxygen diffusion to inner cell layers. Purified liver fractions and single enzyme systems are routinely used in high-throughput systems to identify enzymes involved in the metabolism of new pharmaceutical compounds; although they lack the complete spectrum of gene expression and cellular machinery required for liver-specific functions. In addition, cell lines derived from hepatoblastomas or from immortalization of primary hepatocytes are finding limited use as reproducible, inexpensive models of hepatic tissue. However, such lines are plagued by abnormal levels and repertoire of hepatic functions [26], perhaps most notably, the divergence of constitutive and inducible levels of cytochrome P450 enzymes [27]. Though each of these models has found utility for focused questions in drug metabolism research, isolated primary hepatocytes are generally considered to be most physiologically relevant for constructing *in vitro* platforms for a multitude of applications. However, a major limitation in the use of primary hepatocytes is that they are notoriously difficult to maintain in culture due to their precipitous decline in viability and liver-specific functions upon isolation from the liver [25]. Accordingly, substantial research has been conducted over the last two decades towards elucidating the specific molecular stimuli that can maintain phenotypic functions in hepatocytes. In subsequent sections, we present examples of strategies that have been employed to improve the survival and liver-specific functions of primary hepatocytes in culture.

In vivo microenvironment of the liver

In order to engineer an optimal microenvironment for hepatocytes *in vitro*, one can utilize as a guide the precisely defined architecture of the liver, in which hepatocytes interact with diverse extracellular matrix molecules, non-parenchymal cells, and soluble factors (i.e., hormones, oxygen) (Fig. 46.2). Structurally, the two lobes of the liver contain repeating functional units called lobules, which are centered on a draining central vein. Portal triads at each corner of a lobule contain portal venules, arterioles and bile ductules. The blood supply to the liver comes from two major blood vessels on its right lobe: the hepatic artery (one-third of the blood) and the portal vein (two-thirds). The intrahepatic circulation consists of sinusoids, which are small tortuous vessels lined by a fenestrated basement membrane lacking endothelium that is separated from the hepatocyte compartment by a thin extracellular matrix region termed the space of Disse. The hepatocytes, constituting $\sim$70% of the liver mass, are arranged in unicellular plates along the sinusoid where they experience homotypic cell interactions. Several types of junctions (i.e., gap junctions, cadherins, and tight junctions) and bile canaliculi at the interface of hepatocytes facilitate the coordinated excretion of bile to the bile duct and subsequently to the gall bladder. Non-parenchymal cells including stellate cells, cholangiocytes (biliary ductal cells), sinusoidal endothelial cells, Kupffer cells (liver macrophages), natural killer cells, and pit cells (large granular lymphocytes) interact with hepatocytes to modulate their diverse functions. In the space of Disse, hepatocytes are

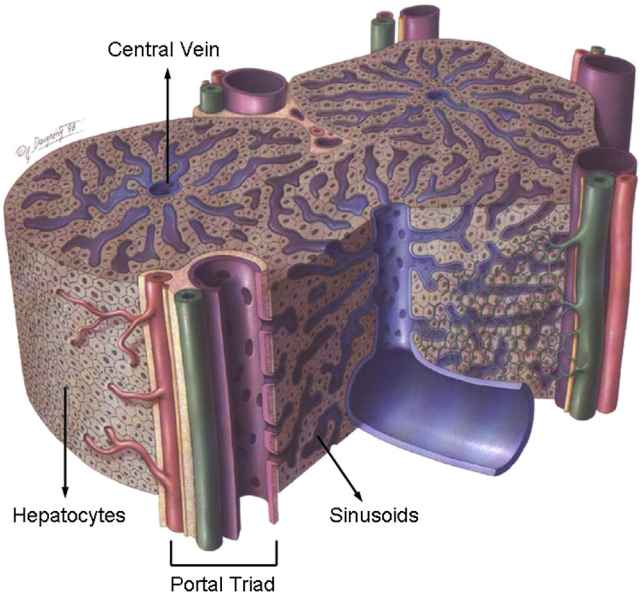

FIGURE 46.2
The precisely defined architecture of the repeating unit of the liver, the lobule. Hepatocytes are arranged in cords along the length of the sinusoid where they interact with extracellular matrix molecules, non-parenchymal cells and gradients of soluble factors. Nutrient and oxygen rich blood from the intestine flows into the sinusoid via the portal vein. After being processed by the hepatocytes, the blood enters the central vein and into the systemic circulation. *(Reproduced with permission from J. Daugherty.)*

sandwiched between layers of extracellular matrix (collagen types I, II, III, IV, laminin, fibronectin, heparan sulfate proteoglycans), the composition of which varies from the portal triad to the central vein [28].

Within the liver lobule, hepatocytes are partitioned into three zones based on morphological and functional variations along the length of the sinusoid (zonation). Zonal differences have been observed in virtually all hepatocyte functions. For instance, compartmentalization of gene expression is thought to underlie the capacity of the liver to operate as a 'glucostat'. Furthermore, zonal differences in expression of cytochrome-P450 enzymes have also been implicated in the zonal hepatotoxicity observed with some xenobiotics [29]. Possible modulators of zonation include blood-borne hormones, oxygen tension, pH levels, extracellular matrix composition, and innervation [30]. Therefore, a precisely defined microarchitecture, coupled with specific cell-cell, cell-soluble factor and cell-matrix interactions allows the liver to carry out its many diverse functions, which can be broadly categorized into protein synthesis (i.e., albumin, clotting factors), cholesterol metabolism, bile production, glucose and fatty acid metabolism, and detoxification of endogenous (i.e., bilirubin, ammonia) and exogenous (drugs and environmental compounds) substances.

Two-dimensional cultures

Numerous studies have improved primary hepatocyte survival and liver-specific functions *in vitro* through modifications in microenvironmental signals including soluble factors (medium composition), cell-matrix interactions as well as heterotypic cell-cell interactions with non-parenchymal cells (Fig. 46.3). The supplementation of culture media with

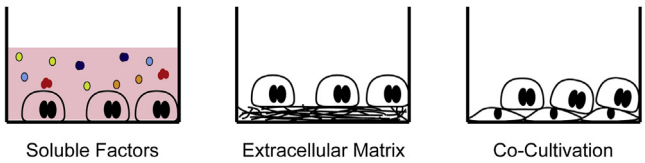

FIGURE 46.3
Cell sourcing of human hepatocytes. Approaches have focused on modulating *in vitro* culture conditions, such as the addition of soluble factors such as growth factors and small molecules, extracellular matrix proteins, heterotypic cell-cell interactions.

physiological factors such as hormones, corticosteroids, growth factors, vitamins, amino acids or trace elements [25,31], or non-physiological small molecules such as phenobarbital and dimethylsulfoxide [32,33] have been shown to modulate hepatocyte function. More recently, oxygen tension has also been shown to modulate hepatocyte function [34]. Certain mitogenic factors like epidermal growth factor and hepatocyte growth factor can induce limited proliferation in rat hepatocytes *in vitro*, but these mitogens result in negligible proliferation of human hepatocytes *in vitro* [35,36]. The impact of extracellular matrix composition and topology on hepatocyte phenotype is widely recognized. A variety of extracellular matrix (ECM) coatings (most commonly collagen type I) have been shown to enhance hepatocyte attachment to the substrate, but this usually occurs concomitantly with hepatocyte spreading and a subsequent loss of hepatocyte function [37]. Studies investigating the benefits of complex mixtures of ECM components have utilized strategies including Matrigel, liver-derived biomatrix [37–40], or bottom-up approaches such as a combinatorial high-throughput ECM microarray, where the latter has revealed specific combinations of ECM molecules that improve hepatocyte function compared to monolayer cultures on collagen I [41]. In contrast to culturing hepatocytes on a monolayer of ECM molecules, hepatocytes have also been sandwiched between two layers of type I collagen gel. In this classic 'sandwich' culture format, hepatocytes exhibit desirable morphology with polarized bile canaliculi, as well as stable functions for several weeks [42,43]. However, phase I/II detoxification processes have been shown to become imbalanced over time in this format [44]. Also, the presence of an overlaid layer of extracellular matrix may present diffusion barriers for molecular stimuli (i.e., drug candidates), and the fragility of the gelled matrix may hinder scale-up within BAL devices or multiwell *in vitro* models.

Heterotypic interactions between hepatocytes and their non-parenchymal neighbors are known to be important at multiple stages *in vivo*. Liver specification from the endodermal foregut and mesenchymal vasculature during development is believed to be mediated by heterotypic interactions [45,46]. Similarly, non-parenchymal cells of several types modulate cell fate processes of hepatocytes under both physiologic and pathophysiologic conditions within the adult liver [47,48]. Substantial work pioneered by Guguen-Guillouzo and colleagues has demonstrated that a wide variety of non-parenchymal cells from both within and outside the liver are capable of supporting hepatocyte function for several weeks in co-culture contexts *in vitro*, even across species barriers, suggesting that the mechanisms responsible for non-parenchymal cell-mediated stabilization of hepatocyte phenotype may be conserved [49,50]. Microfabrication approaches have been employed to control tissue microarchitecture in hepatic co-cultures so as to achieve an optimal balance of homotypic and heterotypic cellular interactions to promote hepatocyte function, which will be described in a later section. Hepatic co-cultures have been utilized to investigate various physiologic and pathophysiologic processes, including the acute phase response, mutagenesis, xenobiotic toxicity, oxidative stress, lipid and drug metabolism [50]. For example, co-cultures of hepatocytes and Kupffer cells have been used to examine mechanisms of hepatocellular damage [51,52], while co-cultures with liver sinusoidal endothelial cells (which are also phenotypically unstable in monoculture upon isolation from the liver) have highlighted the importance of hepatocyte-endothelial cell interactions in the bidirectional stabilization of these cell types [53,56]. Studies focused on the underlying mechanisms of hepatocyte stabilization in co-culture have identified several cell surface and secreted factors that play a role including T-cadherin, E-cadherin, decorin and TGF-β1, which demonstrate the potential for highly functional hepatocyte-only culture platforms [57,58].

Three-dimensional cultures

Culture of hepatocytes on substrates that promote aggregation into three-dimensional spheroids has also been extensively explored. Numerous technologies including non-adhesive surfaces (such as poly(2-hydroxyethyl methacrylate) and positively charged Primaria dishes),

rotational or rocking cultures, and encapsulation in macroporous scaffolds have been developed for hepatocyte spheroid formation [59–64]. Under spheroidal culture conditions, hepatocyte survival and functions are improved over standard monolayers on collagen [65]. Potential mechanisms underlying these effects include an increased extent of homotypic cell-cell contacts between hepatocytes, the retention of a three-dimensional cytoarchitecture, and the three-dimensional presentation of ECM molecules around the spheroids [59]. Some limitations of conventional spheroidal culture include a tendency for secondary aggregation of spheroids and the resultant development of a necrotic core in the larger aggregates due to diffusion-limited transport of nutrients and waste, and the lack of control over the cell numbers within each spheroid [25]. A variety of methods are being developed to prevent secondary aggregation of initially-formed spheroids and control cell-cell interactions, including microfabricated scaffolds, bioreactor systems, encapsulation techniques and synthetic linkers [26,66,67–73], which are discussed in further detail in the following sections.

Bioreactor cultures

A wide range of small-scale bioreactor platforms have been developed for *in vitro* liver applications. For example, perfusion systems containing hepatocellular aggregates exhibit desirable cell morphology and liver-specific functions for several weeks in culture [26,68,74–75], and the incorporation of multiple reactors in parallel has been explored as an approach for high-throughput drug screening studies [76–79]. In order to promote oxygen delivery while protecting hepatocytes from deleterious shear effects, gas-permeable membranes with endothelial-like physical parameters, grooved substrates and microfluidic microchannel networks have been integrated into several bioreactor designs [76,80–82]. *In vivo*, hepatocyte functions are heterogeneously segregated along the hepatic sinusoid, and this zonation is thought to be modulated by gradients in oxygen, hormones, nutrients and extracellular matrix molecules. Using a parallel-plate bioreactor, it was demonstrated that steady state oxygen gradients characteristic of those found in liver sinuosoids could contribute to a heterogeneous expression of drug metabolism enzymes CYP2B and CYP3A in hepatocyte-non-parenchymal co-cultures, which mimics the expression gradients present *in vivo* (Fig. 46.4) [83]. Furthermore, the localization of acetaminophen-induced hepatic toxicity in the region experiencing low oxygen in this model system recapitulates the perivenous location of toxicity *in vivo*. A zonal microbioreactor array based on microfluidic serpentine mixing regions feeding into several microbioreactors in parallel has also been designed for potential *in vitro* liver zonation applications (Fig. 46.4). Overall, the ability to decouple oxygen tension from gradients of other soluble stimuli and cell-cell interaction effects within this platform represents an important tool for the systematic investigation of the role of extracellular stimuli in zonation.

Bioreactors may also be used to study more dynamic physiological processes than is possible in conventional culture platforms. For example, a recent bioreactor device describes the ability to monitor invasion of metastatic cells into hepatic parenchyma by recreating relevant features of the liver tissue such as fluid flow and length scales [84]. Collectively, by providing dynamic control over hepatocyte culture parameters and hence hepatocyte function, bioreactors will continue to be useful in studying liver biology and facilitating drug development applications.

Microtechnology tools to optimize and miniaturize liver cultures

The ability to control tissue architecture along with cell-cell and cell-matrix interactions on the order of single cell dimensions represents another important tool in the investigation of mechanisms underlying tissue development, and ultimately, the realization of tissue-engineered systems. Semiconductor-driven microtechnology tools enable micrometer-scale control over cell adhesion, shape and multicellular interactions [85,86]. Thus, over the last decade, microtechnology tools have emerged both to probe biomedical phenomena at relevant length scales and to miniaturize and parallelize biomedical assays (e.g., DNA microarrays, microfluidics).

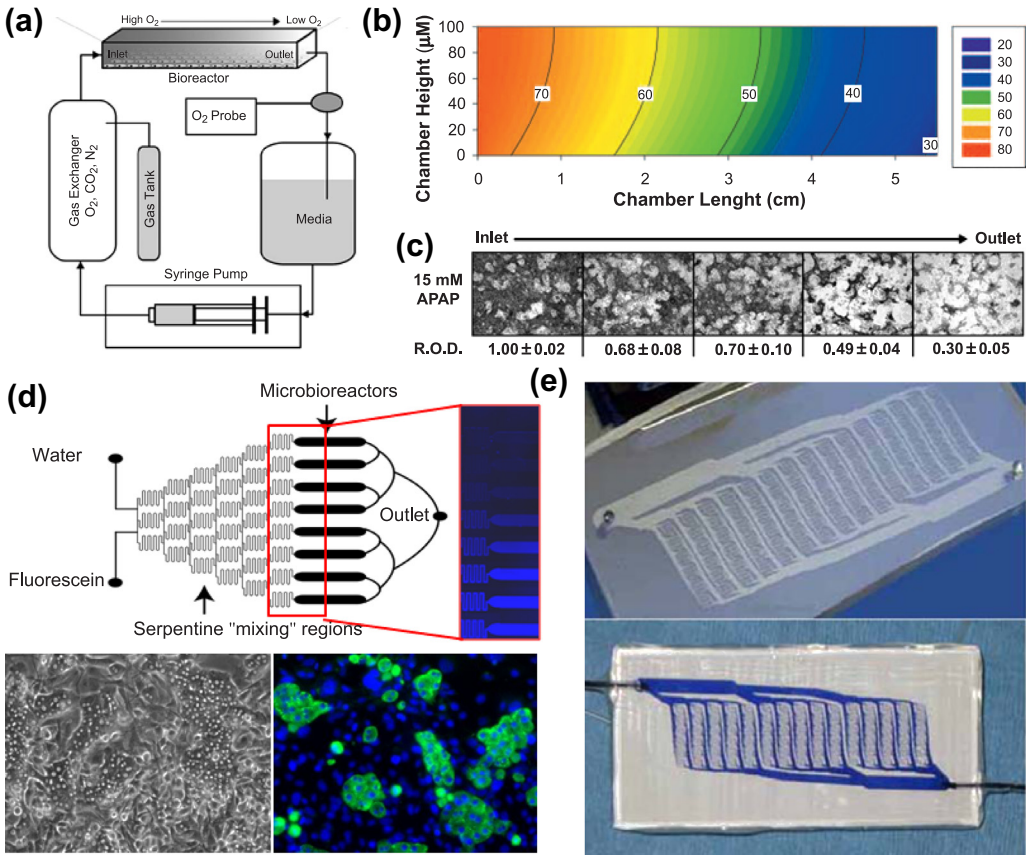

FIGURE 46.4

Bioreactors for *in vitro* liver applications. (a) Zonation and toxicity in a hepatocyte bioreactor. Co-cultures of hepatocytes and non-parenchymal cells are created on collagen-coated glass slides and placed in a bioreactor circuit where the oxygen concentration at the inlet is held at a constant value. Depletion of oxygen by cells creates a gradient of oxygen tensions along the length of the chamber, similar to that observed *in vivo*. (b) Two-dimensional contour plot of the medial cross section of the reactor depicting the cell surface oxygen gradient formed with inlet pO2 of 76 mmHg and flow rate of 0.3 mL/min. (c) Bright-field images of perfused cultures treated with 15 mM acetaminophen (APAP, a hepatotoxin) and stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as a measure of cell viability from five regions along the length of the bioreactor. The intensity of MTT staining is reported as relative optical density (R.O.D) values. The zonal pattern of APAP toxicity seen here is consistent with that observed *in vivo* [83]. (d) A zonal microbioreactor array that incorporates serpentine mixing regions and two sources is able to create a gradient of fluorescein (shown in blue on right) in an array of microbioreactors containing random co-cultures of hepatocytes and non-parenchymal cells (bottom). (e) A bilayer microfluidics device designed with a microchannel network that mimics liver vasculature so as to support the large metabolic needs of hepatocytes contained within an adjacent chamber. *(Figure panels reproduced with permission from [82].)*

In the context of the liver, a photolithographic cell patterning technique ('micropatterned co-culture') has enabled the optimization of liver-specific functions in co-cultures via engineering of the balance between homotypic (hepatocyte-hepatocyte) and heterotypic (hepatocyte-non-parenchymal) cell-cell interactions [50]. Specifically, micropatterned co-cultures were created in which hepatocyte islands of controlled diameters were surrounded by supporting non-parenchymal cells (Fig. 46.5). This pattern was miniaturized into a multiwall format using soft lithography techniques and has resulted in micropatterned co-cultures optimized for human hepatocyte function [14]. The maintenance of hepatic functions has been shown to be highly dependent upon precise optimization of island size in systems utilizing both rat and human hepatocytes. This platform has been utilized extensively for both drug development and pathogen model systems, as discussed in detail in the next section.

As described above, several culture techniques have been explored for the formation of hepatic spheroids. These methods exhibit certain limitations, including an inability to immobilize the spheroids at defined locations and the heterogeneity of the structures, which can result in

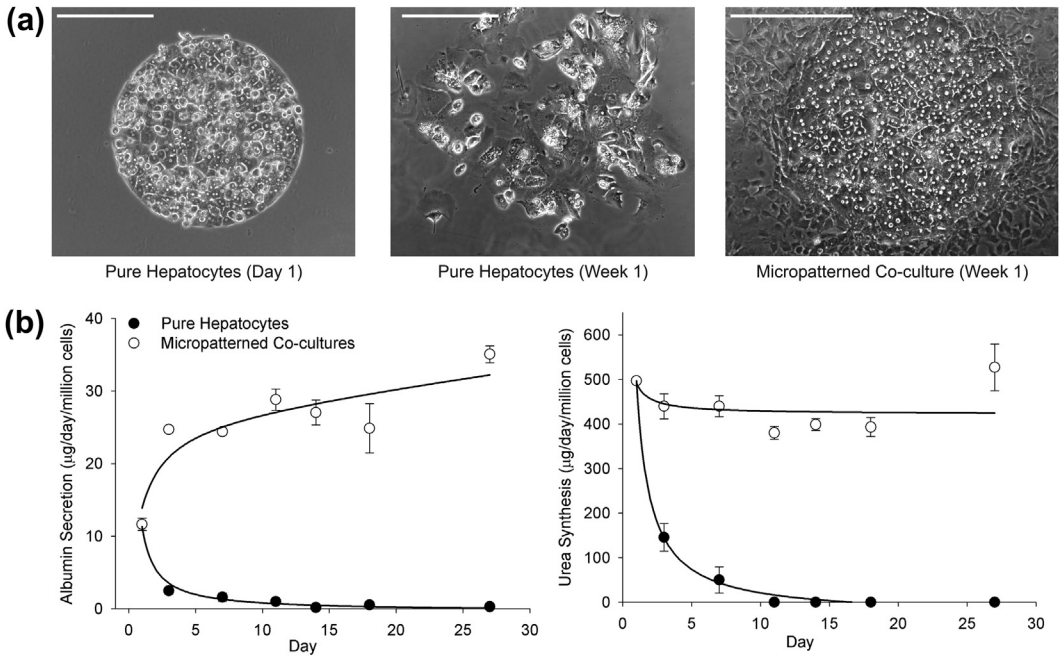

FIGURE 46.5
Micropatterned co-cultures. (a) Morphology of micropatterned co-cultures containing primary human hepatocytes (representative images at day 1 and week 1). Scale bars, 250 μm. (b) Albumin secretion and urea synthesis in micropatterned co-cultures and pure hepatocyte cultures [14].

cell necrosis at the core of large and coalesced spheroids due to depletion of oxygen and nutrients. Recently, microcontact printing, robot spotting techniques and micromolded hydrogels have been used to fabricate immobilized microarrays of hepatic cells or spheroids [41,73,87–90]. Hepatocytes in these platforms typically retain a liver-specific phenotype as assessed by the expression of liver-enriched transcription factors, secretion of albumin, and the presence of urea cycle enzymes. Microtechnological tools have also been applied to the analysis of dynamic processes related to hepatocyte biology. For example, microfluidic devices that recreate physiologically relevant fluid flows and lengths scales have been used to study drug clearance, toxicity and inflammation-mediated gene expression changes [78,91–93], and mechanically actuated, microfabricated substrates have been used to deconstruct the role of contact and short-range paracrine signals in interactions between hepatocytes and stromal cells or liver endothelial cells [53,94]. Overall, the fine spatial and temporal control of molecular signals provided by microtechnological approaches continue to reveal important mechanisms in liver biology and accelerate the development of therapeutic strategies.

Drug and disease model systems

Advances in the development of liver cell-based culture systems have begun to provide important insights into human-specific liver processes that were not previously accessible with standard *in vitro* models. Specifically, engineered tissue systems which promote long-term functional stabilization of human hepatocytes *in vitro* have enabled novel studies into drug-drug interactions and hepatocellular toxicity. For example, micropatterned co-culture-based platforms have been demonstrated to support human hepatocyte phenotypic function for several weeks, including maintenance of gene expression profiles, canalicular transport, phase I/II metabolism, and the secretion of liver-specific products [14,95]. Furthermore, drug-mediated modulation of CYP450 expression and activity, and resultant changes in toxicity profiles were observed, illustrating the utility of the platform for human ADME/Tox (adsorption, distribution, metabolism, excretion, and toxicity) applications (Table 46.3).

TABLE 46.3 Generation of major human drug metabolites by different *in vitro* systems [95,254]

	In Vivo	Microsomes[a]	S-9[b]	Hepatocyte suspension[b]	Micropatterned co-culture	
					48 h	7 Days
Excretory metabolites > 10% of dose						
All excretory metabolites	39	19 (49)	22 (56)	25 (64)	27 (69)	32 (82)
Metabolites arising by phase 1 reactions only	29	17 (59)	19 (66)	19 (66)	20 (69)	24 (83)
Metabolites arising by a phase 2 reaction	10	2 (30)	3 (30)	6 (60)	7 (70)	8 (80)
Metabolites that are one reaction from parent (primary)	16	12 (69)	11 (69)	12 (75)	13 (81)	15 (94)
Metabolites that are two or more reactions from parent (secondary)	23	7 (48)	11 (48)	13 (57)	14 (61)	17 (74)
Circulatory metabolites > 10% of total drug-related material						
All circulating metabolites	40	17 (43)	19 (48)	21 (53)	28 (70)	30 (75)
Metabolites arising by a phase 1 reactions only	31	14 (52)	16 (52)	14 (45)	22 (71)	23 (74)
Metabolites arising by a phase 2 reaction	9	3 (33)	3 (33)	7 (78)	6 (67)	7 (78)
Metabolites that are one reaction from parent (primary)	16	11 (69)	11 (69)	12 (75)	12 (75)	14 (88)
Metabolites that are two or more reactions from parent (secondary)	24	6 (25)	8 (33)	9 (38)	16 (57)	16 (67)

Several other engineered model systems incorporating 3D hepatocellular aggregates or perfusion methods have also been designed for drug screening purposes and tested with either rodent or human hepatocytes [26,68,96,97].

Engineered *in vitro* liver models can also facilitate studies into the behavior of pathogens that exclusively target human hepatocytes, including two such pathogens with profound global health implications, hepatitis C virus (HCV) and malaria. Early experiments examining HCV replication *in vitro* employed carcinoma cells stably transfected with a subgenomic viral replicon [98]. While these studies provided important information on viral replication and potential small molecule inhibitors of viral replicative enzymes, the entire viral life cycle could not be completed due to the absence of structural proteins. Furthermore, prior to 2005, there was no known viral genotype that could complete the viral life cycle *in vitro*. Following the identification of a genotype-2a strain of HCV responsible for fulminant hepatitis in a Japanese patient, termed JFH-1 [99], it was demonstrated that JFH-1 and a chimeric variant could complete a full viral life cycle in the Huh7 carcinoma cell line [100–102]. Subsequently, recent approaches have made it possible to examine HCV infection in primary human hepatocytes [16,103,104]. In particular, the stabilization of human hepatocytes in a micropatterned co-culture platform was demonstrated to allow the recapitulation of the full viral life cycle *in vitro* [16] (Fig. 46.6). In this platform, human hepatocytes expressed all known entry factors for HCV and supported viral replication for several weeks, illustrating the potential of such *in vitro* systems for screening drug candidates that suppress HCV replication, and the ultimate identification of non-hepatotoxic anti-HCV compounds. Furthermore, a recent study demonstrating HCV infection of human iPS-derived hepatocyte-like cells establishes a foundation for the development of personalized disease models that allow the study of the impact of host genetics on viral pathogenesis [105].

In vitro culture platforms have also been explored for the study of hepatocyte infection by *Plasmodium* sporozoites in order to model the liver stage of malaria. To date, the primary cell

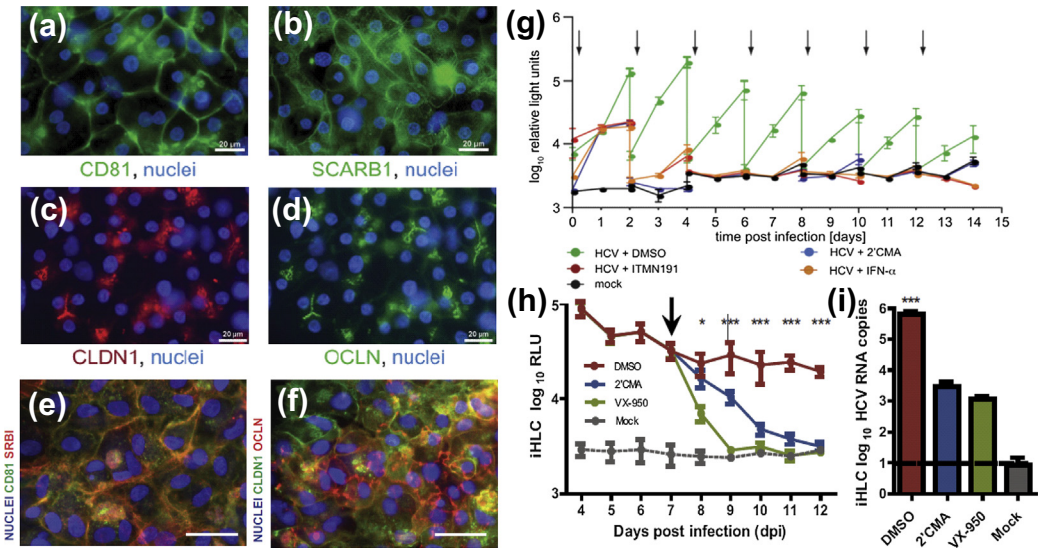

FIGURE 46.6

Modeling hepatotropic pathogenic diseases with human liver platforms. Primary human hepatocyte in micropatterned co-cultures express host entry factors for HCV (a) CD81, (b) SCARB1, (c) CDLN1 and (d) OCLDN, and (g) can be persistently infectible with HCV (green line), and is decreased by various inhibitors (orange, red, blue lines). Human iPS cell-derived hepatocyte-like cells (iHLCs) express host entry factors for HCV like (e) CD81, SRB1, (f) CLDN1 and OCLN. iHLCs are infectible with HCV (red) as determined by (h) a luciferase reporter assay and (i) RT-PCR, and infection can be inhibited with a polymerase inhibitor 2'CMA (blue) and a protease inhibitor VX-950 (green) [16,105].

culture models that have been utilized include primary human hepatocyte monolayers and human hepatoma cells [106–108], and have provided important information regarding hepatocyte invasion and the role of SR-BI and CD81 [109], and potential targets for attenuating parasite growth [110]. Recently, micropatterned co-cultures which support long-term functional maintenance of primary human hepatocytes have been shown to recapitulate the liver stage of two human malaria species, *Plasmodium falciparum* and *Plasmodium vivax, in vitro* [111]. By enhancing the efficiency and duration of *Plasmodium* infection *in vitro*, such engineered hepatocyte culture platforms could form the basis for drug screening and vaccine validation assays against *Plasmodium* in an era of renewed interest and focus on global malaria eradication.

The field of hepatic tissue engineering continues to evolve towards creating an optimal microenvironment for liver cells *in vitro*. Overall, it is evident that many different culture conditions can preserve at least some phenotypic features of fully functional hepatocytes. Since detailed differences can exist in these model systems, current strategies have focused on selecting a platform that is appropriate for a particular application. Although much progress has been made, further work is required to obtain a more complete picture of the molecular signals that provide phenotypic stability of liver cultures. Indeed, it is likely that many pathways contribute to the differentiated state of the hepatocyte via a systems-level network. Stimulation of this network via different inputs (e.g., using different culture systems) may result in distinct signal transduction trajectories, each of which could lead to a 'phenotypically stable' hepatocyte. It is also possible that differences between such stabilized hepatic populations will emerge as new methods to probe phenotype and function in greater detail (e.g., via epigenetics, proteomics, metabolomics) are utilized. Future challenges, therefore, will be to identify such differences and understand them in the context of network-level (*in vivo*) hepatic phenotype. The development of highly functional and systems-level [112,113] *in vitro* liver platforms will ultimately facilitate both the clinical effectiveness of cell-based therapies as well as enable high-throughput screening of candidate drugs for liver-specific metabolism and toxicity earlier in the drug discovery pipeline and for human hepatotropic infectious diseases such as HCV and malaria.

EXTRACORPOREAL BIOARTIFICIAL LIVER DEVICES

One promising approach for cell-based therapies for liver diseases is the development of extracorporeal support devices, which would process the blood or plasma of liver failure patients. These devices are principally aimed at providing temporary support for patients suffering from acute or acute-on-chronic liver failure, to enable sufficient regeneration of the host liver tissue, or serve as a bridge to transplantation. Early extracorporeal device designs utilized primarily non-biological mechanisms such as hemoperfusion, hemodialysis, plasmapheresis, and plasma exchange [114]. Hemoperfusion removes toxins, but also captures other useful metabolites, by passage of blood or plasma through a charcoal column. A modification of this approach, referred to as hemodiadsorbtion, reduces direct contact with charcoal components through the utilization of a flat membrane dialyzer containing charcoal and exchange resin particles [115]. In general, charcoal perfusion systems have been the most extensively studied non-biological configuration, including clinical evaluation in patients with acute liver failure, although no clear survival improvement has been observed [116]. More recent configurations of artificial support systems have focused on the elimination of albumin-bound toxins utilizing a method termed albumin dialysis. These devices, such as the Molecular Adsorbent Recirculating System (MARS®) and the Prometheus platform, clear albumin-bound toxins through interaction with an albumin impregnated dialysis membrane [117]. They have been shown to offer some clinical benefit by reducing plasma bile acids, bilirubin, and other albumin-bound toxins [118], but additional randomized controlled trials are required to fully evaluate their effectiveness.

In order to provide a more complete array of synthetic, metabolic, and detoxification functions, which are lacking in strictly artificial support systems, biological approaches including cross hemodialysis, whole liver perfusion, and liver slice perfusion have been explored [116]. While these methods have shown minor improvements for patients with acute hepatic failure, they have not demonstrated any clinically meaningful survival benefits. Consequently, substantial efforts have been placed towards the development of extracorporeal BAL devices containing hepatic cells. BAL devices can be categorized into four main types, summarized in Fig. 46.7: hollow fiber devices, flat plate and monolayer systems, perfusion bed or porous matrix devices, and suspension reactors. Broadly, several criteria have emerged as central to the design of an effective BAL device; these include issues of cell sourcing, maintenance of cell viability and hepatic functions, sufficient bidirectional mass transfer, and scalability to therapeutic levels. In this section, we discuss these issues and review recent advances in BAL device design.

Cell sourcing

As discussed earlier, the sourcing of hepatic cells is a fundamental challenge for all liver cell-based therapies. Most BAL devices are forced to employ xenogeneic sources (primarily porcine) or immortalized human hepatocyte cell lines [119–122]. Porcine hepatocytes are well studied, easily sourced, and offer a functional profile quite similar to human hepatocytes, but are limited by safety concerns, such as the possibility of transmitting pathogens like porcine endogenous retrovirus (PERV), across species. However, patients treated with porcine-derived therapies have all been negative for PERV infection [123]. Human hepatocyte cell

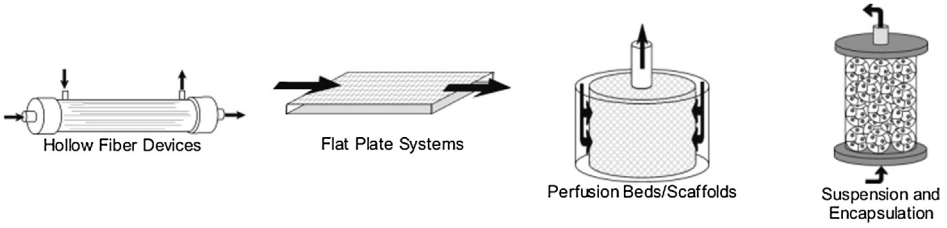

FIGURE 46.7
Schematics of cell-based bioreactor designs. The majority of liver cell-based bioreactor designs fall into these four general categories, each with inherent advantages and disadvantages [253].

lines, while expandable, exhibit an abnormal repertoire of liver functions, which limits their clinical impact. Primary human hepatocytes present the best functional output, but are scarce and rapidly lose viability and phenotype after procurement and isolation, although recent advances in cryopreservation, fetal cell procurement and stem cell differentiation have helped primary human cells become increasingly available [124–127]. Magnifying the cell sourcing challenge are scalability requirements for translation, which will be discussed in detail in the scale-up section of this chapter.

Cell viability and function

The development of effective BAL systems is dependent on the incorporation of appropriate environmental and organizational cues, which enable maximal survival and function of the hepatocellular component. Hollow fiber devices are the most common BAL design and contain hepatic cells within cartridge units similar to those utilized in hemodialysis systems [128]. The hollow fiber membranes serve as a scaffold for cell attachment and compartmentalization, although adequate nutrient transport and proper environmental stimuli are potentially limited in these configurations. Multiple modifications aimed at optimizing cellular performance have been explored. In particular, due to the enhanced function of hepatocyte spheroids relative to dispersed cells, many device configurations contain either attached or encapsulated hepatocyte aggregates [128]. For example, the original HepatAssist system previously developed by Circe Biomedical was a hollow fiber device containing microcarrier-attached porcine hepatocyte aggregates within the extracapillary space [129]. Collagen gel entrapment of hepatocyte aggregates has also been added to some hollow fiber designs to improve function, and many perfusion bed systems integrate hepatocyte aggregates within a polymeric network of pores or capillaries [120]. Furthermore, encapsulation of pre-formed hepatocyte spheroids within calcium-alginate beads has been explored as a means of promoting hepatocyte stability while simultaneously providing an immunoisolation barrier [128]. In the modular extracorporeal liver support (MELS) system (Charite, Germany) hepatocytes are aggregated in co-culture with non-parenchymal cells resulting in the formation of tissue-like organoid structures [130]. Bile canaliculi and bile duct structures as well as matrix deposition have been observed in this system, which utilized both porcine cells and primary human hepatocytes isolated from livers unsuitable for transplant [121]. Platforms based on collagen gel 'sandwich culture', a stable hepatocyte-only culture configuration, have also been developed recently [131]. Moreover, exposure of hepatocytes to the plasma or blood of a sick patient may necessitate alterations in hepatocyte culture conditions. Specifically, pre-conditioning with physiologic levels of insulin, lower than that present in normal culture medium, has been shown to prevent fat accumulation in hepatocytes upon exposure to plasma [132]. Additionally, the supplementation of plasma with amino acids has been demonstrated to increase albumin and urea synthesis [133].

Mass transfer

Bidirectional mass transfer is another primary consideration in the design of BAL systems and is required to provide vital nutrients to the incorporated cells and simultaneously allow export of therapeutic cellular products. Device configuration determines both the convective and diffusive properties of the system, thereby dictating the exchange of soluble components. In particular, diffusion resistance is often a major constraint in BAL devices. Factors commonly limiting diffusive transport are membrane structures, collagen gels, and non-viable cells. For example, semipermeable membranes are often utilized in BAL devices in order to enable selectivity in the size of exchanged factors. Inherent to most hollow fiber devices, but also utilized in some flat plate and perfusion bed systems, such membranes with a designated molecular weight cutoff act to prevent the transport of immunologic components and larger xenogeneic substances while maintaining transport of carrier proteins such as albumin. Although clearly not ideal, in order to maximize mass transfer, immunologic barriers have been eliminated from some device designs [134], with the assumption that the short duration

of contact with xenogeneic cells will result in minimal immunological complications. Perfusion bed systems allow for enhanced mass transfer due to the direct contact with the perfusing media, although fluid flow distribution is highly contingent on the type of packing material. Encapsulation of dispersed or aggregated cells represents another strategy for immunoisolation but can increase diffusion resistance [135,136]. The development of novel methods for the microencapsulation of hepatocytes is an active research area and is discussed in more detail in the implantable applications section of this chapter.

Oxygen tension is an important mediator of hepatocyte function [137–139]; thus, the improved regulation of oxygen delivery is a major goal of many recently developed BAL platforms. Strategies have included the incorporation of additional fiber compartments that carry oxygen directly into the device [140,141], and more recently, the incorporation of bovine red blood cells to the circulating culture medium [142]. Studies have confirmed that hepatocyte viability and function are more effectively maintained in devices with enhanced oxygen transport [143,144]. In contrast with other designs, flat plate geometries can be perfused in a relatively uniform manner, although this configuration may result in the exposure of cells to shear stress causing deleterious effects on cellular function [131,145–147]. Several approaches for minimizing shear stress exposure in the flat plate geometry have been explored, including the fabrication of grooved substrates for shear protection as well as the integration of adjacent channels separated by a gas-permeable membrane as a means to decouple oxygen exchange and volumetric flow rate [80,147,148]. Notably, as discussed earlier, oxygen has been implicated in the heterogeneous distribution of hepatocyte functions along the liver sinusoid, and this zonation can be recapitulated *in vitro* within a bioreactor system [149]. The eventual incorporation of oxygen gradients, as well as gradients of other diverse stimuli such as hormones and growth factors, into BAL designs could provide a means to more closely simulate the range of hepatocyte functions exhibited *in vivo* and further enhance the effectiveness of BAL devices.

Scale-up

The successful clinical implementation of any BAL device is dependent on the ability to scale the device to a level that provides effective therapy. It is estimated that such BAL devices would require approximately 1×10^{10} hepatocytes representing roughly 10% of total liver weight [150], although distinct categories of liver disease, such as acute liver failure, end stage cirrhosis, inherited metabolic disorders, will have varied scale requirements. Hepatocyte transplantation experiments in humans as well as rat models have demonstrated some improvement in various blood parameters following the transplantation of cell numbers representing 1–10% of total liver mass [151–154]. BAL devices that have been tested clinically have used approximately 0.5×10^9 to 1×10^{11} porcine hepatocytes or 4×10^{10} C3A hepatoblastoma cells [120]. Ultimately, to achieve clinical efficacy, approaches will require a) scaled-up systems with efficient nutrient transport and b) expandable cell sources, as discussed in earlier sections of this chapter. Accordingly, increasing cartridge size and use of multiple cartridges have been utilized as a means to scale-up hollow fiber-based systems. Perfusion systems [155] or devices utilizing encapsulated cells can be scaled fairly easily to the desired size; however such expanded configurations normally present a large priming or 'dead' volume. Stacked plate designs have been suggested as a means of scaling-up flat plate systems, although these modifications may introduce channeling effects and heterogeneous flow distribution [114]. Overall, the development of BAL devices exhibiting therapeutic levels of function is a major challenge and modifications aimed at further improving the capacity and efficiency of these systems is a central goal in the field.

Regulation and safety

Similar to other tissue-engineering-based therapies, the regulation of BAL devices is complex, due to the hybrid nature of these systems. Currently, BAL devices are being regulated as drugs

through the Center for Biologics Evaluation and Research of the Food and Drug Administration. The primary safety concerns for BAL systems are similar to those for other cell-based therapies, and include the escape of tumorigenic cells, immune reactions to foreign antigens and xenozoonosis. Other potential complications generally associated with extracorporeal blood treatment include hemodynamic, metabolic, and hypothermia-related abnormalities, as well as problems linked with catheterization and anticoagulation [118]. In order to explicitly prevent escape of tumorigenic cells, such as the C3A cell line used in the ELAD system, downstream filters have been added to BAL designs [119], an approach which is generally acknowledged as an adequate precautionary measure.

With regard to the utilization of porcine hepatocytes, there is some evidence for the presence of antibodies directed against porcine antigens in the serum of patients treated with BAL devices [156]. However, the clinical significance of these findings remains unclear, since high titers are not observed until one to three weeks, for IgM and IgG isotypes, respectively. These results suggest that immune rejection may not be a significant problem in the context of BAL therapy, except in cases of repetitive treatments. Still, appropriate modifications through cell sourcing or device design aimed at limiting immunologic complications would likely be important in expansion of BAL treatment options to chronic liver disease patients and patients with repetitive episodes of acute decompensation. As mentioned earlier, exposure to porcine cells can also represent a risk of xenozoonotic transmission of PERV, ubiquitous in the genome of bred pigs. PERV has been shown to infect human cell lines *in vitro* [157], although studies examining transmission to BAL-treated patients have not demonstrated any evidence of infection [158]. While specific transmission of PERV may not occur during the course of BAL therapy, the use of xenogeneic cells in BAL devices will always incur a note of caution.

Ongoing clinical trials

A number of BAL devices have been tested clinically, and the characteristics of these systems are provided in Table 46.4. Important practical issues include the use of whole blood versus plasma, and the type of anticoagulation regimen. The use of whole blood exhibits the advantage of including oxygen-containing erythrocytes; however, undesirable leukocyte activation and cell damage may arise. In contrast, perfusion of plasma prevents hematopoietic cell injury, but the solubility of oxygen in plasma devoid of oxygen carriers is quite low. Furthermore, heparin coagulation is normally used in BAL systems, although deleterious effects of heparin exposure on hepatocyte morphology and function have been suggested [159].

The design of clinical trials for BAL devices poses a significant challenge. In particular, liver failure progression is highly variable and etiology dependent. Additionally, hepatic encephalopathy, one of the major manifestations of liver failure is difficult to quantify clinically. As a result, patients in clinical trials must be randomized while still controlling for the stage at which support was initiated as well as individual etiology. Similarly, the determination of the relevant control therapy can be difficult. Ideally, in order to minimize non-specific effects of extracorporeal treatment, a non-biological control such as veno-venous dialysis would be utilized. Another challenge is the choice of clinical end point. Most clinical trials utilize end points of 30-day survival and 30-day transplant-free survival; however, trials can often be confounded by the fact that acute liver failure patients receive transplants variably depending on organ availability and the eligibility criteria in place at a given center. Furthermore, interpretation of the specific role of incorporated, live, functional hepatocytes can be complicated by the presence of non-biological adjuncts such as charcoal perfusion in some designs. A direct comparison of the effect of non-biological systems alone, dead or non-hepatocyte cells, and live hepatocytes would provide substantial insight concerning the effectiveness of the cellular components, particularly given that dead hepatocytes and non-hepatocyte cells have been shown to offer some survival benefit in various animal models of

TABLE 46.4 Characteristics of eight bioartificial liver systems

BAL system	Configuration	Cell source / amount	Trial phase	Comments
HepatAssist (Arbios, Waltham, MA)	Hollow fiber, polysulphone; microcarrier attached	Cryopreserved porcine (7×10^9)	II/IIIPrimary endpoint not reached	Plasma, citrate anticoagulation, 0.15–0.20 μm pore size, 7 h/session, daily
ELAD (Vital therapies, San Diego, CA)	Hollow fiber, polysulfone; cellulose acetate; large aggregates	C3A human cell line (200–400g)	II/III	Plasma, heparin anticoagulation, 0.20 um pores, continuous up to 107 h
MELS (Charite Virchow, Berlin, Germany)	Hollow fiber, interwoven, multicompartment; tissue organoids	Freshly isolated porcine or human (up to 650g)	I/II	Plasma, heparin anticoagulation, 400 kDa cutoff, continuous up to 6d
BLSS (Excorp Medical, Oakdale, MN)	Hollow fiber, cellulose acetate; collagen gel entrapped	Freshly isolated porcine (70–120 g)	II/III	Whole blood, heparin anticoagulation, 100-kDa cutoff, 12 h/session for up to 2 session
RFB-BAL (Univ. of Ferrara, Italy)	Radial flow bioreactor; aggregates	Freshly isolated porcine (200–230 g)	I/II	Plasma, heparin/citrate anticoagulation, 1 um polyester screen, 6–24 h treatments
AMC-BAL (Univ. of Amsterdam, Netherlands)	Non-woven polyester matrix, spirally wound; aggregates	Freshly isolated porcine ($10–14 \times 10^9$)	I	Plasma, heparin anticoagulation, direct cell-plasma contact, up to 18 h/session for up to 2 sessions
LiverX-2000 (Algenix Inc., Minneapolis, MN)	Hollow fiber; collagen entrapped	Freshly isolated porcine (40–80g)	I	Whole blood, heparin anticoagulation
HBAL (Nanjing Univ., Nanjing, China)	Hollow fiber; polysulfone; adsorption column	Freshly isolated porcine (10×10^9)	I	Plasma, 100k Da cutoff, one to two 60 h treatments

acute liver failure [160]. Notably, the ability to more accurately assess viability and function of cells during BAL treatment would be a major advance. Such information would be crucial in determining treatment time and the potential requirement for device replacement, both important considerations due to the instability of hepatocellular function in many contexts and the demonstrated detrimental effect of plasma from liver failure patients on cultured hepatocytes [161]. Finally, even if clinical trials of current BAL devices do not prove efficacy, information obtained from these studies coupled with improvements in cell sourcing and functional stabilization will represent the foundation for the next generation of devices.

IMPLANTABLE TECHNOLOGIES FOR LIVER THERAPIES AND MODELING

In addition to temporary extracorporeal support, the development of cell-based therapies for liver treatment aimed at the eventual replacement of damaged or diseased tissue is an active area of investigation. In many cases, hepatocytes have been injected into animal hosts and exhibit substantial proliferative capacity as well as the ability to replace diseased tissue and correct metabolic liver deficiencies in those models [162–165]. However, the clinical efficacy of these procedures is currently limited due in part to technical hurdles in cell delivery and animal models. These limitations might be addressed in part by engineering three-dimensional liver tissue *ex vivo* prior to implantation. Here we detail the state-of-art in cell transplantation in the context of applicable animal models as well as in the construction and application of engineered liver tissue.

Cell transplantation and animal models

Hepatocytes have been transplanted into rodents via injection into the spleen or splenic artery, intraperitoneal space, peripheral veins, or portal vein. Cell transplantation can improve host survival in animals with acute liver failure induced both chemically and surgically as well as end stage liver failure due to cirrhosis [165–169]. Transplantation also can correct metabolic deficiencies in several models of liver-based metabolic diseases [165,166]. Despite encouraging results in cell transplantation in animal models, translation of cell transplantation to the clinic has failed to result in sustained improvements in patient outcome. These results demonstrate the need for further improvements in cell delivery (e.g., through modalities such as tissue engineering) and/or better animal model systems.

Models of acute liver failure include administration of clinically relevant toxic doses of carbon tetrachloride (CCl_4) or acetaminophen, which can induce localized centrilobular necrosis, or surgical resection of two thirds of the liver in a partial hepatectomy model [170]. In these models, animals develop severe liver injury, but if animals can be kept alive for as little as 72 hours following injury, host regeneration can rapidly correct the damage. This observation is unlike the human situation, in which the repair of damage due to acute liver failure in patients has been estimated to take weeks or months [171,172]. This discrepancy could in part explain contradictory results in which animals can be 'cured' following acute liver failure after hepatocyte transplantation whereas humans are not; it is possible that injected hepatocytes are able to provide short-term support to animals but not the long-term replacement of hepatic function necessary for clinical therapy. Of note, parabiotic systems have demonstrated that after partial hepatectomy, factors regulating liver cell proliferation are present in the circulation [173,174]. Consequently, this model may serve as a well-controlled system to examine the importance of regenerative cues in the engraftment and proliferation of hepatic constructs implanted in extrahepatic sites.

Chronic liver failure represents a more substantial clinical burden, as outlined above. The most common animal models of experimental fibrosis are toxic damage due to CCl_4 administration and bile duct ligation [175]. In a rat cirrhosis model induced by CCl_4 treatment, hepatocytes transplanted into the spleen result in improved survival over controls for a period of months [176–178]. To date, hepatocyte transplantation has resulted in improvements in liver function and encephalopathy in some patients with acute and chronic liver failure, but no change in outcome and survival of these patients has been observed [179]. Engineering strategies to improve engraftment and survival in extrahepatic sites may improve the efficacy of these therapies.

Finally, hepatocytes have been transplanted into several animal models of metabolic liver diseases. In most cases, transplantation only partially corrected the genetic abnormalities. However, in the fumaryl acetoacetate hydrolase (FAH)-deficient mouse model of familial tyrosinemia [180], the albumin-uPA transgenic mouse [181], and in the transgenic mouse model of human alpha-1-antitrypsin deficiency [182], the inherited metabolic defect results in reduced survival of host hepatocytes and transplanted hepatocytes are able to engraft and replace the hepatocytes in the host liver over the course of weeks or months. These findings led to the generation of 'chimeric' mice, in which a substantial portion of the liver is composed of human hepatocytes (Fig. 46.8). For this reason, FAH-deficient and albumin-uPA transgenic mice are the most frequently used animal models for studying hepatocyte engraftment in host liver. Additionally, such mice promise to be useful as model systems for investigating human-specific drug metabolism and toxicity as well as infectious diseases that exhibit human tropism. The most convincing data for the therapeutic use of hepatocyte transplantation to date has been demonstrated in patients, primarily pediatric, with liver-based metabolic disorders. Patients with certain disorders such as urea cycle disorders experience greater improvement than others, but even in these cases, the function of hepatocytes deteriorates with time such that liver transplantation is typically necessary by six months [183].

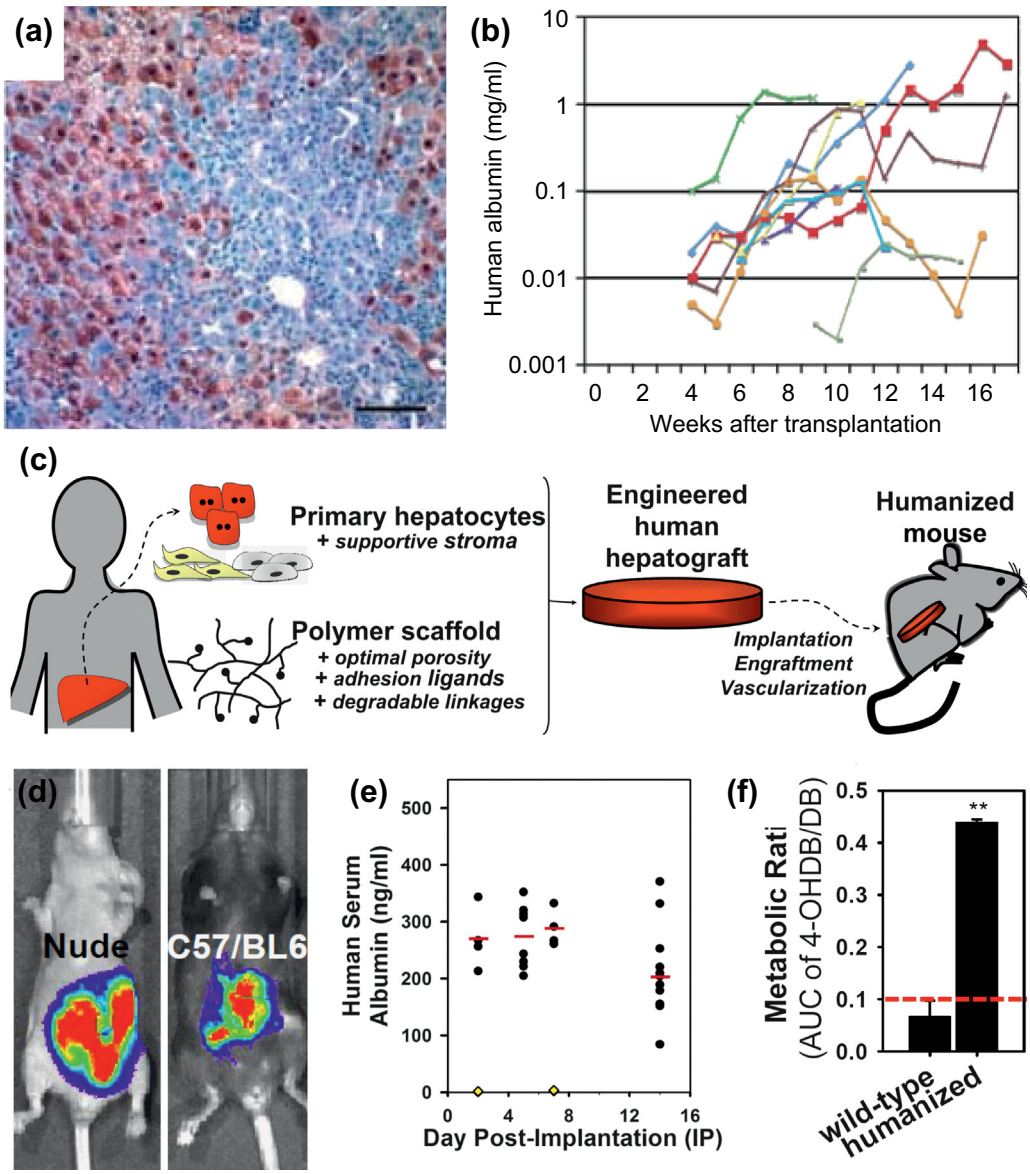

FIGURE 46.8

Mice with humanized livers. (a) FAH-positive human hepatocytes injected into the spleen of Fah$^{-/-}$ mice can engraft and integrate in the mouse livers, resulting in mice with 'humanized' livers. (b) Human albumin can be detected in the blood of these mice. (c) Engineered human liver tissue ('ectopic liver tissue') could also be constructed *ex vivo* and then implanted into mice. (d) Ectopic liver tissue can survive and function in mice that are both immunocompromised (left) and immunocompetent (right). (e) Human albumin can be detected in the blood of these mice. (f) Mice with ectopic human liver tissue can be used to identify disproportionate human drug metabolites that would not have been identified by wild-type mice. *(Reproduced with permission from [15,180].)*

In all of these animal models, a regenerative stimulation is provided by transgenic injury, partial hepatectomy, portocaval shunting, or the administration of hepatotoxic agents prior to cell transplantation. However, as evidenced by recent clinical trials that have seen limited success, these approaches are difficult to adapt to a clinical setting. Major limitations of isolated cell transplantation include the inefficient engraftment and limited survival of transplanted hepatocytes, which has been collectively reported at only 10–30% of injected cells [184]. Several studies have demonstrated methods to improve engraftment and enhance the selective proliferation of transplanted hepatocytes [185–187], although analogous to regeneration models, the clinical utility of these approaches remains to be determined. The time for engraftment and proliferation of transplanted hepatocytes also can create a

substantial lag time (48 hours in one study [152]) before clinical benefit is observed, which could restrict the utility of cell transplantation for certain clinical conditions, such as fulminant hepatic failure.

In summary, clinical trials of hepatocyte transplantation have recently demonstrated long-term safety, but donor hepatocyte engraftment and restoration of failing host livers has not been adequate to obviate the need for organ transplantation [188–190]. Additionally, only partial correction of metabolic disorders has been accomplished to date in the clinic [188–190]. Future studies that improve cell delivery, survival, and engraftment as well as reduce the time required for integration of the grafted cells with the host could greatly improve the effectiveness of cell-based therapies and animal model systems.

Implantable engineered tissue for humanized mouse models

Implantation of human engineered liver tissue into animal hosts may also provide alternative *in vivo* model systems for human disease. Despite their promise as model systems for investigating human-specific drug responses and infectious diseases with human tropism, current humanized mouse models (e.g., $FAH^{(-/-)}$ and albumin-uPA models detailed above) are limited in that animals must be both immunodeficient and exhibit significant host liver injury. Additionally, the process of human hepatocyte injection, homing to the liver, and expansion can take weeks to months; creating humanized mice using 'classic' cell transplantation is therefore tedious and time-consuming [15]. As one example of a candidate alternative, a recent study generated humanized mice by implanting hepatocytes and supporting non-parenchymal cells within a three-dimensional hydrogel scaffold into the intraperitoneal space of uninjured mice [15] (Fig. 46.8). The engineered human liver tissue synthesized human liver proteins as well as human-specific drug metabolism, drug-drug interaction, and drug-induced hepatocellular toxicity. The polyethylene glycol (PEG)-based engineered tissue was shown to survive and function within immunocompetent hosts for a period of time after implantation, suggesting that encapsulation of cells in this material system may have the potential to delay immune rejection and enable studies that require both human liver systems and intact immune processes.

Implantable therapeutic engineered liver tissue

The development of implantable engineered hepatic tissue is a promising strategy for the treatment of liver disease due to its potential to mitigate the limitations in current cell transplantation strategies, including inefficient seeding and engraftment, poor long-term hepatocyte survival, a required donor cell repopulation advantage, and the inherent lag phase before clinical benefit is attained [165,191]. Implantable engineered hepatic tissues are typically fabricated by immobilizing or encapsulating hepatic cells in biomaterial scaffolds in conjunction with strategies to optimize hepatocyte survival and function, leading to the generation of liver-like tissue *in vitro* prior to *in vivo* implantation.

Design criteria for implantable systems

To achieve therapeutic levels of liver function to treat liver failure, the development of engineered hepatic tissues that contain high densities of stable and functional hepatocytes with efficient transport of nutrients and secreted therapeutic factors is necessary. Furthermore, integration of the engineered tissue with the host upon implantation is critical in ensuring its long-term survival. The potential tunability of engineered implantable systems offers attractive prospects for the optimization of hepatocyte survival and function as well as subsequent host integration. Scaffold parameters that are customizable include porosity, mechanical and chemical properties, and three-dimensional architecture. Additionally, relevant microenvironmental cues like paracrine and juxtacrine cell-cell interactions, cell-matrix interactions and soluble factors can be incorporated into implantable engineered hepatic tissue

by translating either biological or biomimetic strategies from *in vitro* culture models so as to recapitulate important aspects of the *in vivo* hepatocyte microenvironment.

Natural scaffold chemistry and modifications

A wide variety of naturally-derived material scaffolds have been explored for liver tissue-engineering applications, including materials like collagen, peptides, fibrin, alginate, chitosan, hyaluronic acid, cellulose, decellularized liver matrix and composites of these [70,192–197] (Table 46.5). The choice of material determines the physicochemical and biological properties of the scaffold. For example, early efforts in developing implantable hepatic constructs utilized collagen-coated dextran microcarriers that enabled hepatocyte attachment since hepatocytes are known to be anchorage-dependent cells. The intraperitoneal transplantation of these hepatocyte-attached microcarriers resulted in successful replacement of liver functions in two different rodent models of genetic liver disorders [198]. Subsequently, collagen-coated or peptide-modified cellulose [196,199], gelatin [200], and gelatin-chitosan composite [201] microcarrier chemistries have also been explored for their capacity to promote hepatocyte attachment. On the other hand, materials that are poorly cell adhesive like alginate [70] have been exploited for their utility in promoting hepatocyte-hepatocyte aggregation (e.g., spheroid formation) and hence hepatocyte stabilization within these scaffolds. Collectively, the size of engineered tissues created by these approaches is limited by oxygen and nutrient diffusion to only a few hundred microns in thickness. To address this constraint, recent work has sought to use decellularized whole organ tissue as a matrix for liver tissue engineering (Fig. 46.9). The decellularization process removes cells from donor tissues but preserves the structural and functional characteristics of much of the tissue microarchitecture and vasculature of the underlying extracellular matrix. Decellularized liver matrices can be seeded with hepatocytes and vascular cells, exhibit liver-specific functions, and survive after transplantation into rodents [197,202]. Future work in this area will likely focus on improving cell seeding protocols, which to date have achieved only 20–40% of endogenous liver mass after recellularization, as well as co-seeding with a both hepatocytes and the various non-parenchymal cell types found in liver (e.g., stellate cells, Kupffer cells). In general, the advantages of biologically-derived materials include their biocompatibility, naturally occurring cell adhesive moieties, and, in the case of decellularization, native architectural presentation of extracellular matrix molecules.

Synthetic scaffold chemistry

In contrast to biologically-derived material systems, synthetic materials enable precisely customized architecture (porosity and topography), mechanical and chemical properties, and degradation modality and kinetics. Synthetic materials that have been explored for liver tissue engineering include poly(L-lactic acid) (PLLA), poly(D,L-lactide-co-glycolide) (PLGA), poly (ε-caprolactone) (PCL), and poly(ethylene glycol) (PEG) [15,71,203–207]. Polyesters like PLLA and PLGA are the most common synthetic polymers utilized in the generation of porous tissue-engineering constructs. These materials are biocompatible, biodegradable, and have been used as scaffolds for hepatocyte transplantation [203,208]. A key advantage of PLGA is the potential to finely tune its degradation time due to differences in susceptibility to

TABLE 46.5 Scaffolds utilized for hepatocellular constructs

Category	Composition
Natural	Collagen, chitosan, collagen/chitosan composites, alginate, alginate composites, liver-derived biomatrix, peptides, hyaluronic acid, fibrin, gelatin, agarose
Synthetic	PLLA, PLGA, PLLA/PLGA composite, PGA, PEG, PCL, PET, PVA

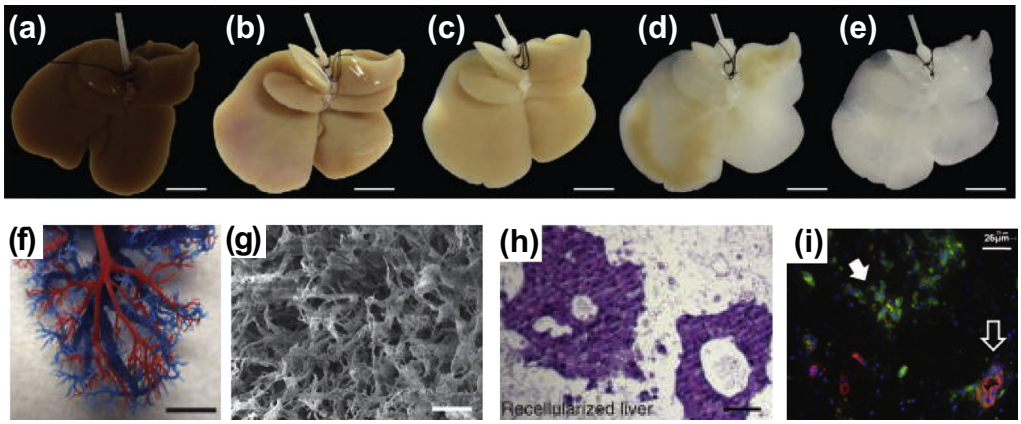

FIGURE 46.9

Decellularized liver scaffolds. Representative photographs of ischemic rat livers during decellularization at (a) 0 h, (b) 18 h, (c) 48 h, (d) 52 h and (e) 72 h. (f) Corrosion cast model of a decellularized liver with portal (red) and venous (blue) vasculature demonstrates that vasculature is intact. (g) SEM images of extracellular matrix within the parenchyma, with hepatocyte-size free spaces. (h) Decellularized livers can be re-cellularized to a total cell mass of 20—40% of native liver mass. (i) Recellularization with human fetal liver cells can result in self-organization of hepatocytes (green) and biliary cells (red). *(Reproduced with permission from [197,202].)*

hydrolysis of the ester groups of its monomeric components (lactic acid and glycolic acid). However, the accumulation of hydrolytic degradation products has been shown to produce an acidic environment within the scaffold which initiates peptide degradation and stimulates inflammation, which may affect hepatocyte function [209]. Consequently, as alternatives to macroporous scaffold systems, approaches aimed at the efficient and homogeneous encapsulation of hepatocytes within a fully 3D structure have been explored. In particular, hydrogels that exhibit high water content and thus similar mechanical properties to tissues are widely utilized for various tissue-engineering applications including hepatocellular platforms. Synthetic, PEG-based hydrogels have been increasingly utilized in liver tissue-engineering applications due to their high water content, hydrophilicity, resistance to protein adsorption, biocompatibility, ease of chemical modification, and the ability to be polymerized in the presence of cells, thereby enabling the fabrication of 3D networks with uniform cellular distribution [210]. PEG-based hydrogels have been used for the encapsulation of diverse cell types, including immortalized and primary hepatocytes and hepatoblastoma cell lines [15,206,207]. The encapsulation of primary hepatocytes requires distinct material modifications (e.g., 10% w/v polyethylene glycol (PEG) hydrogel, inclusion of RGD adhesive motifs) as detailed below, as well as, analogous to 2D co-culture systems, the inclusion of non-parenchymal supporting cell types such as fibroblasts and endothelial cells [207].

Modifications in scaffold chemistry

The relatively inert nature of synthetic scaffolds allows for the controlled incorporation of chemical/polymer moieties or biologically active factors to regulate different aspects of cellular function. Chemical modifications like oxygen plasma treatment or alkali hydrolysis of PLGA [211,212], or the incorporation of polymers like poly(vinyl alcohol) (PVA) or poly(N-p-vinylbenzyl-4-O-β-D-galactopyranosyl-D-glucoamide) (PVLA) into poly(lactic-co-glycolic acid) (PLGA) or poly-l-lactide acid (PLLA) scaffolds [203,213,214] have improved hepatocyte adhesion by modulating the hydrophilicity of the scaffold surface [215]. Biological factors may include whole biomolecules or short bioactive peptides. Whole biomolecules are typically incorporated by non-specific adsorption of extracellular matrix molecules like collagen, laminin or fibronectin [211,216] and covalent conjugation of sugar molecules like heparin [217,218], galactose [71,219], lactose [217] or fructose [220], or growth factors like EGF [221]. Alternatively, short bioadhesive peptides that interact with cell surface integrin receptors have been extensively utilized to promote hepatocyte attachment in synthetic scaffolds.

For example, conjugation of the RGD peptide to PLLA has been shown to enhance hepatocyte attachment [222], whereas RGD modification significantly improved the stability of long-term hepatocyte function in PEG hydrogels [15,207]. The additional incorporation of adhesive peptides that bind other integrins may serve as a way to further modulate and enhance hepatocyte function within synthetic polymer substrates. Moreover, although not yet applied to hepatocellular systems, the integration of matrix metalloproteinase-sensitive peptide sequences into hydrogel networks as degradable linkages has been shown to enable cell-mediated remodeling of the gel [223–225]. The capacity to modify biomaterial scaffold chemistry through the introduction of biologically active factors will likely enable the finely tuned regulation of cell function and interactions with host tissues important for implantable systems.

Porosity

A common feature of many implantable tissue-engineering approaches is the use of porous scaffolds that provide mechanical support often in conjunction with cues for growth and morphogenesis. Collagen sponges, various alginate and chitosan composites, and PLGA are the most commonly used porous scaffolds for hepatocyte culture, and are generally synthesized using freeze-dry or gas-foaming techniques. Pore size has been found to regulate cell spreading and cell-cell interactions, both of which can influence hepatocyte functions [192], and may also influence angiogenesis and tissue ingrowth [226]. Porous, acellular scaffolds are normally seeded using gravity or centrifugal forces, capillary action, convective flow, or through cellular recruitment with chemokines, but hepatocyte seeding is generally heterogeneous in these scaffolds [227,228].

Controlling 3D architecture and cellular organization

Another approach to improving the functionality of tissue-engineered constructs is to more closely mimic *in vivo* microarchitecture by generating scaffolds with a highly defined material and cellular architecture, which would provide better control over the 3D environment at the microscale.

A range of rapid prototyping and patterning strategies have been developed for polymers using multiple modes of assembly including fabrication using heat, light, adhesives, or molding, and these techniques have been extensively reviewed elsewhere [229]. For example, 3D printing with adhesives combined with particulate leaching has been utilized to generate porous PLGA scaffolds for hepatocyte attachment [230], and microstructured ceramic [231] and silicon scaffolds [232,233] have been proposed as platforms for hepatocyte culture. Furthermore, molding and microsyringe deposition have been demonstrated to be robust methods for fabricating specified 3D PLGA structures towards the integration into implantable systems [234].

Microfabrication techniques have similarly been employed for the generation of patterned cellular hydrogel constructs. For instance, microfluidic molding has been used to form biological gels containing cells into various patterns [235]. In addition, syringe deposition in conjunction with micropositioning was recently illustrated as a means to generate patterned gelatin hydrogels containing hepatocytes [236]. Patterning of synthetic hydrogel systems has also recently been explored. Specifically, the photopolymerization property of PEG hydrogels enables the adaptation of photolithographic techniques to generate patterned hydrogel networks. In this process, patterned masks printed on transparencies act to localize the UV (ultraviolet) exposure of the pre-polymer solution, and thus, dictate the structure of the resultant hydrogel. The major advantages of photolithography-based techniques for patterning of hydrogel structures are its simplicity and flexibility. Photopatterning has been employed to surface pattern biological factors [237], produce hydrogel structures with a range of sizes and shapes [238,239], as well as build multilayer cellular networks [240,241].

Consequently, hydrogel photopatterning technology is ideally suited for the regulation of scaffold architecture at the multiple length scales required for implantable hepatocellular constructs. As a demonstration of these capabilities, photopatterning of PEG hydrogels was utilized to generate hepatocyte/fibroblast co-culture hydrogels with a defined 3D branched network, resulting in improved hepatocyte viability and functions under perfusion [206]. More recently, a 'bottom-up' approach for fabricating multicellular tissue constructs utilizing DNA-templated assembly of 3D cell-laden hydrogel microtissues demonstrates robust patterning of cellular hydrogel constructs containing numerous cell types [242]. Also, the additional combination of photopatterning with dielectrophoresis-mediated cell patterning enabled the construction of hepatocellular hydrogel structures organized at at the cellular scale (Fig. 46.8). Overall, the ability to dictate scaffold architecture coupled with other advances in scaffold material properties, chemistries, and the incorporation of bioactive elements will serve as the foundation for the future development of improved tissue-engineered liver constructs that can be customized spatially, physically, and chemically.

Host interactions

Further challenges in the design of therapeutic implantable liver devices are more specifically associated with the interactions with the host environment. These include issues related to vascularization, remodeling, the biliary system, and immunologic considerations. Notably, a significant challenge for the design of implantable liver constructs is the need to overcome transport limitations within the grafted construct due to the lack of functional vasculature. Within the normal liver environment, hepatocytes are supplied by an extensive sinusoidal vasculature with minimal extracellular matrix and a lining of fenestrated (sinusoidal) endothelial cells. Together these features allow for the efficient transport of nutrients to the highly metabolic hepatocytes. Strategies to incorporate vasculature into engineered constructs include the microfabrication of vascular units with accompanying surgical anastomosis during implantation [232,243]. For example, polymer molding using microetched silicon has been shown to generate extensive channel networks with capillary dimensions [244]. The incorporation of angiogenic factors within the implanted scaffolds has also been explored. Specifically, integration of cytokines important in angiogenesis, such as VEGF [208], basic fibroblast growth factor (bFGF) [245], and Vascular endothelial growth factor (VEGF) in combination with Platelet-derived growth factor (PDGF) [246], has been shown to promote the recruitment of host vasculature to implanted constructs. Furthermore, preimplantation of VEGF releasing alginate scaffolds prior to hepatocyte seeding was demonstrated to enhance capillary density and improve engraftment [247].

In addition to interactions with the vasculature, integration with other aspects of host tissue will constitute important future design parameters. For instance, incorporation of hydrolytic or protease-sensitive domains into hepatocellular hydrogel constructs could enable the degradation of these systems following implantation. Of note, liver regeneration proceeds in conjunction with a distinctive array of remodeling processes such as protease expression and extracellular matrix deposition. Interfacing with these features could provide a mechanism for the efficient integration of implantable constructs. Similarly to whole liver or cell transplantation, the host immune response following the transplant of tissue-engineered constructs is also a major consideration. Immunosuppresive treatments will likely play an important role in initial therapies, although stem cell-based approaches hold the promise of implantable systems with autologous cells. Furthermore, harnessing the liver's unique ability to induce antigen-specific tolerance [248] could potentially represent another means for improving the acceptance of engineered grafts. Finally, incorporation of excretory functions associated with the biliary system will ultimately be required in future designs. Towards this end, current studies are focused on the development of *in vitro* models which exhibit biliary morphogenesis and recapitulate appropriate polarization and bile canaliculi organization [249–251], as well as platforms for the engineering of artificial bile duct structures [252].

CONCLUSION

Although many challenges remain for the improvement of tissue-engineered liver therapies, substantial progress has been made towards achieving a thorough understanding of the necessary components. The parallel development of highly functional *in vitro* systems as well as extracorporeal and implantable therapeutic devices is based on contributions from diverse disciplines including regenerative medicine, developmental biology, transplant medicine, and bioengineering. In particular, novel technologies such as hydrogel chemistries, high-throughput platforms, and microfabrication techniques represent enabling tools for investigating the critical role of the microenvironment in liver function and, subsequently, the development of structurally complex and clinically effective engineered liver systems.

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PART 12

Hematopoietic System

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CHAPTER 47

Hematopoietic Stem Cells

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INTRODUCTION

Hematopoietic stem cells (HSCs) are characterized by their extensive self-renewal capacity and pluripotency. The probabilities of asymmetric versus symmetric division of HSCs can be stochastically determined or influenced by external signals. There are many *in vitro* systems involving either bone marrow (BM) stromal support or provision of a combination of recombinant hematopoietic growth factors, or both, that can maintain HSC proliferation and differentiation over many weeks. However, the goal of significant expansion of the HSC population *in vitro* has proved more elusive. There has been an explosive increase in knowledge of the cellular and molecular bases of HSC regulation with the identification of pathways implicating Notch, Wnt, and Hedgehog as well as cytokine signaling through the c-Kit, Flt3, IL-6-R, Mpl and Tie-2 receptors and downstream pathways involving Jak/STAT and homeobox proteins. There is considerable redundancy in pathways regulating HSCs, and both additive and synergistic interactions between different pathways determine the balance between self-renewal and differentiation. With the identification of specific niches within the BM including endosteal and endothelial, it is now recognized that intimate interactions between HSC and regulatory components of the marrow microenvironment (osteoblasts, osteoclasts, granulocytes, macrophages, mesenchymeal cells, endothelium) determine HSC proliferative status, pool size, differentiation and mobilization. The migration of HSCs between different niches and the vascular compartment is regulated by the chemotactic action of the stromally-derived chemokine SDF-1(CXCL12) acting through its receptor CXCR4, in combination with CD44 and hyaluronic acid. The release of various proteases within the marrow environment leads to cleavage of stromal and HSC-associated adhesion molecules, receptors, cytokines and chemokines, providing a further level of regulation.

HISTORICAL BACKGROUND

For more than a century, the nature and developmental potential of stem cells within the hematopoietic system has been debated. The concept of a self-renewing, pluripotent hematopoietic stem cell achieved experimental validation through the pioneering work of Till and McCulloch in the early 1960s, using the spleen colony-forming assay (CFU-s) in irradiated mice (reviewed in reference [1]). These authors undertook secondary passage of individually excised spleen colonies and observed a high variability in numbers of secondary colonies generated which fitted a skewed (gamma) distribution. The probability that a single CFU-s upon division would generate a new CFU-s (self-renewal) was calculated as 0.6 while production of a differentiated progenitor cell was 0.4. The conclusion was that this process was random or stochastic. Other studies at this time indicated the existence of 'hematopoietic inductive

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microenvironments' that influenced the differentiation of HSCs. The relevance of stochastic processes versus external inductive signals in the regulation of HSC self-renewal and differentiation is still debated.

PROPERTIES OF HEMATOPOIETIC STEM CELLS (HSCs)

Asymmetric division

Recent genetic studies have provided important insight into the relation between the cell cycle activity and stemness of HSCs (reviewed in reference [2]). In HSCs, the cell cycle is tightly regulated by cyclins, cyclin-dependent kinases (CDKs), the retinoblastoma protein family, the transcription factor E2F and CDK inhibitors. Most, but not all, of these regulators are necessary for the maintenance of HSCs, with abnormal activation or suppression of the cell cycle resulting in HSC exhaustion [2,3]. HSC proliferation is also regulated by the ubiquitin proteasome pathway and by external factors such as cytokines, chemokines and morphogens produced by niche cells. The ability to isolate purified HSCs has led to the detailed analysis of their transcriptional and epigenetic status (reviewed in reference [4]). The undifferentiated state of an HSC is characterized by dynamic maintenance of chromatin structures and epigenetic plasticity, and the epigenetic chromatin states uniquely define the developmental plasticity of murine HSCs (reviewed in references [5,6]). HSCs can divide either symmetrically or asymmetrically depending on environmental, developmental or stochastic factors (Fig. 47.1). Using a Notch reporter mouse, in which green fluorescent protein (GFP) acts as a sensor for differentiation, imaging studies show that they undergo both symmetric and asymmetric division [7]. The balance between these divisions is not hardwired, but responsive to extrinsic and intrinsic signals. Murine HSCs in a pro-differentiation environment preferentially divide asymmetrically, whereas those in a pro-renewal environment primarily divide symmetrically [7]. The balance between the probability of symmetric versus asymmetric changes between fetal, neonatal, early postnatal (three weeks mouse, ~three months human), and adult hematopoiesis is biased toward symmetric HSC divisions allowing for expansion of the HSC population to match the expansion of the total lympho/hematopoietic system [8–10]. Asymmetric division can be governed by intrinsic partitioning of regulators of cell fate or by asymmetric exposure to extrinsic cues (reviewed in reference [11]). Symmetric divisions occur during hematopoietic expansion during development and in hematopoietic regeneration following myelodepletion or HSC transplantation. In the latter situations, there

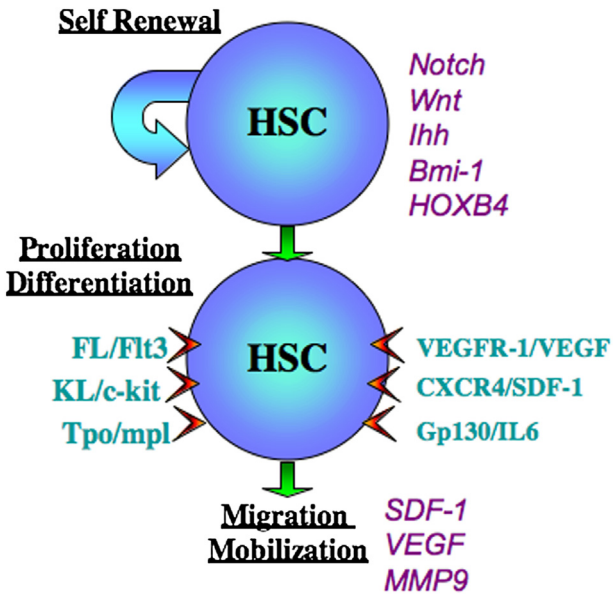

FIGURE 47.1
Model of HSC self-renewal and differentiation illustrating the role of key cytokines — Flt3 ligand (FL), c-Kit ligand (KL, also termed stem cell factor or Steel factor), thrombopoietin (Tpo) and Interleukin-6 (IL-6). The chemokine receptor CXCR4 and its ligand SDF-1 and the VEGF Receptor-1 (VEGFR-1) and its ligands are implicated in HSC migration and mobilization. The Notch, Wnt and Indian Hedgehog (Ihh) signaling pathways have also been implicated in HSC self-renewal as have the Polycomb group transcriptional repressor Bmi-1 and the HOX homeodomain protein HOXB4.

is well-documented data showing HSC expansion in murine models. For example after successive serial transfers of HSCs in irradiated mice, a cumulative 8,400-fold increase in HSC number was observed (reviewed in reference [12]). Extensive expansion of human HSCs was also observed following prolonged *in vitro* culture of hematopoietic precursors from umbilical cord blood [13]. There is less evidence of symmetrical HSC division either *in vitro* or *in vivo* in adult humans [14]. Beckmann et al. [15], identified four proteins (CD53, CD62L/L-selectin, CD63/lamp-3, and CD71/transferrin receptor) that segregate differentially in about 20% of human HSCs that divide in stroma-free cultures, indicating that HSCs/HPCs have the intrinsic capability to divide asymmetrically. Three of these proteins, the transferrin receptor and the tetraspanins CD53 and CD63, are endosomally-associated proteins, suggesting a link between the endosomal compartment and the process of HSC asymmetric cell division.

An intact p53 positively regulates HSC self-renewal independently from apoptosis, preventing gammaH2AX foci accumulation and ensuring optimal HSC self-renewal [16–18]. Constitutive DNA methylation is an essential epigenetic mechanism for protecting HSCs from premature activation of predominant differentiation programs, and methylation dynamics may determine HSC self-renewal [19]. Alternative functional programs of HSCs are governed by gradual differences in DNA methylation levels [20]. HSCs from mice with reduced DNA methyltransferase 1 activity cannot suppress key myeloerythroid regulators and thus can differentiate into myeloerythroid, but not lymphoid, progeny [20]. HSCs were thought to segregate chromosomes asymmetrically during self-renewing divisions so that older ('immortal') DNA strands are retained in daughter HSC whereas newly synthesized strands segregate to differentiating cells. Using definitive markers, Kiel et al. [21] demonstrated that HSCs do not asymmetrically segregate chromosomes or retain bromo-2-deoxyuridine (BrdU) and do not retain older DNA strands during division.

A promyelocytic leukemia (PML)-peroxisome proliferator-activated receptor δ (PPAR-δ) —fatty-acid oxidation (FAO) pathway has been identified which controls the asymmetric division and maintenance of HSCs [22]. PML exerts an essential role through regulation of PPAR signaling and FAO. Loss of PPAR-δ or inhibition of mitochondrial FAO induced the loss of HSC maintenance, whereas treatment with PPAR-δ agonists improved HSC maintenance [22]. The endocytic protein AP2A2, an agonist of mouse HSC activity, segregates asymmetrically during mitosis [23]. *In vivo* RNA interference has been used in mouse HSCs to inhibit a panel of genes with a role in HSC polarity and asymmetric cell division. Four important determinants of HSC behavior were identified, three being upregulated- Musashi2 (*Msi2)*, polarity-specifying PAR protein (*Pard6a)* and atypical protein kinase C homolog (*Prkcz*) and one downregulated (*Prox1*, an atypical homeodomain-containing transcription factor) [22]. A number of recent studies have convincingly shown a role for Msi2 independent of Msi1 in normal [24,338] and malignant [24,339] hematopoiesis. It was shown, using gain-of-function and loss-of-function strategies in mouse and human models, that Msi2 positively regulates HSC proliferation and, when overexpressed, impairs differentiation into mature blood cells [24]. Msi2 and the closely related Msi1 bind consensus motifs in mRNAs to inhibit their transcription. Msi2 has a role in determining binary cell fates during development by inhibiting Notch and Hedgehog signaling (reviewed in reference [25]). Known targets include developmental transcriptional factors, cell cycle regulators and Numb, a membrane-bound protein that has been shown to be a negative regulator of Notch and Hedgehog signaling via ITCH E3-mediated ubiquitination (Ub) and subsequent proteasome-mediated degradation of Notch intracellular domain (NICD) and the zinc finger transcription factor Gli that mediates Hedgehog signaling [22,25,338]. ITCH belongs to the HECT (homologous to E6AP C terminus) family of E3 ligases. It is proposed that it has more than 20 cellular targets including Notch (reviewed in reference [26]). In $Itch^{-/-}$ mice, the self-renewal capacity and absolute number of HSCs are increased without an increase in mature cell numbers. Under the physiological stress of myeloablation, $Itch^{-/-}$ cells show enhanced hematopoietic recovery. Knockdown of Notch, a known target of Itch, in

Itch-deficient progenitors has demonstrated a partial rescue, suggesting that the Itch$^{-/-}$ phenotype may in part result from increased stability of active intracellular Notch (ICN) [26]. It therefore appears that Itch, similar to c-Cbl, functions as a negative regulator of HSC self-renewal and proliferation. The HoxA9 binding element in the Msi2 promoter induces Msi2 reporter activity in HSCs and HoxA9$^{-/-}$ HSCs exhibited a substantial loss of proliferative capacity leading to diminished repopulation ability, very similar to what was found with Msi2$^{-/-}$ animals (reviewed in reference [25]). In this Hox—Msi2—Notch pathway, HoxA9 activates the expression of Msi2, which in turn inhibits the translation of Numb mRNA, resulting in activation of the Notch pathway. As NICD does not possess any DNA binding activity, it associates with recombination signal binding protein J (RBPJ), the primary transcriptional mediator of canonical Notch signaling (reviewed in reference [25]). By inhibiting the translation of Numb mRNA, Msi2 acts as a master switch that is 'on' in normal HSCs and leukemic stem cells (LSCs) [22,24,25,338]. After HSC asymmetric division, one daughter cell remains an HSC whereas the other becomes a non-self-renewing progenitor, with MSI2 downregulated and Numb upregulated (reviewed in reference [25]).

HSC transcriptome

The ability to isolate purified HSCs has led to the detailed analysis of their transcriptional and epigenetic status (reviewed in reference [4]). This detailed cellular picture of murine hematopoiesis combined with robust genetic approaches is beginning to unlock the molecular and biochemical pathways that underlie HSC function (reviewed in references [27,221]). Accumulating evidence indicates that the undifferentiated state of HSCs is characterized by dynamic maintenance of chromatin structures and epigenetic plasticity (reviewed in references [5,6]). Recent genetic studies in mice have provided insights into the regulation of the cell cycle in stem cells, including its potential modulation by the stem cell niche (reviewed in reference [27]). Bioinformatic analysis revealed HSCs were more transcriptionally active than their progeny, and identified a 'lineage fingerprint' of ~100—400 genes uniquely expressed in HSCs, with Wnt signaling being particularly enhanced [28]. Deneault et al. [29] developed a novel gain-of-function screen to identify 18 nuclear factors that were novel effectors of HSC activity. Overexpression of ten of these factors resulted in an increased repopulating activity compared to unmanipulated cells. Interestingly, at least four of the 18 factors, Fos, Tcfec, Hmgb1, and Sfpi1, show non-cell-autonomous functions. Novershtern et al. [30] reported a comprehensive transcriptome analysis of 38 distinct purified populations of human hematopoietic cells, combined with sophisticated bioinformatics analysis and high-throughput DNA binding data for multiple transcription factors. Large numbers of transcription factors were differentially expressed across hematopoietic states suggesting a more complex regulatory system for hematopoiesis than had been previously assumed. A genome-wide comparison of CpG methylation in human HSCs revealed a genome-wide undermethylation dip around the transcription start site and a hierarchical epigenetic plasticity [31]. Inhibition of chromatin condensation by chemical treatment (5-azacytidine, trichostatin A) enhanced the self-renewal of 'stimulated' HSCs in reconstituting BMs but not 'steady state' HSCs in stationary phase BM [31].

The gene expression profiles of steady state BM HSCs has been compared to HSCs treated with mobilizing drugs that expand the HSC pool and induce egress from the marrow, and to non-self-renewing multipotent progenitors [32]. The transcription factor Egr1, a regulator of HSC migration and location, was highly downregulated in mobilized HSCs. Likewise, the adhesion molecule Esam1, which was identified as a highly selective HSC marker [32]. Cell cycle and G_0 analysis indicates that the largest category of transcripts selective for normal HSC fall in the 'unknown' categories in cellular location and molecular function. Meta-analysis of differentially expressed gene sets defined a unique expression profile highly selective for quiescent HSC [32]. These 'HSC signature genes' likely include active regulators of controlled self-renewing divisions and interactions with the microenvironment which function to actively suppress differentiation, proliferation, and transformation.

HSC-associated genes

Aldehyde dehydrogenase (ALDH) was reported to be a key regulator of HSC differentiation [33]. Inhibition of ALDH with diethylaminobenzaldehyde (DEAB) delayed the differentiation of human HSCs that otherwise occurred in response to cytokines. Moreover, short-term culture with DEAB caused a 3.4-fold expansion in HSC [33]. The effects of DEAB on HSC differentiation could be reversed by the co-administration of the retinoic acid receptor agonist, all-trans-retinoic acid, suggesting that the ability of ALDH to generate retinoic acids is important in determining HSC fate. High levels of the aldehyde dehydrogenase isoform ALDH1A1 are expressed in HSCs, but loss of ALDH1A1 does not affect them [34]. ALDH1A1 deficiency is associated with increased expression of the ALDH3A1 isoform, suggesting its potential to compensate for ALDH1A1. Mice deficient in both ALDH3A1 and ALDH1A1 have reduced numbers of HSCs as well as aberrant cell cycle distribution, increased reactive oxygen species levels, p38 mitogen-activated protein kinase activity and sensitivity to DNA damage [34]. These findings demonstrate that ALDH3A1 can compensate for ALDH1A1 and both genes ALDH1A1 and 3A1 are important in HSC metabolism of reactive oxygen species and reactive aldehydes.

The matricellular protein Nephroblastoma Overexpressed (Nov, CCN3) is essential for HSC functional integrity [35]. Forced expression of Nov or addition of recombinant Nov protein both enhance HSC and/or progenitor activity. Scl and Lyl1 encode two related basic-helix-loop-helix transcription factors, and single and double knockout studies in mice showed that expression of at least one of these factors is essential for maintenance of adult HSC function [36]. Lineage-specific transcription factors must be precisely regulated during HSC self-renewal and lineage commitment decisions. During embryogenesis, both Runx1/AML1 and Evi-1 are essential for HSCs, whereas in the adult Runx1 and Evi-1 regulate HSCs negatively and positively, respectively (reviewed in reference [37]). The tumor suppressor and mediator of cell contact inhibition Nf2/merlin is critical for maintaining the normal structure and function of the HSC niche [38]. HSCs in Nf2-deficient mice were increased in number and demonstrated a marked shift in location to the circulation, and this was associated with an increase in vascular endothelial growth factor (VEGF) and in both bone and vascular components [38]. Nf2 limits bone and vascular niche components, thereby constraining HSC number and position. Disruption of the c-myb proto-oncogene specifically in adult bone marrow resulted in a critical depletion of the HSC pool with impaired hematopoiesis and profound reductions of neutrophilic, monocytic, B lymphoid, erythroid, and megakaryocytic cells [39]. This transcription factor is an essential regulator of self-renewal and multilineage differentiation of adult long-term and short-term HSCs and multi-potential progenitors. The coexpression of lymphoid and myeloid genes is an early event detected in multipotent progenitors, but also in ~30% of short-term HSCs [40].

The proliferating cell nuclear antigen-associated factor (Paf) is highly expressed in cycling BM HSCs and plays a critical role as an essential regulator of early hematopoiesis [41]. Mice lacking Paf exhibited reduced BM cellularity and leukopenia with reduced numbers of HSCs and committed progenitors. These phenotypes are caused by a cell-intrinsic blockage in the development of long-term (LT)-HSCs into multipotent progenitors and preferential loss of lymphoid progenitors caused by markedly increased p53-mediated apoptosis [41]. In addition, LT-HSCs from Paf(−/−) mice had increased levels of reactive oxygen species (ROS), failed to maintain quiescence, and were unable to support long-term hematopoiesis.

Focal adhesion kinase (Fak) is a non-receptor protein tyrosine kinase that plays an essential role in many cell types, where its activation controls adhesion, motility, and survival. Fak expression is relatively increased in HSCs compared to progenitors and mature blood cells [42]. Widespread Fak inactivation in the hematopoietic system induces an increase in the numbers of activated HSCs, probably due to alterations in the reciprocal interactions between HSCs and their microenvironment [42]. The endoplasmic reticulum chaperone protein GRP94 has been reported to be essential for the expression of specific integrins, and is required for maintaining HSC interactions with the adult BM niche [43]. There was an increase in HSCs

and granulocyte-monocyte progenitors in Grp94$^{-/-}$ BM associated with loss of HSC quiescence and an increase in proliferation. This expansion of the HSC pool can be attributed to the impaired interaction of HSCs with their niche, as indicated by severely compromised HSC homing and lodging ability, and enhanced mobilization [43].

Endoglin is a transforming growth factor-beta (TGF-beta) accessory receptor recently identified as being highly expressed in long-term repopulating HSCs [44]. However, little is known regarding its function in these cells. Transplantation of BM enriched in HSCs and progenitor cells revealed that neither endoglin suppression nor endoglin overexpression affected the ability of stem cells to repopulate recipient marrow in either the short or long term [44]. Decreasing endoglin expression increased the clonogenic capacity of early blast-forming unit-erythroid progenitors, whereas overexpression compromised erythroid differentiation at the basophilic erythroblast phase, suggesting a pivotal role for endoglin at key stages of adult erythropoietic development [44].

It has recently been demonstrated that protein regulation via the ubiquitin proteasome system (UPS) is crucial for normal HSC function (reviewed in reference [26]). Casitas B cell lymphoma (c-Cbl), Itch and Fbw-7 have been termed the 'gatekeepers of quiescence' of the HSC population [26]. The proto-oncogene, c-Cbl, is a RING finger E3 ubiquitin ligase that is the cellular homolog of v-Cbl, a retroviral transforming gene c-Cbl is thought to regulate approximately 150 proteins either directly or indirectly and is a negative regulator of the activity of Notch1, c-Kit and signal transducer and activator of transcription (STAT)5 (an activator of c-myc expression) all of which contribute to HSC maintenance [26]. Cbl knockout mice exhibit a cell autonomous increase in the HSCs without an increase in mature cell output. Cbl$^{-/-}$ HSCs have increased concentrations of phospho-STAT5 and Myc mRNA suggesting Cbl deficiency stabilizes active STAT5. The E3 ligase activity of c-Cbl keeps the proliferative capacity of HSCs in check with loss of activity leading to enhanced self-renewal and aberrant proliferation in a normally quiescent adult HSC compartment [26]. Itch belongs to the HECT (homologous to E6AP C terminus) family of E3 ligases and, similar to c-Cbl, functions as a negative regulator of HSC self-renewal and proliferation via Notch, a known regulator of hematopoiesis [26]. Fbw-7 is a RING finger E3 ligase that plays a role in regulation of approximately 20 proteins including c-myc and Notch. Germline deletion of Fbw7 results in embryonic lethality around E10.5 due to vascular defects, which are attributed to the stabilization of Notch4 in the embryo. Conditional deletion of Fbw7 leads to an increase in the frequency of actively cycling HSCs with their eventual loss. Fbw7$^{-/-}$ HSC defects are cell autonomous and involve downregulation of genes involved in HSC quiescence, with global loss of quiescent characteristics [26].

USP1 is a cysteine protease DUB that deubiquitinates FANCD2 (Fanconi anemia group D2) which is a regulator of the Faconi anemia (FA) pathway. It is able to protect HSCs from DNA damage during the divisions that are necessary to maintain the stem cell pool (reviewed in reference [26]). RelB and nuclear factor κB (NF-κB2), main effectors of NF-κB non-canonical signaling, positively and intrinsically regulate HSC and progenitor cell self-renewal [45].

HSC assays

Recent developments and validation of phenotypic and functional assays for both murine and human HSCs have been extensively reviewed [4,46–50]. Human HSCs are enriched in Lin-CD34+CD38− cord blood (CB) and BM and express CD90. The Lin-CD34+CD38− fraction of cord blood and bone marrow can be subdivided into three subpopulations; CD90−CD45RA+, CD90−CD45RA+ and CD90+CD45RA−. In CB, the latter subpopulation is highly enriched in HSCs, with 10 purified cells detected in *in vitro* or *in vivo* HSC assays [51]. CB CD34+CD38−CD90+ long-term repopulating HSCs were further enriched by selection for integrin α2 receptor expression [53]. Primitive, highly proliferative potential progenitor (HPP-CFC) culture systems have been used as surrogate HSC assays [52]. More recent assays

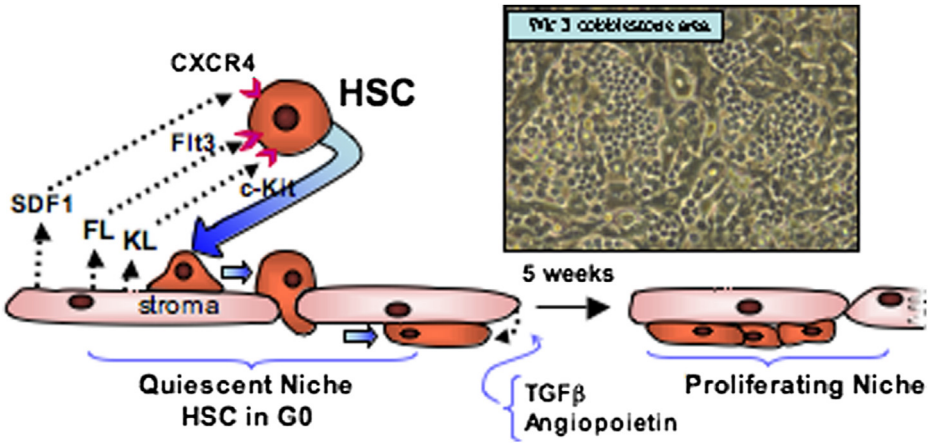

FIGURE 47.2
A model illustrating the concept of the 'late' cobblestone area -forming cell (CAFC)' and its interaction with bone marrow stromal elements. SDF-1 produced by the marrow stroma establishes a gradient that attracts CXCR4+ HSC that adhere via VLA-4-VCAM-1 mediated adhesion and then migrate beneath the stroma. Stromal membrane-associated c-Kit ligand (KL) and Flt3 ligand (FL) provide survival and proliferation signals to the HSC while negative regulatory influences from the stroma (TGFβ, Angiopoietin) override proliferative signals and place the HSC in a G0 state that persists for some weeks. Escape from quiescence may be a stochastic process or driven by an alteration in the balance between proliferation stimulating and inhibiting factors. At this stage the HSC proliferates and differentiates, forming colonies of 5–100 phase-dark cells by week 5. At this stage each cobblestone area contains on average of 1–4 HSC and 5–20 progenitors (CFU-GM, BFU-E).

involve the development of cobblestone areas of phase-dark cells that develop beneath BM stroma following 2–3 weeks (murine) or > 5 weeks (human) co-culture with BM or CD34+ cells [54] (Fig. 47.2). These cobblestone area forming cells (CAFCs) have self-renewal and pluripotent differentiation potential. A modification of this assay involves the quantitation of secondary, progenitor erythroid and myeloid colony formation (CFC) after five weeks (human) or three weeks (mouse) of stromal or cytokine-dependent culture (long-term culture-initiating cell assay – LTC-IC). The *in vivo* assays for murine HSCs involve limiting dilution, competitive (e.g., with addition of a genetically distinct marrow population) repopulation assay in irradiated recipients, with evaluation after 3–6 months. The immunodeficient SCID or NOD/SCID mouse supports human hematopoiesis following intravenous injection of $2 \times 10^4 - 2 \times 10^5$ CD34+ cells, and quantitation of engraftment by measuring human CD45+ cells in the murine femoral BM at five weeks and beyond can be used under limiting dilution conditions to quantify human HSCs (SCID Repopulating Cells – SRCs) (reviewed in reference [55]). Human HSCs with long-term engraftment of NOD/SCID mice are in the CD38−, CD34+ fraction [56]. Mazurier et al. [57] identified a new class of human HSC by direct intrafemoral injection in NOD-SCID mice. This CD34+, $CD38^{low}$, CD36− HSC was termed a 'rapid SCID repopulating cell' (R-SRC), since it rapidly generate high levels of human myeloid and erythroid regeneration within the injected femur and migrates to the circulation and colonizes individual bones within two weeks of transplantation. While the presence of CD34 and the absence of CD38 provide reliable markers for human HSCs, murine HSCs are routinely isolated as c-Kit+, Sca-1+, lineage negative (Lin−) populations with additional features such as Hoechst dye exclusion. CD34+ is expressed on long-term repopulating HSC of the murine fetus and neonate but decreases with age so that HSC of 10-week-old mice are CD34− [58,59]. The observations clearly demonstrate that CD34 expression reflects the activation/kinetic state of HSC, and that expression is reversible. In the murine system, the majority of HSCs are CD38+, and there is a reciprocal relationship between CD34 and CD38 expression [58]. Cell surface receptors of the SLAM family are differentially expressed among functionally distinct HSC and progenitor populations of the mouse and HSC are CD150+, CD244−, CD48−, while multipotent progenitors are CD244+, CD150−, CD48− [60].

HSC numbers and proliferative status

It has been suggested that HSC numbers are conserved in mammals, with cats, mice and rats having comparable numbers (~11–12,000) [19,61]. If extrapolated to humans, the frequency of HSCs would be 0.7–1.5/10^8 marrow cells, a frequency that is 20-fold less than estimated by the NOD/SCID repopulating assay. Granulocyte telomere length measurements have been used to estimate human HSC kinetics, and the results showed that replication is infrequent, on average once every 45 weeks, substantially slower than the average rates estimated for mice (once per 2.5 weeks) or cats (once per 8.3–10 weeks) [62]. Catlin et al. [63] analyzed the changes in this ratio with age of maternal/paternal X-chromosome phenotypes in blood cells from females and concluded that human HSCs replicate on average once every 40 weeks (range 25–50 weeks). They also provide evidence that the human HSC pool increased from birth until adolescence and then plateaued, and that the ratio of contributing to quiescent HSCs in humans significantly differs from that in the mouse. Long-term HSCs (LT-HSCs) can fully reconstitute all the hematopoietic lineages of a lethally irradiated host for the life span of that host, and represent 1/10^4 to 1/10^5 cells in the BM [27,64]. In addition, these cells can be serially transplanted, although, for reasons that are unclear, repopulation capacity is entirely lost after four to five passages through irradiated hosts, even when telomere length is maintained by overexpression of telomerase.

LT-HSCs develop into short-term (ST)-HSCs that are capable of reconstituting all the lineages of the hematopoietic system, but have limited self-renewal capacity, providing 'short-term' reconstituting cells able to sustain clones of differentiating cells for only 4–6 weeks and not able to sustain serial repopulation (reviewed in reference [46]). The Shelterin component Pot1b plays an essential role in HSC survival [65]. Its deletion in mice, in the setting of telomerase haploinsufficiency, results in rapid telomere shortening and increased apoptosis, leading to fatal BM failure due to severe depletion of the HSC reserve [65]. Benzeniste et al. [66] identified a numerically dominant 'intermediate-term' multipotent HSC stage in mice, whose clones persist for 6–8 months before becoming extinct and that are separable from both short-term as well as permanently reconstituting 'long-term' HSCs. The authors proposed that the first step in HSC differentiation consists not in the loss of initial capacity for serial self-renewal divisions, but rather in the loss of mechanisms that stabilize self-renewing behavior throughout successive future HSC divisions.

HSC quiescence

Quiescence of HSCs is critical for sustaining the HSC pool over long periods, and protecting them by minimizing their accumulation of replication-associated mutations (reviewed in reference [67]). The balance between quiescence and proliferation is tightly controlled by both HSC-intrinsic and -extrinsic mechanisms. In recent years, a wide variety of positive and negative regulatory molecules or pathways critical for HSC quiescence regulation have been identified (reviewed in reference [67]). Although HSCs divide infrequently, it is thought that the entire HSC pool turns over every few weeks, suggesting that HSCs regularly enter and exit cell cycle (reviewed in reference [68]). Quiescent mouse HSCs are lin(−) Sca1+ c-Kit+ CD150+ CD48(−) CD34(−) and give rise to an active self-renewing CD34(+) HSC population [68]. Computational modeling suggests that quiescent murine HSCs divide about every 145 days, or five times per lifetime [68]. The quiescent HSC population contains the vast majority of multilineage long-term self-renewal activity that can be rapidly activated to self-renew in response to BM injury or G-CSF stimulation. After re-establishment of homeostasis, activated HSCs return to dormancy, suggesting that HSCs are not stochastically entering the cell cycle but reversibly switch from dormancy to self-renewal under conditions of hematopoietic stress [68]. Quiescent HSCs reside in individual niches that preserve their dormancy via signaling molecules such as thrombopoietin, angiopoietins, and stem cell factor (c-Kit Ligand). A series of intracellular regulatory molecules, including FoxOs, mTORC1, Fbw7, Egr1,

Pbx1, pRb, c-Cbl, Myc, and Bmi1 mediate the processes of HSC dormancy, cycling, self-renewal, differentiation and survival [68].

Deletion of Cited2, a transcriptional modulator involved in fetal liver hematopoiesis, resulted in increased HSC apoptosis, loss of quiescence, and increased cycling, leading to a severely impaired HSC reconstitution capacity [69]. Transcriptional profiling revealed that multiple HSC quiescence- and hypoxia-related genes such as Egr1, p57, and Hes1 were affected in Cited2-deficient HSCs. Because Cited2 is a negative regulator of HIF-1, which is essential for maintaining HSC quiescence, Cited2 and HIF-1α double knockout mice showed partial rescue of impaired HSC quiescence and reconstitution capacity [69]. At the transcriptional level, deletion of HIF-1α restored expression of p57 and Hes1 but not Egr1 to normal levels. Results suggest that Cited2 regulates HSC quiescence through both HIF-1-dependent and HIF-1-independent pathways [69]. Rho GTPase Cdc42 is a critical coordinator of HSC quiescence maintenance [70]. Cdc42$^{(-/-)}$ HSCs enter the active cell cycle, resulting in a significantly increased number and frequency of the stem/progenitor cells in the BM. Cdc42 deficiency also causes impaired adhesion, homing, lodging, and retention of HSCs, leading to massive egress of HSCs from the BM [70]. These effects are intrinsic to the HSCs, and are associated with defective actin organization and deregulated c-Myc, p21(Cip1), N-cadherin and beta1-integrin expression [70]. Vascular niche E-selectin in the vascular niche also regulates HSC self-renewal and chemoresistance [71].

The importance of the p53 protein in the cellular response to DNA damage is well known, but its function during steady state hematopoiesis is less clear. Liu et al. [72] have defined a critical role of p53 in regulating HSC quiescence, especially in promoting the enhanced quiescence seen in HSCs that lack the MEF/ELF4 transcription factor. Transcription profiling of HSCs isolated from wild-type and p53 null mice identified Gfi-1 and Necdin as p53 target genes of importance in regulating HSC quiescence [72]. Necdin, a growth-suppressing protein, functions as a molecular switch in adult hematopoiesis, acting in a p53-like manner to promote HSC quiescence in the steady state [73]. Necdin suppresses p53-dependent apoptosis in response to genotoxic stress via both cell-cycle-dependent and cell-cycle-independent mechanisms, with the latter occurring in a Gas2L3-dependent manner [73]. Necdin (Ndn) plays an important role in restricting excessive HSC proliferation during hematopoietic regeneration and is preferentially expressed in HSC by gene expression profiling [74]. Mice deficient in Necdin had no overt abnormality in steady state hematopoiesis but show accelerated recovery of the hematopoietic system after myelosuppressive injury [74]. These findings suggest that Necdin functions in a negative feedback loop that prevents excessive proliferation of HSCs during hematopoietic regeneration. The Mixed Lineage Leukemia (MLL) gene is a homolog of Drosophila Trithorax which is commonly rearranged in infant leukemia. It is essential for embryonic and adult HSC and progenitor development, with fatal BM failure occurring within three weeks of Mll deletion [75,76]. Mll plays a dual role within the hematopoietic system, maintaining quiescence in HSCs and promoting proliferation in progenitors. Mll-deficient cells fail to self-renew even in an intact BM environment and exhibit ectopic cell cycle entry, resulting in the depletion of quiescent HSCs [75,76]. Committed lymphoid and myeloid cells no longer require Mll, defining the early multipotent stages of hematopoiesis as Mll dependent.

BM regeneration critically depends on the release of soluble factors from cells such as stromal cells, a process regulated by proteases. Two proteolytic systems, the fibrinolytic system and the matrix metalloproteinases (MMPs), have recently been shown to be involved in this process [77]; reviewed in reference [78]. The plasminogen/plasmin system is mostly recognized for its fibrinolytic activity, but it is also involved in processes such as cell invasion, chemotaxis, growth factor activity modulation, and tissue remodeling (reviewed in reference [78]). Plasminogen (Plg), a classical fibrinolytic factor, is a key player in controlling the hematopoietic stress response (e.g., myelosuppression) [77,78]. Deletion of Plg in mice prevented HSCs from

entering the cell cycle and undergoing multilineage differentiation after myelosuppression, leading to the death of the mice. Activation of Plg by administration of tissue-type plasminogen activator promoted matrix metalloproteinase-mediated release of Kit ligand from stromal cells, thereby promoting HSC/HPC proliferation and differentiation (reviewed in reference [78]).

The expression of thioredoxin-interacting protein (TXNIP) is essential for maintaining HSC quiescence and the interaction between HSCs and the BM niche. It is decreased during HSC activation and in Txnip$^{(-/-)}$ mice, the long-term reconstituting HSC population is decreased and progressively exhausted and its capacity to repopulate is rapidly lost [79]. These effects are associated with stress-induced cell cycling, reduced p21 expression and hyperactive Wnt signaling. Txnip deficiency reduced the CXCL12- and osteopontin-mediated interaction between HSCs and the BM, and impaired homing and retention in the osteoblastic niche, resulting in mobilized HSCs [79].

Hypoxia signaling in HSC

Although HSCs are thought to reside in hypoxic niches, the significance of hypoxia signaling in HSCs is still under active investigation. In particular, the question of how chronic local hypoxia influences HSC fate and how the cells adapt to a low oxygen environment needs to be addressed. HSCs are distributed in the BM according to regional hypoxia and are predominantly located at the lowest end of an oxygen gradient, with the implication that regionally defined hypoxia plays a fundamental role in regulating their function [80,81]; reviewed in reference [82]. The slow-cycling/quiescence balance of HSCs is related to the physiological gradient of oxygen [83], and hypoxic *in vitro* conditions maintain the quiescent phenotype of mouse HSCs [81]. Culture of human CD34$^+$ cells at low O_2 concentrations ($O_2 < 3\%$) maintains stem cell engraftment potential better than at 20% O_2 (NOD/Scid xenograft model) [84]. Very low O_2 levels ($< 1\%$) induce CD34$^+$ quiescence but progenitors disappear from cultures. Hypoxia also induced hypophosphorylation of pRb and increased the expression of p27^{KIP1}, the two proteins that have a major role in the control of G_0 and G_1 to S-phase transition [84]. HSCs in the hypoxic niche have low concentrations of ROS, and these increase as proliferating and differentiating HSC move out of the niche. ROS is a DNA damage signal that leads to the activation of p53 (reviewed in reference [82]). MDM-2 is a RING finger E3 ligase that targets p53 for degradation, as well as represses the accumulation of p53 by binding to its N terminus, inhibiting its transcriptional activity (reviewed in reference [26]). Loss of MDM-2 activity leads to stabilized p53, which induces cell cycle arrest, senescence and ultimately cell death in HSCs and progenitors. This is a cell-intrinsic defect that is completely dependent on p53 and is alleviated by treatment with antioxidants, demonstrating the necessity of ROS to induce p53. HSCs express multiple isoforms of NADPH oxidase, and a positive feedback mechanism of NOXs activation enables fine tuning of the ROS levels and redox-mediated signaling involved in growth and differentiation of HSCs [85].

HIF-1a is an essential regulator of HSC quiescence and strict regulation of HIF-1a is pivotal for the long-term maintenance of HSCs [86,87]. Hypoxia regulates the metabolic state of HSCs and protects their integrity by controlling HIF-1a (an alpha subunit of HIF-1). Adult HSCs have elevated levels of HIF-1a and increased expression of hypoxia-inducible genes, including those controlling glycolysis [87]. As a consequence, HSCs adapt to the hypoxic microenvironment within stem cell niches by altering their metabolism. They utilize glycolysis with increased glucose consumption and lactate production instead of mitochondrial oxidative phosphorylation with decreased oxygen consumption compared to mature BM cells [87]. This metabolic balance promotes HSC maintenance by limiting the production of reactive oxygen species, but leaves HSCs susceptible to changes in redox status (reviewed in reference [88]). Delete of HIF-1a in HSC results in loss of quiescence, increased cell cycling rate and progressive loss of long-term repopulation capacity in serial transplantation assays [86]. Interestingly, although HIF-1a is critical for HSC maintenance under conditions of hematopoietic stress,

under physiological conditions HIF-1a-deficient HSCs sustain hematopoiesis for a prolonged period. The homeodomain protein Meis1, which is essential for hematopoiesis and markedly enriched in LT-HSCs, directly activates HIF-1a expression [87]. Meis1 regulates the metabolic phenotype and oxidant defense of HSCs [89]. Loss of Meis1 resulted in down-regulation of both Hif-1alpha and Hif-2alpha in HSCs. This resulted in a shift to mitochondrial metabolism, increased reactive oxygen species production, and apoptosis of HSCs.

The effect of Meis1 knockout on HSCs is entirely mediated through reactive oxygen species since treatment of the *Meis1* knockout mice with the scavenger N-acetylcystein restores HSC quiescence and rescues HSC function [89]. Von Hippel-Lindau (VHL) protein is an E3 ubiquitin ligase that mediates HIF-1a degradation, and its monoallelic deletion stabilized HIF-1a and increased the fraction of quiescent HSCs [86] resulting in their expansion in transplanted recipients. However, ablation of both alleles of VHL and overstabilization of HIF-1a enhanced the quiescence of HSCs, but resulted in loss of HSC activity upon transplantation [86]. Conditional deletion of Cited2, a negative regulator of HIF-1a mediated transcription, results in dramatic loss of HSCs [90]. A direct target gene of HIF-1, Foxo3a, is essential for the quiescent phenotype of HSCs [91]. In addition to genes regulating energy metabolism, HIF-1 controls transcription of more than 100 genes involved in multiple cellular processes including the cell cycle, differentiation, autophagy and survival, suggesting that hypoxia signaling may also be critical for HSC fates other than quiescence (reviewed in reference [92]). A central role for the Lkb1 (Stk11) metabolic sensor has been identified in restricting HSC entry into the cell cycle and in broadly maintaining energy homeostasis in hematopoietic cells through a novel metabolic checkpoint (reviewed in references [93–96]). Upon *Lkb1* ablation there was an immediate loss of HSC quiescence and increased HSC/multipotent progenitor cell proliferation [96]. The overall increase in progenitor numbers was transient and was followed by a progressive depletion of HSCs and eventual pancytopenia [96]. The effect of *Lkb1* deletion is cell autonomous, as mutant HSCs failed to rescue lethally irradiated recipients and were outcompeted by *Lkb1*-replete HSCs in competitive reconstitution assays. The metabolic profiles of $Lkb1^{-/-}$ HSCs displayed elevated fatty acid levels, suggesting LKB1 regulates additional aspects of cellular metabolism [95]. $Lkb^{-/-}$ HSCs displayed decreased AMP-activated protein kinase (AMPK) activity resulting from loss of AMPKα phosphorylation, with increase in phospho-S6 levels and mTORC1 activity. LKB1 regulates HSC quiescence through an AMPK–, mTORC1–, and FoxO–independent mechanism. Transcriptome analysis of $Lkb1^{-/-}$ HSCs revealed enrichment for genes involved in the PPARγ metabolic pathway, with downregulation of PPARγ coactivators *Pgc-1*α and *Pgc-1*β [94]. LKB1-deficient cells exhibited defects in mitosis, characterized by supernumerary centrosomes and aberrant mitotic spindles. These cells became aneuploid and underwent apoptosis in an AMPK-independent manner [96], suggesting that LKB1–mediated regulation of mitosis and genome stability account for HSC loss.

ONTOGENY OF HSCs

Yolk sac (YS) phase

The ontogeny of hematopoiesis has been studied extensively in mice and man and also in the xenopus and zebrafish. A number of recent reviews are available [97–100]. In the mouse, hematopoiesis is initiated in the visceral yolk sac (YS) with generation of a non-self-renewing progenitor with limited multilineage differentiation potential termed the erythromyeloid progenitor (EMP). These progenitors develop before definitive HSCs, and are the source of primitive erythroblasts, megakaryocytes, and macrophages, which emerge on embryonic day (E)7.0 [101,102]. The YS blood islands have long been recognized as the first site for blood cell emergence during embryonic development. Much of our understanding of how blood islands form is derived from studies conducted in chick embryos. Similar processes are thought to be active during murine blood island development. But, the strength of the evidence that the process of blood island formation in the chick coincides with that documented in the

mouse (reviewed in reference [103]). The onset of definitive hematopoiesis at E8.25 in the murine conceptus is marked by high-level CD41 co-expressed with CD31, CD34, and Flk1 in some intraluminal, round, hematopoietic cells that appeared to arise from flattened endothelial cells lining YS capillary vessels [104]. CD41 expression marks the onset of primitive and definitive hematopoiesis in the murine conceptus, and persists as a marker of some HSC and progenitor cell populations in the fetal liver and adult marrow, suggesting novel roles for this integrin. CD41 serves as the earliest marker of primitive erythroid progenitor cells at E7.0 in the YS and high-level expression identifies essentially all E8.25 YS definitive hematopoietic progenitors [104]. Some definitive hematopoietic progenitor cells in the fetal liver and BM also express CD41. HSC competitive repopulating ability is present in CD41(dim) and CD41(lo/−) cells isolated from BM and fetal liver cells, however, activity is enriched in the CD41(lo/−) cells [104]. Pure populations of primary endothelial cell monolayers isolated from E9.5 YS and P-Sp were cocultured with murine BM Sca-1+c-K-it+lin- cells for seven days resulting in a three- to 10-fold expansion of long-term (> 6 months) HSC with competitive repopulating ability [105].

Aorta-Gonad-Mesonephros (AGM) and onset on intra-embryonic hematopoiesis

The first mouse adult-repopulating (definitive) HSCs emerge in the aorta-gonad-mesonephros (AGM) region at E10.5. Their numbers in this region increase thereafter and begin to decline at E12.5, thus pointing to the possible existence of both positive and negative regulation of emerging HSCs (reviewed in reference [100]). At E8.5, expression of Runx1, a pivotal transcription factor required for HSC generation in the vascular regions of the mouse conceptus, is observed in hematopoietic and endothelial cells within the blood islands of the YS (reviewed in reference [100]). A strong lymphoid potential of the pre-circulation embryo proper suggests that definitive HSC precursors segregate *in situ* from the para-aortic-splanchnopleuric (P-Sp) mesoderm that precedes the appearance of the aorta hemogenic endothelium (reviewed in references [106,107]). Slightly later, Runx1 expression is seen in the endothelial cells and underlying mesenchymal cells of the ventral portion of the dorsal aorta and the endothelial cells of the vitelline and umbilical arteries and placenta (reviewed in reference [100]). With further maturation, Runx1 expression appears in the hematopoietic cells that closely adhere to these endothelial structures. Runx1 is also expressed at high levels in the hematopoietic cells that develop within the fetal liver, spleen and bone marrow in later stage embryos and adult animals.

Several lines of evidence suggest that definitive HSCs derive from the transitory subset of endothelial cells, termed hemogenic endothelium, that express VE-cadherin, a specific marker of endothelial lineage, and Runx1 [108]. In the mouse, lineage tracing of endothelium, via an inducible VE-cadherin Cre line, indicated an endothelial origin of HSCs in the AGM region [108]. These HSCs subsequently migrated to the fetal liver, and later, to the BM and were capable of expansion, self-renewal, and multilineage hematopoietic differentiation [108]. AGM mesenchyme, as selectively traced via a myocardin Cre line, was reported to be incapable of hematopoiesis.

It has been thought that HSCs emerge from vascular endothelial cells through the formation of intra-arterial clusters, and that Runx1 functions during the transition from 'hemogenic endothelium' to HSCs (reviewed in reference [97]). Boisset and Robin [98] reviewed their use of time-lapse confocal imaging and a new dissection procedure to visualize the dynamic *de novo* emergence of phenotypically defined HSCs (Sca1+, c-kit+, CD41+) emerging from murine ventral aortic hemogenic endothelial cells at E10.5 and budding directly into the lumen from the aortic wall. Compared to the zebrafish, hematopoietic cell emergence in the mouse aorta was rare, with just 1.7 events per cultured E10.5 embryo over the course of 15 hr (as opposed to three events/hr in zebrafish). Previous quantification of c-kit+ cells in the

dorsal aorta of mouse embryos has identified hundreds of these cells in small and larger clusters (reviewed in reference [97]). In the light of recent demonstrations that blood flow augments Runx1 expression and blood formation [109] (reviewed in reference [97]), the low number of events observed *ex vivo* may be due to the absence of flow in the culture system. Selective deletion of Runx1 in VE-cadherin+ cells resulted in the ablation of all hematopoietic progenitors including HSCs with a complete lack of development of fetal liver hematopoiesis [110]; reviewed in reference [97]. Haploinsufficiency of Runx1 results in a dramatic change in the temporal and spatial distribution of HSCs, leading to their early appearance in the AGM and also prematurely in the E10 YS [110]; reviewed in reference [100]. E10 Runx1+/− YS cells are fully potent HSCs that can be serially transplanted, and can repopulate all hematopoietic tissues and lineages of adult recipients. The appearance of abundant HSC activity in the YS may be explained if this site is capable of autonomous HSC generation, producing more HSCs under non-physiologic Runx1 concentrations. Alternatively the YS could be seeded prematurely by HSCs generated in the AGM region [110]. E9.5 P-Sp/AGM explant cultures fail to produce definitive HSCs [110], an observation that is not consistent with a role for the P-Sp/AGM region as a site for *de novo* HSC origin. The earliest definitive HSCs are also found in other vascular territories at similar frequencies and at the same time as in the AGM region. These observations imply that there exists an early anatomical source of definitive HCS that enter the P-Sp after E9.5. This source might be located in the visceral YS, as was first proposed by Moore in avian [111–114] and murine [113,115] studies (reviewed [116,336]). High levels of persistent adult marrow chimerism were first demonstrated, using sex chromosome markers, in marmosets and freemartin cattle (reviewed in reference [116]). In both species, synchorial twinning and embryonic interchange of blood are of frequent occurrence. In avian studies using fertile double yolk eggs or vascular parabiosis established between chick embryos at of opposite sex at day 6–10 of development, Moore and Owen [111,113,114] undertook a cytogenetic analysis of primary lymphoid organs (thymus, bursa of Fabricius) and the hematopoietic organs (spleen and bone marrow) at intervals up to the time of hatching. This revealed extensive chimerism, with approximately equal proportions of male and female metaphases, indicating that these organs were all colonized by circulating HSC/HPCs that equilibrated between the embryos. The YS was proposed as the unique initial source of the HSCs that colonized the developing lymphohematopoietic organs and persisted through hatching and into adult life [114]. Moore and Metcalf [115] used whole E7–7.5 mouse conceptus cultures to monitor *in vitro* the transition between YS hematopoiesis and onset of intra-embryonic hematopoiesis over a period of 3–4 days. When embryos were cultured after removal of the developing YS, intra-embryonic hematopoiesis was not initiated, and neither CFU-S nor CFC was detected. In contrast, a culture of YS in the absence of the embryo resulted in robust hematopoiesis with CFU-S and CFC production and expansion. Subsequent studies concluded that, in addition to its well-known role in the establishment of 'primitive' hematopoiesis, the visceral YS served as an independent source of some 'definitive' hematopoietic stem/progenitor cells but not adult-type HSCs as defined by ability to repopulate adult, irradiated mice [117,118]. Cell-transplantation studies [119,118] have shown that YS precursor cells contribute to adult hematopoiesis. Yoshimoto et al. [120] showed the existence of T cell-restricted progenitors in the E9.5 YS that directly engraft in recipient immunodeficient mice. T cell progenitors were also produced *in vitro* from both YS and para-aortic splanchnopleura hemogenic endothelial cells, and these T cell progenitors repopulated the thymus and differentiated into mature T cell subsets *in vivo* on transplantation [120].

Cell tracing experiments have reported direct cell-lineage relationship between the YS precursor cells and adult hematopoiesis [121]. YS cells expressing Runx1 at embryonic day 7.5 developed into fetal lymphoid progenitors and adult HSCs. During midgestation (E7.5) the labeled YS cells colonized the umbilical cord, the AGM region and subsequently the embryonic liver. This indicated that at least some HSCs associated with major embryonic vasculature are derived from YS precursors. In more recent studies by this group to address the

issue of ultimate origin of HSCs, Tanaka et al. [122] designed an embryo rescue system in which the key hematopoietic factor Runx1 is reactivated in Runx1-null conceptuses at specific developmental stages. These investigators concluded that specification of the HSC lineage takes place early in ontogenesis, immediately after the start of gastrulation. The demonstration of this VE-cadherin+ definitive hematopoietic precursor in the proximal YS is consistent with a close ontogenic relationship between extra-embryonic hemogenic endothelium and hemangioblasts or nascent lateral mesoderm as a major source of the HSC precursors [122–124]. These findings must be compared with data on avian hematopoietic ontogenesis [125]; reviewed in reference [106]. The histogenesis of the bursa of Fabricius and of bone marrow was studied by a biological cell marking technique based on differences in the nuclear structure of two species of birds; the Japanese quail and chick [126]. By grafting bursal rudiments and limb buds of the quail into the chick, and the reverse, it was possible to demonstrate that the whole hematopoietic population of the bursa of Fabricius and of the BM was derived from blood-borne extrinsic stem cells. It remains to be determined whether mammalian and avian *de novo* hematopoiesis differ significantly, or whether a closer look at the presumed sites of blood cell generation in chickens would reveal a more prominent role for the avian YS [127], as first suggested by Moore and Owen [111,113,114]. An alternative concept, based on analysis of morphologically distinct chick/quail hybrids, relegated the YS to be a transient source of hematopoiesis of no relevance to subsequent *de novo* emergence of HSCs in the AGM region of the embryo [126,128,129]; reviewed in reference [100]. Specifically, HSC appeared to arise by budding from the floor of the dorsal aorta and other major arterial regions of the mouse embryo (reviewed in reference [97]).

It has been proposed that during embryonic development hematopoietic cells arise from a mesodermal progenitor with both endothelial and hematopoietic potential called the hemangioblast [130,131]; reviewed in reference [106]. An alternative theory is that hematopoietic cells originate from a specific type of endothelium designated as hemogenic. Indeed, transient, time-specific, hemogenesis arising at the floor of the aortic endothelium is prominent in embryos of many vertebrate species. Support for the hemangioblast concept was initially provided by the identification, during mouse embryonic stem cell differentiation to the blast colony-forming cell (BL-CFC) stage, of the development of blast colonies with both endothelial and hematopoietic components [130]. A VEGF-R2-positive progenitor, capable of differentiating into either endothelial or hematopoietic cells, depending on the growth factors in the culture medium, can indeed be isolated from the caudal mesoderm of the gastrulating avian embryo [133], or gastrulating mouse embryo [134]. Lancrin et al. [124] demonstrated that the hemangioblast, likely of mesenchymal or mesodermal origin, generates hematopoietic cells through the formation of a hemogenic endothelium intermediate. At the molecular level, the transcription factor Tal1 (also known as Scl) is indispensable for the establishment of this hemogenic endothelium and the upregulation of c-Kit and Tie2, whereas the core binding factor Runx1 (also known as AML1) is critical for generation of definitive hematopoietic cells from hemogenic endothelium characterized by CD41 expression and Tie2 downregulation [124]. It would appear that development of HSCs from an aortic hemangioblast (although this cell so far has proven elusive) must occur in a linear fashion that involves an endothelial intermediate, similar to what was recently reported for embryonic stem (ES) cell-derived blood cells [124,133]. Three-dimensional whole embryo immunostaining and live-imaging techniques have allowed quantitation and cartographic mapping of intravascular hematopoietic cell clusters developing in association with the midgestation mouse aorta, umbilical and vitelline arteries [135]. The data indicate that definitive HSCs and early progenitors (visualized as clusters) are generated from hemogenic endothelium. By continuous, long-term, single-cell observation of mouse mesodermal cells generating endothelial cell and blood colonies, it was possible to detect hemogenic endothelial cells giving rise to blood cells [133]. Living endothelial and hematopoietic cells were identified by simultaneous detection of morphology and multiple molecular and functional markers. Detachment of nascent blood cells from endothelium was not directly linked to asymmetric cell division, and hemogenic

endothelial cells were specified from cells already expressing endothelial markers [133]. Although some authors have presented evidence that HSCs may arise directly from the aortic floor into the dorsal aortic lumen, others support the notion that HSCs first emerge within the underlying mesenchyme (reviewed in reference [101]).

Zebrafish have been used to determine the function of a gene during embryonic-to-adult transition of hematopoiesis, since bloodless zebrafish embryos can develop normally into the early larval stage by obtaining oxygen through diffusion (reviewed in references [136,137]). Runx1 truncation mutant zebrafish embryos developed CD41+ HSCs in the AGM region, which later migrated to the kidney, the site of adult hematopoiesis, suggesting that in zebrafish adult HSCs can be formed in the absence of a functioning Runx1 [136]. The zebrafish embryo can be imaged directly using combinations of fluorescent reporter transgenes, confocal time-lapse microscopy and flow cytometry. This method was used to demonstrate aortic hemogenic endothelium in the ventral wall of the dorsal aorta transitioning to nascent HSCs [101,102]. These authors also used a permanent lineage tracing strategy to demonstrate that the HSCs generated from hemogenic endothelium are the lineal founders of the adult hematopoietic system, forming a large majority of kidney marrow cells in adult zebrafish, consistent with what was previously demonstrated in mouse (reviewed in references [97,108,137,138]). Kissa and Herbomel [139] showed by non-invasive, high-resolution imaging of live zebrafish embryos that HSCs emerge directly from the aortic floor, through a stereotyped process that does not involve cell division but a strong bending process involving a single endothelial cell on the floor of the dorsal aorta that is polarized not only in the dorso-ventral but also in the rostro-caudal versus medio-lateral direction, and depends on Runx1 expression. This single endothelial cell then egresses from the aortic ventral wall into the sub-aortic space, assumes a rounded morphology and transforms into a hematopoietic cell, apparently without a cell division. This new type of cell behavior was termed 'endothelial hematopoietic transition' [139]. These nascent hematopoietic cells transiently occupy the sub-aortic space and then migrate to and subsequently enter the caudal vein, and then migrate to and colonize the kidney, which is a hematopoietic site in zebrafish. Lam et al. [140] used time-lapse microscopy of Runx1-enhanced GFP transgenic zebrafish embryos to capture a subset of cells within the ventral endothelium of the dorsal aorta as they acquire hemogenic properties and directly emerge as presumptive HSCs. Cell tracing showed that these cells enter the circulation via the caudal vein and subsequently populate the sites of definitive hematopoiesis (thymus and kidney), consistent with an HSC identity [140].

In parallel to the AGM region, a number of intra and extra-embryonic sites in the E10.5–E11.5 mouse harbor definitive HSCs, as defined by long-term, high-level, multilineage reconstitution and self-renewal capacity in adult recipients. The murine placenta contains a large population of HSCs during midgestation within the vascular labyrinth region [129,141,142]. Tracking developing HSCs via their expression of Runx1-lacZ and CD41 revealed that they emerge in large vessels in the placenta and appear as early there as they do in the AGM region and before any HSCs had colonized the liver or were circulating in the blood. The placental HSC pool continues to expand until E12.5–E13.5, ultimately containing 15 times more HSCs than the AGM. As the placental HSC population declines, the liver HSC pool expands, suggesting that the placenta may be a major source of the HSCs that seed the liver [142]. However, these studies did not determine whether the placenta is capable of producing HSCs *de novo* or whether it functions solely as a niche for the maturation and expansion of HSCs originating from other sites. Once a heartbeat is initiated at E8.5, any cell within the vasculature may be released into circulation. Although free distribution of progenitors is delayed until E10.5 [118], adult repopulating HSCs are found only after this time, and may therefore have circulated. The emergence of HSCs in the placental vasculature was reported to occur in the absence of a circulation [143].

Probing the hematopoietic potential of cells from different anatomic locations in mammalian conceptuses has led to conflicting views on blood origin (reviewed in

reference [100]). Other vascular regions of the conceptus, such as the umbilical and vitelline arteries and the vascular labyrinth region of placenta, also harbor the earliest HSCs (reviewed in references [97,141]) although their role in the generation of these stem cells is less clear. Earlier studies on quail-chick chimeras showed that the avian allantois is a source of definitive hematopoietic cells [144]. Recently, the hematopoietic potential of the mouse chorionic and allantoic mesoderm was assessed by *in vitro* culture of the tissues explants that were harvested prior to chorioallantoic fusion and circulation [129,145]. Strikingly, these studies documented myeloerythroid hematopoietic potential in both the allantoic and chorionic mesoderm, supporting the hypothesis that HSCs may be generated in the placenta. Yet, these studies did not define lymphoid potential of the rudiments. Of note, one study in 1979 described B lymphoid potential in the midgestation placenta, however, the origin of these cells was not defined [146]. Hematopoiesis is also initiated in the midgestation E10.5—E11.5 mouse head, as indicated by the appearance of intravascular cluster cells and the blood-forming capacity of a sorted endothelial cell population preceding the entry of HSCs into the circulation [147]. A spatially restricted VE-cadherin-Cre lineage labeling system revealed a physiological contribution of cerebrovascular endothelium to postnatal HSCs and multilineage hematopoiesis [147].

To define the sites of origin of the HSC, it has been necessary to develop methods to circumvent the onset of blood cell redistribution via the circulation. Two novel model systems have been utilized to address the problem. The first method involves the use of the VE-cadherin$^{-/-}$ embryo lacking proper development of vasculature [148]. While the heart does beat in these embryos, the lack of interconnection between the embryo and the yolk sac via the vitelloembryonic stalk prevents the mixture of cells. The number of primitive erythrocytes present in the VE-cadherin$^{-/-}$ YS was not significantly different from wild-type. The presence of multipotent hematopoietic stem/progenitors in the YS in the absence of a contribution of cells from the embryo suggested the YS as their site of origin. These results were validated by lineage-specific gene expression analysis [148]. There was no evidence of lymphoid potential through E10.5 in this model system. An alternative approach is to leave the vascular network intact, but block the initiation of the fetal heartbeat. Ncx1 is a sodium calcium exchanger initially expressed exclusively in the fetal heart that facilitates cardiac muscle fiber contraction. When the Ncx1 gene was disrupted by targeted gene knockout, the resultant phenotype of homozygous mutant offspring was found to be unchanged until day 8.25 at which time the heart failed to commence beating, a phenotype which is ultimately fatal [118]. As Ncx1 is not expressed in cells thought to contribute to hematopoietic development, the mutant phenotype should only effect cellular distribution via the circulation. At E9.5, Ncx1$^{-/-}$ embryos had an intact vascular system, both *intra-* and *extra*-embryonically, in contrast to E9 wild-type embryos that had vessels full of primitive erythrocytes [118]. Cross sections of the E9.25 mutant embryo proper revealed an intact vasculature devoid of luminal blood cells. Plating of cells in blood colony-forming assays revealed the presence of both primitive and definitive progenitor lineages in the YS of the Ncx1 mutant, but both were nearly absent in the embryo proper [118]. The total number of hematopoietic progenitors in the conceptus was not significantly altered, suggesting that cells that would typically be distributed to the embryo proper by the circulation remained in the YS.

These results support a model in which both the mammalian primitive as well as the definitive hematopoietic progenitor cells originate entirely in extra-embryonic tissues and it is this population of myeloerythroid progenitor cells that first seeds the fetal liver [118]. A caveat to conclusions based on either the Ncx1$^{-/-}$ or VE-cadherin$^{-/-}$ model is that HSCs (or possibly hemogenic endothelium or hemangioblasts) may migrate considerable a distance outside the vasculature, as was shown in zebrafish where CD41+, cmyb+ hematopoietic precursors colonize the pronephros by a novel extravascular migration route along the pronephric tubules to initiate adult hematopoiesis in the developing kidney, the teleostean equivalent of mammalian BM [1]. In this context, during early embryogenesis in mice,

primordial germ cells (PGCs) arise in an extra-embryonic region near the YS, at E7–7.25. They then migrate to the endodermal epithelium of the hindgut as embryogenesis advances, and then separate from the gut epithelium to enter the dorsal mesentery, through which they finally migrate to form the gonadal anlage at E10.5 [150]. The PGCs move actively by amoeboid movements to transit their migratory pathway in response to attraction by chemotactic factors, substrate-guidance, and interaction with extracellular matrix molecules. This migration occurs over the same time period as the development of HSCs in the YS and establishment of definitive hematopoiesis in the AGM region at E10.5. In marked difference from the mammalian model, in birds, PGCs use the anterior vitelline veins to enter the embryonic circulation and migrate to the gonadal ridges. In both avian and murine systems, migration is driven by the CXCR4/CXCL12 gradient [150].

Analysis of the hematopoietic potential in extra-embryonic and intra-embryonic tissues in *Ncx1* mutant embryos verified myeloid and lymphoid potential not only in the AGM and the placenta but also the YS, suggesting that all three sites may independently generate HSCs [143]. These data, combined with evidence from literature [117,118,151,152], support a model of at least three waves of hematopoiesis during embryogenesis. The first, primitive wave, occurs in the YS, and generates a burst of primitive erythroid cells. The second wave involves production of transient definitive progenitors and is initiated in the YS, after which the progenitors circulate into the liver and give rise to definitive erythroid and myeloid cells that are the first mature blood cells released by the liver. The third wave, formation of HSCs, occurs in the large arteries, but is not only confined to the AGM but most likely also occurs within the umbilical and vitelline arteries, the placenta and the YS. These programs are regulated in part by different mechanisms since Notch1 signaling is not required for generation of the primitive or the transient definitive progenitors in the YS, but it is essential for formation of HSCs in the P-Sp/AGM [151]. Yoshimoto et al. [153] used the $Ncx1^{(-/-)}$ mice to demonstrate that the E9 YS and the intra-embryonic P-Sp tissues independently give rise to AA4.1(+)CD19(+)B220 (lo-neg) B progenitor cells that preferentially differentiate into innate type B-1 and marginal zone (MZ) B cells but not into B-2 cells upon transplantation. They further demonstrated that these B-1 progenitor cells arise directly from YS and P-Sp hemogenic endothelium. These results document an initial wave of innate B lymphopoietic progenitor cells available for seeding the fetal liver at E11. The results of these studies expand understanding of hemogenic endothelial sites specifying distinct B-1 and MZ cell fates apart from B-2 cells and independent of an HSC origin during development. Analysis of $Ncx1^{(-/-)}$ embryos, verified that HSC development is initiated in the placental vasculature independent of blood flow [142]. However, fewer CD41+ hematopoietic cells were found in $Ncx1^{(-/-)}$ placentas than in controls, implying that some HSCs/progenitors colonize the placenta via circulation and/or HSC emergence is compromised without blood flow [142]. Interestingly, the $\mathbf{Ncx1}^{-/-}$ placentas frequently displayed large aggregates of CD31+ cells that were protruding into the lumen, possibly precursors to definitive hematopoietic cells that were stalled during their emergence into the vascular lumen. Indeed, the CD31+ aggregates consist of round cells that are morphologically similar to hematopoietic cells, and cells in one of the clusters in the umbilical cord also expressed CD41+. As these aggregates were not found in the controls, it is likely that they form due to lack of developmental signals normally conveyed by blood flow [142]. Alternatively, they may represent endothelial cells that are unable to organize properly in the absence of blood flow. Mechanical forces created by circulation promote a response through mechanosensory receptor complexes in the endothelial cells to release angiogenic factors, such as VEGF [154,155], which are also essential for hematopoiesis. Therefore the absence of blood flow may impair both vasculogenesis and hematopoiesis. Of note, newly formed HSCs are not equivalent to adult HSCs, as they require a maturation process before they can engraft into adult BM and self-renew (reviewed in references [104,105,118,120,153,156]). Furthermore, in contrast to relatively quiescent adult HSCs, fetal HSCs are highly proliferative as they expand to establish a supply of HSCs for adult life [8,157,158].

Human hematopoietic ontogeny

Hematopoiesis in the human conceptus progresses similarly to that in the mouse conceptus; in a wave-like manner in several different embryonic sites in a sequence of hematopoietic migratory events. Blood generation begins at day 16 of development in the YS with the production of primitive erythroid cells. At day 19, the intra-embryonic splanchnopleura becomes hematopoietic. The emergence of multipotent progenitors and HSCs, organized in clusters of cells closely adherent to the ventral wall of the dorsal aorta, starts at day 27 in the developing splanchnopleura/AGM region. Starting at day 30, the first erythroid progenitors (BFU-E, burst forming unit-erythroid) are found in the liver, with multilineage hematopoietic progenitors (CFU-Mix or -GEMM; colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte) appearing in this tissue at week 13. The human placenta contains fetal-derived immature hematopoietic progenitors and HSCs from gestation week 6 onwards, and these differentially express CD34 through ontogeny [143,159]. Furthermore, pericyte-like mesenchymal stromal cells, isolated from human placenta throughout development, can support the *in vitro* maintenance of human cord blood hematopoietic progenitors [143]. Also, in cultures supporting osteogenic differentiation, placenta mesenchymal lines (second trimester and term) were positive for alkaline phosphatase and mineralization, and most cell lines also could be differentiated into adipocytes. This placenta mesenchyme also possessed endothelial potential, forming endothelial tubules in Matrigel [160]. Thus, the human placental cell lines have the same mesenchymal potential as reported previously in hematopoietic supportive AGM [161]. Robin et al. [160] showed, over a range of developmental times including term, that the human placenta contains hematopoietic progenitors and HSCs. Moreover, stromal cell lines generated from human placenta at several developmental time points are pericyte-like cells and support human hematopoiesis. Immunostaining of placenta sections during development localizes hematopoietic cells in close contact with pericytes/perivascular cells [160]. Thus, the human placenta is a potent hematopoietic niche throughout development.

Mammalian fetal liver

Johnson and Moore [162] first demonstrated the migration of HSCs from the YS and p-Sp region to the fetal liver. At E12, HSC were first detected in the murine liver (50 HSC repopulating units per liver) [163]. The number of HSC per liver increased 10-fold and 33-fold by day 14 and day 16 of gestation, and decreased thereafter, suggesting a single wave of HSC development in the fetal liver. Robin et al. [164] performed a complete *in vivo* transplantation analysis with YS, aorta, placenta, and fetal liver cells, sorted based on CD41 expression level. The data show that the earliest emerging HSCs in the aorta express CD41 in a time-dependent manner. In contrast, placenta and liver HSCs are CD41-negative. Thus, differential and temporal expression of CD41 by HSCs in the distinct hematopoietic territories suggests a developmental/dynamic regulation of this marker throughout development.

Zeb2 (Sip1/Zfhx1b) is a member of the zinc finger E-box-binding (ZEB) family of transcriptional repressors that regulate epithelial-to-mesenchymal transition processes during embryogenesis and tumor progression and high Zeb2 mRNA expression levels were reported in HSCs [165]. Conditional deletion of Zeb2 showed it to be dispensable for hematopoietic cluster and HSC formation in the AGM region but is essential for normal HSC/HPC differentiation [165]. In addition, Zeb2-deficient HSCs fail to properly colonize the fetal liver and/orBM.

Ghiaur et al. [166] studied the role of the Rho GTPases Rac1 in HSC migration during ontogeny and seeding of fetal liver. Using a triple-transgenic approach, they deleted Rac1 in HSC during very early embryonic development and showed a decrease in circulating HSCs in the blood of E10.5 embryos, while YS definitive hematopoiesis was quantitatively normal. Intra-embryonic hematopoiesis was significantly impaired in Rac1-deficient embryos, with absence of intra-aortic clusters and fetal liver hematopoiesis. These data suggest that Rac1 plays

an important role in HSC migration during embryonic development and is essential for the emergence of intra-embryonic hepatic hematopoiesis.

Late fetal/neonatal hematopoiesis

Lin28b is specifically expressed in mouse and human fetal liver and thymus, but not in adult BM or thymus. Ectopic expression of Lin28 reprograms HSCs from adult BM, endowing them with the ability to mediate multilineage reconstitution that resembles fetal lymphopoiesis, including increased development of B-1a, marginal zone B, gamma/delta (γδ) T cells, and natural killer T (NKT) cells [167].

The Sox17 transcriptional regulator is specifically expressed in fetal and neonatal but not adult HSCs and is required for the maintenance of fetal hematopoiesis, thus distinguishing fetal/neonatal HSC transcriptional regulation from that of adult HSCs [157]. Germline deletion of Sox17 led to severe fetal hematopoietic defects, including a lack of detectable definitive HSCs. Conditional deletion of Sox17 from hematopoietic cells led to the loss of fetal and neonatal but not adult HSCs [157]. HSCs stopped expressing Sox17 approximately four weeks after birth. During this transition, loss of Sox17 expression correlated with slower proliferation and the acquisition of an adult phenotype by individual HSCs [157].

MIGRATION, MOBILIZATION AND HOMING OF HSC

Bone marrow (BM) homing of HSCs

Large numbers of HSC move into and out of the circulation on a daily basis. Homing of HSC to the BM is rapid (hours) involving cells traversing across the BM sinusoidal endothelium that presents adhesion molecules and chemokines facilitating the process (reviewed in reference [168]). These circulating HSCs develop low affinity contacts with cell adhesion molecules E- and P-selectin on the endothelium in a process called 'rolling' that precedes firm adhesion via ICAM-1 and VECAM-1 (reviewed in reference [169]). Upon egress from the vasculature, HSC reside in, or adjacent to, specialized microenvironments in the BM. The endothelial or (peri)vascular niche is adjacent to BM endothelial sinuses and its perivascular stromal cells produce the HSC chemo-attractant CXCL12 while the endothelium provides the HSC survival and proliferation-inducing factor c-Kit ligand/SCF (Figs. 47.3, 47.4). The seeding efficiency of HSC to the BM as determined by secondary passaging has generally been accepted as 10—20% by 24 hours. However, Benveniste et al. [170] using competitive repopulation and tracking of a single purified HSC in mice concluded that HSCs engraft with near absolute efficiency. This view has been challenged by Camargo et al. [171], who reported that individual HSC (SP cells) could only engraft one in three mice. The transition from quiescence to active cell cycling of HSC was associated with significant loss of engraftment potential [172] that was not associated with loss of expression of the integrins VLA-4, VLA-5 or the chemokine receptor CXCR4.

The SDF-1/CXCR4 chemokine pathway in HSC homing and migration

The chemokine SDF-1 (CXCL12) is expressed on BM vascular endothelium, immature osteoblasts in the endosteal region and marrow stromal cells, while its receptor, CXCR4, is expressed on HSC and progenitors (Figs. 47.2, 47.3). The SDF-1/CXCR4 pathway plays a major role in regulating the mobilization, migration and retention of HSCs (reviewed in reference [168,173]). This chemokine pathway is essential for HSC seeding from the fetal liver to the BM during development, however, CXCR4 deficient HSCs can, with reduced efficiency, engraft adult irradiated mice [174]. Elevation of plasma levels of SDF-1 occurs following intravenous injection of an adenovector expressing SDF-1, and the consequent reversal of the SDF-1 gradient from blood to marrow leads to the mobilization of HSCs and progenitors [175]. Overexpression of CXCR4 on human CD34+ cells by gene transfer increased their proliferation, migration and NOD/SCID engraftment potential (reviewed in reference [168]). CXCR4 neutralization abolished human intravenous or intrafemoral CD34+ engraftment in

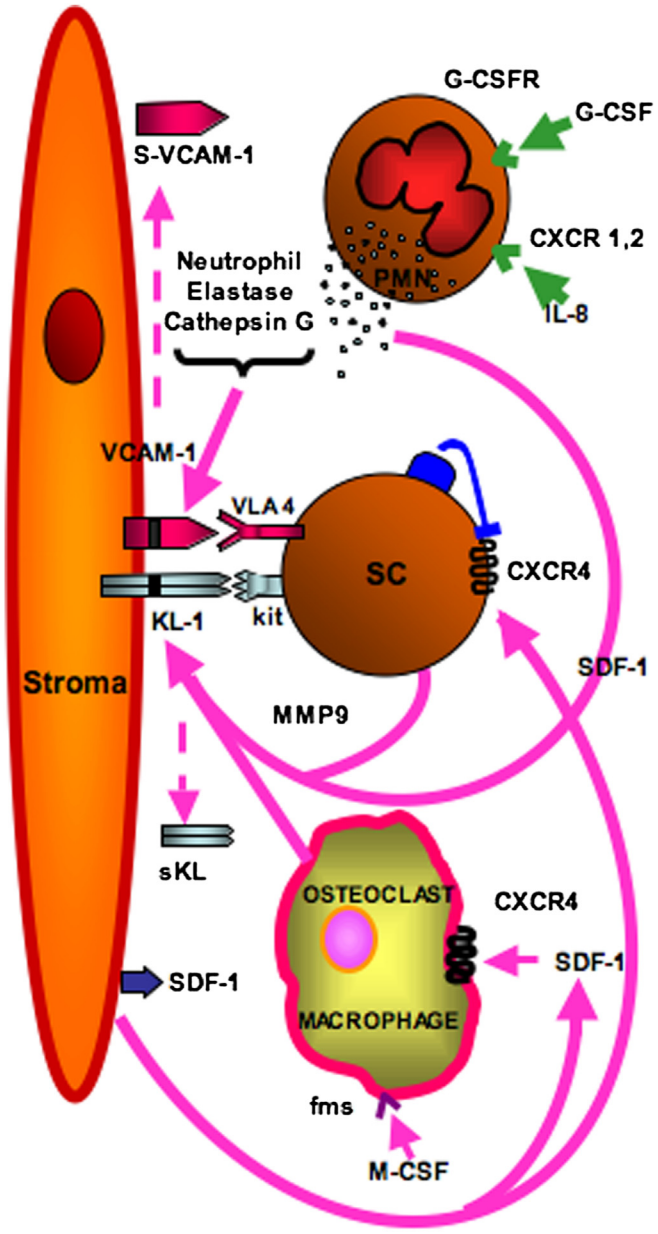

FIGURE 47.3
Model illustrating HSC adherence to bone marrow stromal cells via VLA4 binding to VCAM-1 and c-Kit binding transmembrane c-Kit ligand (KL-1). SDF-1 produced by the stroma induces matrix metalloproteinase-9 (MMP9) production by HSC and macrophages and this in turn cleaves the transmembrane KL-1, releasing soluble active ligand (sKL). The HSC mobilizing role of G-CSF is explained in part by increased numbers and activation of neutrophils within the bone marrow with secretion of a number of proteases (elastase, cathepsin G) that cleave VCAM-1 and 'untether' HSC. Protease activity is also implicated in cleavage and inactivation of SDF-1. HSC express the membrane-bound ectopeptidase CD26 that removes dipeptides from the amino-terminus of proteins and acts as a negative regulator of the SDF-1-CXCR4 axis.

NOD/SCID mice, indicating the essential role of this receptor in BM seeding and colonization, as well as in homing (reviewed in reference [168]).

The adhesion receptor CD44 and its major ligand, hyaluronic acid, are essential for HSC homing to BM and spleen. HSCs express CD44 and migrate on hyaluronic acid towards a gradient of SDF-1, acquiring a polarized morphology with CD44 concentrated at the leading edge of pseudopodia [176]. Since hyaluronic acid is expressed on both BM endothelium and endosteum, it is likely that CD44 plays a key role in SDF-1-mediated trans-endothelial migration and localization in specific BM niches.

HSC mobilization

Beginning in the mid 1980s, autologous peripheral blood was used for transplantation, particularly after chemotherapy at the rebound in hematopoietic regeneration. The availability of two hematopoietic growth factors, G-CSF and GM-CSF, in recombinant form, led to their use

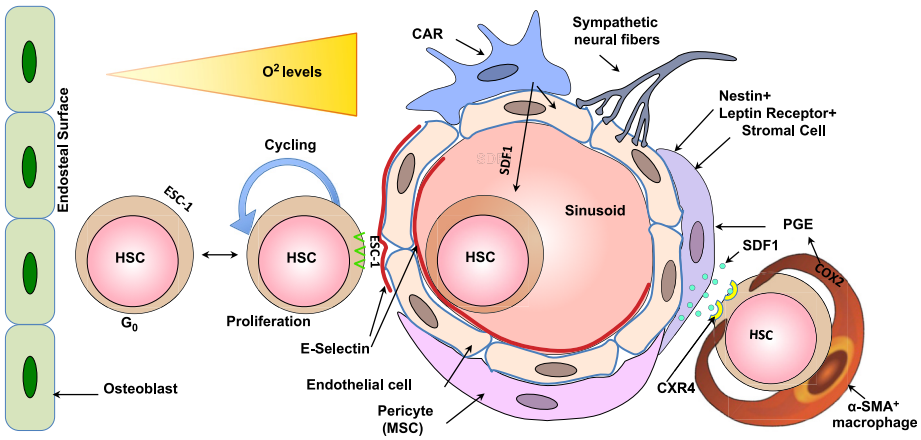

FIGURE 47.4

Hematopoietic stem cells in the bone marrow vascular niche. The endothelial sinusoid is surrounded by mesenchymal stromal cells (MSCs) identified as pericytes, and a subpopulation that can be further identified by expression of Nestin and/or Leptin receptors. CAR cells are MSCs characterized by high expression of the chemokine CXCL12 that is a chemo-attractant for CXCR4-expressing hematopoietic stem cells (HSCs). Sympathetic neural fibers also connect with the niche and may provide negative proliferative signals [184]. E-selectin is expressed exclusively on the endothelial cells of the niche and can bind HSCs expressing an appropriate ligand. ESC-1 is shown here as a candidate ligand that is binding to E-selectin is required for circulating HSC to attach and roll on the sinusoidal endothelium prior to their firm attachment and egress. In addition, the attachment induces HSC proliferation, self-renewal and chemo- and radio-sensitivity [71]. HSC close to the bone endosteal surface are in a hypoxic environment and are in a quiescent state. The extent of migration of these quiescent HSC into the proliferation-inducing environment of the E-selectin-expressing vasculature niche is dependent on demand for production of mature blood cells. Macrophages express α-smooth muscle actin are shown preserving HSCs in the BM adjacent to sinusoids [292].

as HSC and progenitor mobilizing agents [177]. G-CSF treatment for mobilization of HSC, peaking at 5–6 days, is currently the most efficient strategy for clinical HSC collection. A number of chemokines (IL-8, MCP-1, MIP-1α, Groβ, SDF-1) also induce rapid HSC mobilization in murine models with peak HSC and progenitor mobilization in a few hours, in contrast to the days required with CSF's [79,175,178]. Movement of HSCs between endosteal, perivascular and intravascular sites is influenced by G-CSF activation of marrow neutrophils with resulting protease release and cleavage and inactivation of SDF-1 (Fig. 47.3). One mechanism proposed to explain the ability of G-CSF treatment to mobilize HSCs involves a reported reduction in SDF-1, possibly due to degradation by neutrophil protease activity and an increase in its receptor CXCR4 in the bone marrow [177]. Activated neutrophils release matrix metalloproteinase 9 (MMP-9) and lactoferrin from specific granules, and neutrophil elastase, cathepsin G and proteinase 3 from azurophilic granules (Fig. 47.3) (reviewed in reference [179]). The release of multiple proteases within the BM can lead to cleavage or degradation of vascular cell adhesion molecule 1 (VCAM-1), membrane-bound c-Kit Ligand (KL), SDF-1(CXCL12) and its receptor CXCR4 – all events that can 'untether' HSC's from their niches and facilitate their proliferation, differentiation and mobilization (Fig. 47.3) [180]. Studies in genetic models of protease deficiency, including models with multiple prostease deficiencies, surprisingly did not show impaired G-CSF-induced HSC mobilization (reviewed in reference [179]). Thus, the contribution of a single protease to mobilization may not be critical, but multiple proteases acting in a cell-context-specific manner appear important. Targeting and reduction of SDF-1, or its receptor, may be common denominators of proteases implicated in HSC mobilization. HSCs express the membrane-bound ectopeptidase CD26 that removes dipeptides from the amino-terminus of proteins and CD26 null mice have an attenuated response to G-CSF [181].

CD26 expression on HSCs negatively regulated their homing and engraftment, and by inhibiting or deleting CD26 it was possible to greatly increase the efficiency of engraftment [181]. CD26 expression may regulate HSC engraftment potential by inactivating SDF-1 produced by marrow stroma. CXCR4 antagonists such as AMD3100 have also been shown to be

effective in mobilized HSCs with long-term repopulating capacity in mice [182], humans and non-human primates [183]. Signals from the sympathetic nervous system also regulate HSC egress from bone marrow [184]. Pharmacologic or genetic ablation of adrenergic neurotransmission resulted in failure of HSC and progenitors to egress the marrow following G-CSF stimulation while a β2 adrenergic agonist enhanced HSC mobilization in both control and norepinephrine-deficient mice [184]. Kollet et al. [168,178] have linked bone remodeling with regulation of stress-induced mobilization of HSC and progenitor cells. They showed that an increase in osteoclast numbers is associated with mobilization. RANK ligand (RANKL) is a member of the TNF family of cytokines that is necessary for the production of osteoclasts. It is a product of osteoblasts and is responsible for the balance between bone production and destruction in skeletal remodeling [178]. RANKL altered osteoclast expression of MMP-9 and cathepsin K, which cleaved membrane-bound KL resulting in decreased osteoblast KL and the production of osteopontin [168,178]. Inhibition of osteoclasts with calcitonin reduced G-CSF stem/progenitor mobilization. Mice deficient in the protein tyrosine phosphatase-ε (PTP-ε) have dysfunctional osteoclasts and HSC in these mice did not mobilize upon treatment with either RANKL or G-CSF [178].

Grassinger et al. [185] analyzed the proportion and number of murine HSC in the endosteal and central bone marrow regions after four days of G-CSF administration. They demonstrated that the number of HSCs, defined as CD150+CD48- LSK cells, increased within the central BM region in response to G-CSF, but not within the endosteal BM region. This study provided evidence that HSC in the BM respond differently to G-CSF and this is dependent on their location [185]. These findings may lead to development of new agents to specifically mobilize HSC from the endosteal BM region-an HSC population with significantly greater hematopoietic potential compared to their phenotypically identical counterparts located in other regions of the BM [185].

The Rho guanosine triphosphatases, Rac1 and Rac2, play distinct roles in actin organization, hematopoietic cell survival and proliferation. Deletion of both Rac1 and Rac2 murine alleles led to a massive egress of HSC from the marrow to blood, and Rac1−/− mice showed profound defects in engraftable HSC [186]. Rac2 deficient HSC showed defective adhesion, motility and interaction with the hematopoietic microenvironment and had a significant competitive repopulation defect [187].

HSC PROLIFERATION AND EXPANSION *IN VITRO*

The *in vitro* maintenance and potential expansion of HSCs has been the subject of extensive investigation over the last 30 years, beginning with murine long-term bone marrow co-cultures with murine bone marrow stroma ('Dexter cultures') [188] and subsequent adaptation of this system to primates [189] and humans [14,190–194], using both human and murine stroma or endothelium [195]. With the discovery of a number of hematopoietic growth factors (interleukins, colony stimulating factors) and their availability as recombinant proteins, *in vitro* culture systems were developed that supported extensive cell and progenitor expansion in the absence of stroma but in the presence of combinations of cytokines [13,14,52].

Stromal-based cultures

With adult human long-term bone marrow stromal co- culture (LTBMC), hematopoiesis was generally limited to 5–8 weeks and there was continuing decay in repopulating HSC and progenitors over time [14,193]. Several protocols for the expansion of HSCs and progenitor cells *in vitro* have been developed, such as the use of cytokine cocktails, co-culture with mesenchymal stem cells as feeder cells, and cell culture in bioreactors (reviewed in reference [199]). Frequent re-addition of fresh media and cytokines ranging from one exchange per week up to total daily feeding resulted in increased CFC and LTC-IC output and led to the development of clinical scale flat-plate perfusion bioreactors [196].

Unfortunately, the use of media exchange alone has not translated into an expansion of repopulating adult HSCs. CB CD34+ cells have been expanded in transwell culture systems (non-contact cultures) above AFT024 mouse fetal liver stromal cells with additional cytokines (Flt3 ligand [FL], c-Kit Ligand [KL], thrombopoietin [Tpo] and IL-7) [197]. In stromally supported cultures of CB CD34+ cells and cytokine (FL, Tpo, KL +/−IL-6), enhanced production of CAFC (11−89-fold over 6 weeks) was noted, while NOD/SCID repopulating cells increased 10−14-fold by 2 weeks but were absent by week 5 [198]. Expanded cells engrafted primary and secondary NOD/SCID mice and day 7 expanded cells engrafted primary, secondary and tertiary retransplanted fetal sheep. The murine OP9 stromal cell line derived from osteopetrotic op/op mice has been used to support long-term murine hematopoiesis in the absence of added cytokines, however it is not supportive of human CD34+ cells [193]. The addition of Tpo to the stromal co-culture system or transduction of OP9 with an adenovector expressing Tpo permitted very efficient long-term (16+ weeks) human hematopoiesis initiated with cord blood CD34+ cells, with sustained generation and extensive cumulative expansion of LTC-IC [13,200]. In a comparable system of co-culture of OP9 transduced with adenovectors expressing Tpo, KL and FL, but using adult bone marrow or G-CSF-mobilized blood CD34+ cells, hematopoiesis was sustained for 12 weeks but a much lesser degree of LTC-IC expansion was noted (3−4-fold) [193]. In the latter system addition of human (but not murine) M-CSF or CD14+ monocyte/macrophages to the cultures substantially decreased progenitor and HSC expansion, indicating that accumulation of differentiated cells, particularly macrophages, exerted a negative influence on HSC proliferation. *Ex vivo* expansion of human HSCs has been obtained by the use of osteoblast-differentiated mesenchymal stem cells (MSCs) acting as a feeder layer, and likely operates through the CXCL12 chemokine signaling pathway [194].

Due to progress in the understanding of the molecular and cellular mechanisms regulating HSPCs maintenance and expansion, more recent approaches have involved transcription regulation, cell cycle regulation, telomerase regulation, and chromatin-modifying agents. *In vitro* maintenance of HSC has been reported using Wnt5a-secreting AGM and embryonic liver-derived stromal cell line [201]. Human BM-derived MSCs have been used as feeder cells to expand CB HSCs and progenitors in a serum-free culture system [202]. CD34+ cells increased 43-fold while GM and erythroid progenitor increased 3.3- and 4.7-fold respectively, while HSCs capable of *in vivo* engraftment were maintained [202]. Culturing mouse or human hematopoietic cells on tropoelastin, a uniquely elastic biomaterial substrate, led to a two- to three-fold expansion of murine HSC [203]. Treatment with cytokines in the presence of tropoelastin had an additive effect on this expansion. These biological effects required substrate elasticity, as neither truncated nor crosslinked tropoelastin reproduced the phenomenon. The recent emerging technology which uses nanofibers as a supporting matrix, in combination with growth factors, has made a significant improvement in HSC self-renewal and expansion *in vitro* [205]. FGF-2 was reported to expand murine HSCs and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 downregulation [204].

Cytokine-supplemented cultures

It was found that direct contact between stromal cells and hematopoietic cells was not necessary for long-term *in vitro* hematopoiesis [197] and hematopoiesis could be maintained in cultures fed with media conditioned by stromal feeders. This information suggested the feasibility of using stromal-free cytyokine supplemented cultures for expanding HSCs and progenitors *in vitro*. Over the years, the use of phenotypic and functional assays has identified a number of cytokines that have distinct stimulatory effects on primitive hematopoietic cells, particularly when used in various combinations (Fig. 47.1). These include FL, KL, Tpo, IL-1, IL-3, IL-6, IL-11, IL-12, G-CSF, and GM-CSF [52]; reviewed in references [14,206,207]. Using limiting dilution assays for competitive repopulating cells, modest degrees of HSC expansion have been reported in murine *in vitro* expansion systems using combinations of KL, FL and

IL-11. Optimal growth factor combinations are required to achieve *in vitro* HSC expansion and while KL and FL are sufficient to maintain survival and proliferation, retention of HSC function requires activation of additional pathways, for example gp130, stimulated via IL-6 or IL-11 (reviewed in reference [12]).

Haylock et al. [208] were first to report a CFC expansion of 66 fold in human CD34+ cultures stimulated with IL-1, IL-3, IL-6, G-CSF and GM-CSF. Subsequent studies showed the importance of specific cytokines for expansion of defined progenitor cell types. In a human CD34+ expansion system, IL-6 plus soluble IL-6 receptor (to maximize IL-6 signaling where IL-6 receptor density could be limiting), together with FL, KL and Tpo resulted in a ~4-fold expansion of HSCs, as determined by NOD/SCID limiting dilution repopulation [209]. Both FL and IL-6/sIL-6 are important for expanding CB CD34+-derived LTC-IC, while the addition of KL to either of these factors enhances generation of CFCs [210]. In contrast, FL, KL and IL-3 most efficiently stimulated BM LTC-IC proliferation, whereas the addition of IL-6/sIL-6R or Tpo to this combination was required for expansion of CFCs [210]. Regardless of the cytokine combination used, rigorous limiting dilution engraftment studies in NOD/SCID mice have shown, in the majority of studies, that cytokine-supplemented cultures generally showed a loss, maintenance or modest (2–4-fold) expansion of human SRC [209].

In contrast, Piacibello's group [13], reviewed in reference [14], reported a major expansion of LTC-IC and progenitors (3,000-fold) and a 70-fold expansion of NOD/SCID engrafting HSCs (SRC) in cultures of CB CD34+ cells with FL, KL, Tpo and IL-6. Under similar conditions but with CD34+ cells from adult BM and mobilized peripheral blood (MPB), *ex vivo* expansion was shorter in duration and extent, with SRC expansion of only six-fold by three weeks [13]. In a modified stromal-free culture system with CB-derived CD34+ cells and a cocktail of four cytokines (KL, FL, Tpo and IL-6), with CD34+ re-isolation at monthly intervals, continuous expansion of HSCs was observed over 16–20 weeks by *in vitro* CAFC and LTC-IC assays, and by NOD/SCID engraftment and secondary and tertiary passaging [13] (Table 47.1). Despite extensive proliferation, the telomere length of cultured hematopoietic cells initially increased and it was only at late stages of culture that telomere shortening was detected. Telomere length stabilization correlated with high telomerase levels (Table 47.1).

TABLE 47.1 Stem and progenitor cell production, telomere length and telomerase activity of CB CD34$^+$ cells generated after repeated expansion-isolation procedures 'Fractionated' LTC*

	Input	1st isolation wk 4	2nd isolation wk 8	3rd isolation wk 12	4th isolation wk 16	5th isolation wk 20
CD34+ (x103)	100	380	1,820	6,550	30,100	144,500
CD34+/38/Lin-, x103	11	72	400	1,560	7,600	6,700
CFC, x103	7.9	15.7	20.7	222.7	1,033.4	2,408.3
CFU-GEMM, x103	1.8	3.8	17	43.7	501.7	647.4
LTC-IC, x103	14.3	40	73.5	791	1,063.5	0.082
Telomere Length by flow-FISH, kb	9.1	9.9	9.3	9.3	9.3	7.25
Telomerase Activity, TPG units	41.95	27.06	16.56	11.1	10.1	4.8
Engraftment, no. injected mice/no. positive						
Primary, 3x105 CD34+ infused	6/6	11/11	5/5	7/7	7/7	5/5
Secondary	2/2	3/3	2/2	3/3	2/2	0/1
Tertiary	0/2	1/1	1/1	1/1	1/1	ND
SRC/100,000 CD34+ cells	2.8	9.4	14.4	19	58.3	1.2

**Modified from Gammaitoni et al., 2004.*

In contrast, cytokine-stimulated adult CD34+ cells from BM or G-CSF mobilized blood showed CD34+ and NOD/SCID engrafting HSC expansions of six-fold over 3–4 weeks, with telomere shortening and low levels of telomerase activity. Human fetal liver-derived HSCs with NOD/SCID repopulating capacity have been expanded 10–100-fold (net expansion) over 28 days of culture with FL, Tpo, KL and IL-6 and 8% human AB plasma [211]. The CD133+ G0 cell subpopulation from CB is enriched for HSCs and progenitors (CFC), with an LTC-IC incidence of 1 in 4.2 cells and a CFC incidence of 1:2.8 cells [212]. These cells could be expanded with cytokines in a serum-free, stromal-free culture system for up to 30 weeks, resulting in a 100-million-fold amplification of progenitors. Quantitation of HSC expansion *in vitro* is complicated by divergence in results when *in vitro* HSC assays (CAFC, LTC-IC) are used as an end point, versus *in vivo* (SCR). While this has lead to considerable debate as to the validity of the assays, the issue is probably related to the efficiency of the respective systems. Clearly, the *in vivo* assay requires HSC survival and homing to the HSC niches of the mouse BM and there is evidence that *in vitro* culture or cycling HSC may be less efficient *in vivo* while retaining potential to self-renew and form CAFC/LTC-IC *in vitro*. In this context, Liu et al. [213] cultured CB CD34+ cells for up to five days with a cocktail of cytokines, labeled the cells with 111-Indium and determined recovery at 48 hours following intravenous injection into NOD/SCID mice. Cultured, cycling HSCs and progenitors showed a reduction of marrow homing (from 11.3% to 5.4%) with reduced homing to spleen, liver and lung. Acute cytokine deprivation in the *in vivo* environment and Fas/CD95-mediated cell death may be responsible for the reduced engraftment efficiency of cultured HSCs [213]. *Ex vivo* expansion may be enhanced by manipulating intracellular mediators and morphogen signaling pathways to increase the probability of symmetric division, which favors HSC expansion at the expense of differentiation (reviewed in reference [214]).

Pleiotrophin, a secreted neurite outgrowth factor with no known function in hematopoiesis, efficiently promotes HSC expansion *in vitro* and HSC regeneration *in vivo* [207]. Treatment of murine HSCs with pleiotrophin caused a marked increase in long-term repopulating HSC numbers in culture, as measured in competitive repopulating assays. *In vitro* culture of human CB CD34+CD38-Lin- cells with pleiotrophin also substantially increased the numbers of xenograft SCID repopulating HSCs recovered compared to input and cytokine-treated cultures. The chromatin-modifying agents 5-aza-2′-deoxycytidine (5azaD) and trichostatin A (TSA) have been used to ameliorate epigenetic alteration of CB HSCs during *ex vivo* culture [215]. The sequential addition of 5azaD followed by TSA was crucial for retention of *in vivo* engraftment of cytokine stimulated HSC.

Members of the angiopoietin-like (Angptl) family of growth factors have been shown to expand both murine and human HSCs, and Angptl5 has been implicated in the expansion of human NOD/SCID repopulating cells (SRCs) *ex vivo* [216]. Angptl4 maintained the *in vivo* repopulation capacity of CD34+ human CB cells, and both Angptl4 and Angptl5 can increase the engraftment capacity of SRCs [216]; but more frequently, these factors are associated with maintenance of SRC activity during *ex vivo* culture.

An unbiased screen with primary human HSCs identified a purine derivative, StemRegenin 1 (SR1), that promotes the *ex vivo* expansion of CD34+ cells [217]. Culture of HSCs with SR1 led to a 50-fold increase in cells expressing CD34, and a 17-fold increase in cells that retain the ability to engraft immunodeficient mice. Mechanistic studies show that SR1 acts by antagonizing the aryl hydrocarbon receptor (AHR) [217].

MORPHOGENS AND HSC REGULATION

In addition to the classic hematopoietic growth factors, signaling pathways implicated in general development have also been shown in various models to influence HSC proliferation and differentiation (Fig.47.1).

Notch

The transcription factors hepatic leukemia factor (HLF) and Notch target Hes1 were initially identified by gene expression profiling of $CD34^{+}CD38^{-}$ cells from human fetal liver, CB, and adult BM [218]. Overexpression of Hes1 or HLF conferred increased repopulation potential; however, their activity in clonal assays or serial transfer was not tested [218]. Canonical Notch signaling is initiated by their binding Jagged or Delta-like, which leads to proteolytic cleavage of the intracellular domain of Notch (ICN) by gamma secretase and ICN translocation to the nucleus, where it participates in transcriptional activation [219]. Interestingly, CCN3 (NOV), an extracellular modulator of Notch signaling, is also linked with human HSC function. NOV binds to the extracellular domain of Notch and increases expression of Hes1 [220]. The Notch pathway in murine HSCs has been widely studied. Recent evidence suggests that Notch does not have an obligate function in adult HSCs [341], but constitutive activation of Notch can block differentiation and promote HSC expansion [222]. Thus, while it seems that Notch has a conserved role in mouse and human, additional studies should examine the requirement for Notch in human systems via loss-of-function or dominant-negative approaches.

In differential gene display, Notch1 has consistently been shown to characterize the murine and human HSC. Notch1 null mouse embryos showed defective hematopoietic (and vascular) development and were devoid of HSC. Retrovirus-mediated expression of activated Notch 1 enhanced HSC self-renewal and a similar effect of differentiation inhibition and progenitor/HSC expansion was reported with activated Notch4 (Int3) [222]. The Bernstein group [222,223] showed that incubation of murine BM precursors with the Notch ligand Delta1 extracellular domain fused to the Fc portion of human IgG1, together with cytokines (KL, IL-6, IL-11 and Flt3L) inhibited myeloid differentiation and promoted several log increases in precursors capable of short-term lymphoid and myeloid repopulation. Addition of IL-7 promoted T-lymphocyte development whereas GM-CSF induced myeloid differentiation. It has been shown that Notch signaling mediated by both Delta and Jagged ligands expands the HSC compartment while blocking or delaying terminal myeloid differentiation. The quantitative aspects of Notch signaling in determining hematopoietic precursor fate has been demonstrated by Delaney et al. [223] who showed in CD34+ CB cultures that low densities of the Notch ligand Delta1 enhanced *in vitro* generation of CD34+ cells as well as CD14 and CD7 cells consistent with myeloid and lymphoid differentiation whereas higher concentrations induced apoptosis of CD34+ cells but not CD7 T cell precursors. A role for combinatorial effects of Jagged stimulation of Notch and cytokine-induced signaling pathways (Kit, Flt3, Mpl) was reported in murine HSC cultures, with a 10—20 fold expansion of HSC with long-term repopulating potential [224]. Inhibition of Notch signaling leads to accelerated differentiation of HSC *in vitro* and depletion of HSC *in vivo* [225]. However, using a Cre-LoxP-mediated inactivation system, mice with simultaneous inactivation of Jagged and Notch1 survived normally, even following chemotherapy-induced myelosuppression [226]. HSC regulation is complex, cell-context dependent and plagued by potential compensation systems and while the study excludes an essential role for Jagged1 and Notch1 during hematopoieis, there are 4 Notch receptors and five ligands and the Notch pathway cross-talks with the Wnt pathwy with more than nine frizzled receptors and 12 ligands [225]. The Drosophila hairy and enhancer of split (Hes) 1 basic helix-loop-helix protein is a major downstream effector of the Notch pathway and is expressed at high levels in HSC-enriched CD34+, CD38- subpopulations. Conditional expression of Hes1 in murine and human HSC inhibited cell cycling *in vitro* and cell expansion *in vivo* with preservation of long-term reconstituting function.

Wnt

The Wnt pathway has been implicated in HSC proliferation but its function in HSC biology is still controversial (reviewed in references [227—230]). Notch signaling was required for Wnt-mediated maintenance of undifferentiated HSC but not for their survival or entry into the

cell cycle [225]. Wnt signaling represses glycogen synthase kinase-3β (GSK3β), leading to accumulation of β-integrin but also accumulation of intracellular fragments of Notch and activation of Notch target genes such as Hes1. Hematopoietic repopulation by mouse or human HSCs was augmented by administration of a GSK-3 inhibitor to recipient mice [231]. The inhibitor improved neutrophil and megakaryocyte recovery, mouse survival and enhanced sustained long-term repopulation. The GSK-3 inhibitors modulated gene targets of Wnt, Hedgehog and Notch pathways in primitive hematopoietic cells, without affecting mature cells. Wnt-5A injected into immunodeficient mice enhanced engraftment by human CD34+ cells [232]. Reya et al. [227] showed that overexpression of activated β-catenin expanded the pool of HSC in long-term culture. Activation of Wnt signaling in HSC increases expression of HOXB4 and Notch1, both implicated in HSC self-renewal. Ectopic expression of axin or the frizzled ligand-binding domain, both inhibitors of the Wnt signaling pathway, inhibited HSC growth *in vitro* and reduced *in vivo* engraftment. Wnt3a palmitolylated protein has been purified and shown to induce self-renewal of HSC [233].

Induction of BMP or Wnt in zebrafish hematopoietic cells after irradiation led to increased gene expression of key hematopoietic genes, suggesting that regulation of blood genes may be important for BMP and Wnt effects during regeneration (reviewed, [230]). The ultimate effectors of Wnt signaling pathways are TCF/LEF transcription factors [230]. In hematopoietic development in the early embryo, HSCs are maintained by secretion of Wnt5a by AGM stromal cells [201]. In a 'lineage fingerprint' of ~100–400 genes uniquely expressed in HSCs, Wnt signaling was particularly enhanced [28]. During hematopoietic regeneration, both hematopoietic and stromal cell elements within the bone marrow microenvironment show increased expression of Wnt10b, which can function to enhance the growth of HSCs [234]. In mice, Wnt3a deficiency irreversibly impairs HSC self-renewal and leads to defects in progenitor cell differentiation [340]. Studies in zebrafish showed that Wnt activation in HSCs required prostaglandin E2 (PGE2) [337]. Although a central role for the canonical Wnt signaling pathway has been suggested in HSC self-renewal, as well as in the development of B and T cells, conditional deletion of beta-catenin (which is considered to be essential for Wnt signaling) had no effect on hematopoiesis or lymphopoiesis [235,236]. The combined deficiency of beta- and gamma-catenin in HSCs also did not impair their ability to self-renew and to reconstitute all myeloid, erythroid, and lymphoid lineages [236]. These results would appear to exclude an adult HSC cell autonomous role for canonical Wnt signaling (as mediated by beta- and/or gamma-catenin) during hematopoiesis and lymphopoiesis.

However, there is a distinct mode of Wnt/beta-catenin signal that can stabilize beta-catenin in the BM stromal cells, thus promoting maintenance and self-renewal of HSCs in a contact-dependent manner, whereas direct stabilization in hematopoietic cells caused loss of HSCs [237]. Wnt/β-catenin activity is needed for the emergence but not the maintenance of HSCs in mouse embryos, since it is transiently required in the AGM to generate long-term HSCs and to produce hematopoietic cells *in vitro* from AGM endothelium [238].

Hedgehog

The Hedgehog pathway has been implicated in HSC proliferation but its function in HSC biology is still controversial. Sonic Hedgehog (Shh) treatment in CD34+ cultures induced expansion of human HSCs, and Noggin, a specific inhibitor of bone morphogenic protein-4 (BMP-4) inhibited Shh-induced proliferation, indicating that Shh regulates HSC via mechanisms that are dependent on downstream BMP signals [239]. Indian hedgehog (Ihh)-expressing human stromal cells supported CD34+ cells with markedly enhance production of progenitor CFCs above the stroma, but no change in long-term cobblestone formation [240]. Despite these reports indicating that Hh signaling is an important regulator of HSC maintenance and differentiation, Gao et al. [241] reported that Hedgehog signaling is dispensable for adult HSC function. Gain- and loss-of-function Hh genetic models demonstrate that

conditional Smoothened (Smo) deletion or overactivation has no significant effects on adult HSC self-renewal and function. Transcriptome analysis reveals that silencing of Hh signaling does not significantly alter the HSC-specific gene expression 'signature' [241]. Moreover, there was a lack of synergism between the Notch and Hh pathways in HSC function.

Bone morphogenic protein

CD34+, CD38− HSC express the bone morphogenic protein type 1 (BMP-1) receptors activin-like kinase (ALK-3 and ALK-6) and their downstream transducers SMAD-1, -4 and -5 (reviewed in reference [230]). Like TGFβ, high concentrations of BMP-2, -4 and -7 inhibited HSC proliferation but maintained their long-term survival and repopulating potential whereas low concentrations of BMP-4 induced HSC proliferation and differentiation [242]; reviewed in reference [230].

MOLECULAR PATHWAYS IMPLICATED IN HSC SELF-RENEWAL

Jak-STAT

Transduction of mouse HSCs with constitutively activated STAT3 enhanced HSC self-renewal under stimulated, but not homeostatic conditions, while a dominant-negative form of STAT3 suppressed self-renewal [243]. Cytokine receptor signals through c-Kit and c-Mpl can modulate HSC quiescence and engraftment, acting in part through STAT5, a transcriptional regulator and mitogenic factor in hematopoietic progenitors that is also required for maintenance of HSC quiescence during steady state hematopoiesis [244]. Conditional deletion of STAT5 rapidly decreased quiescence-associated c-Mpl downstream targets (Tie-2, p57), increased HSC cycling, and gradually reduced survival and depleted the long-term HSC pool [244]. The STAT5 pathway is strongly activated following ligand binding to the erythropoietin and IL-3 receptors, and weakly following FL binding to Flt3. However, constitutively activating mutants of Flt3, found in ∼25% of human acute myeloid leukemias, are associated with strong activation of STAT5 (reviewed in reference [14]). A constitutively activated double mutant of STAT5a [STAT5a(1*6)] transduced into CD34+ cells promoted enhanced HSC self-renewal, as measured by CAFC assay, and promoted enhanced erythroid differentiation relative to myeloid [245]. This was causally linked to downregulation of C/EBPα, a transcription factor uniquely associated with granulocytic differentiation.

Polycomb group (PcG) proteins

PcG proteins are required for the maintenance of embryonic as well as a broad range of adult stem cells, including HSCs (reviewed in reference [246]). PcG proteins play a role in the self-renewal capacity of HSCs by repressing tumor suppressor genes through histone modifications and keeping differentiation programs poised for activation by repressing hematopoietic developmental regulator genes (reviewed in references [246,247]). PcG proteins also maintain redox homeostasis to prevent premature loss of HSCs. These findings established PcG proteins as epigenetic regulators of the self-renewal capacity and multipotency of HSCs. (reviewed in reference [246]). The Polycomb group transcriptional repressor gene Bmi-1 has been implicated in HSC maintenance, and loss-of-function studies showed profound defects in HSCs [247,248]. Bmi-1 overexpression downregulated the expression of p16 and p19Arf, which are encoded by ink4a, and enhanced HSC symmetrical division, resulting in the expansion of multipotent progenitors *in vitro* and enhanced HSC repopulation *in vivo* [249]. Enforced expression of Bmi-1 augments the self-renewal capacity of HSCs. Transcriptional comparison of HSCs and MPPs identified a number of transcription factors associated with the HSC state, including ID genes, SOX8, SOX18, and Bmi-1 [247,250].

The polycomb group (PcG) gene Bmi-1 has been identified as one of the key epigenetic regulators of cell fates during different stages of development in multiple murine tissues. In a clinically relevant model, enforced expression of Bmi-1 in CB CD34+ cells resulted in

long-term maintenance and self-renewal of HSC and progenitor cells [247,250]. Long-term culture-initiating cell frequencies were increased upon stable expression of Bmi-1 and these cells engrafted more efficiently in NOD-SCID mice [247,248,250]. Week 5 CAFCs were replated to give rise to secondary CAFCs. Serial transplantation studies in NOD-SCID mice revealed that secondary engraftment was only achieved with cells overexpressing Bmi-1. Importantly, Bmi-1-transduced cells proliferated in stroma-free cytokine-dependent cultures for more than 20 weeks, while a stable population of approximately 1% to 5% of CD34+ cells was preserved that retained colony-forming capacity [247,250]. Control cells lost most of their NOD-SCID engraftment potential after 10 days of *ex vivo* culture in the absence of stroma, NOD-SCID multilineage engraftment was retained by overexpression of Bmi-1. These data indicate that Bmi-1 is an intrinsic regulator of human HSC self-renewal [250,251]. Down-modulation of Bmi-1 in CB CD34+ cells impaired long-term expansion and progenitor-forming capacity, both in cytokine-driven liquid cultures as well as in bone marrow stromal co-cultures [251]. In addition, LTC-IC frequencies were dramatically decreased upon knock-down of Bmi-1, indicating an impaired maintenance of HSC and progenitor cells [251]. The reduced progenitor and HSC frequencies were associated with increased expression of p14ARF and p16INK4A and enhanced apoptosis, which coincided with increased levels of intracellular reactive oxygen species and reduced Foxo3A expression [251]. Bmi-1 overexpression is associated with poor prognosis in leukemia and down-modulation of Bmi-1 in AML CD34+ leukemic stem cells impaired their self-renewal and long-term expansion [251]. In addition to a role in bypassing senescence and the orchestration of the symmetry of HSC, it has recently become clear that Bmi-1 also functions in the protection against oxidative stress [247]. In the absence of Bmi-1, reactive oxygen species accumulate, associated with activation of DNA damage response pathways and increased apoptosis. Bmi-1-mediated control over reactive oxygen species levels can occur independently of the INK4a/ARF pathway, by impaired mitochondrial functions [247]. In human hematopoietic malignancies, Understanding molecular mechanisms by which Bmi-1 affects stem cell fate will increase our insights into the biology of HSCs and will also aid in understanding the process of leukemic transformation and ultimately in the identification of drugable targets that might facilitate the eradication of leukemic stem cells.

The HOX family of hematopoietic regulators

The Homeobox (HOX) transcription factors are important regulators of normal and malignant hematopoiesis because they control the proliferation, differentiation and self-renewal of hematopoietic cells at different levels of the hematopoietic hierarchy. *HOX* genes are expressed at various stages during hematopoietic development. Mounting evidence suggests that *HOXA9* plays an important role in normal hematopoiesis. Targeted disruption of *HOXA9* in mice severely reduces the number of HSC and progenitor cells, while enforced expression of *HOXA9* promotes proliferative expansion of HSC and progenitor cells and subsequently inhibits their differentiation [252]. These data highlight the importance of precise control of HOXA9 protein levels during hematopoiesis.

HOXB4 plays critical role in promoting HSC self-renewal, and engraftment [253]; as reviewed in references [12,14]. Combinations of early acting cytokines increased HOXB4 promoter activity in primitive hematopoietic cells and Tpo acting via Mpl and p38MAPK increased HOXB4 expression 2–3-fold in primitive hematopoietic cells [254]. In $Tpo^{(-/-)}$ mice, hematopoietic HoxB4 expression was 2–5-fold lower than in wild-type animals [254]. Wnt signaling in primitive hematopoietic cells also induces HOXB4 expression [255]. HOXB4 null mice had reduced numbers of HSC and progenitor cells due to impaired proliferative capacity, but did not show perturbed lineage commitment. Retroviral-mediated ectopic expression of HOXB4 resulted in a rapid increase in proliferation of murine HSC both *in vivo* (1,000-fold increase in transduced HSC in a murine transplant model), and *in vitro* (40-fold expansion of murine HSC), with retention of lympho-myeloid repopulating potential and enhanced

regenerative capability in mice (reviewed in reference [12]). However, high levels of HOXB4 expression in human CB $CD34^+$ cells were recently reported to either increase the proliferation of HSCs and inhibit differentiation [253], or direct the cells toward a myeloid differentiation program, rather than increasing proliferation [256]. HoxB4 has been ectopically expression in human embryonic stem cells (hESCs) and did not affect ESC features [257]. However, HoxB4-expressing, hESC-derived, CD34+ hematopoietic cells generated higher numbers of erythroid and blast-like colonies than controls. The number of CD34+ cells increased but CD45+ and KDR+ cell numbers were not significantly affected. When the hESC-derived CD34+ cells were transplanted into NOD/SCID beta $2m^{(-/-)}$ mice, the ectopic expression of HoxB4 did not alter their repopulating capacity [257]. Ectopic expression of HOXB4 in combination with MS-5 stroma exerts different effects in HSC-enriched, primitive (CD34+ CD38-) and HSC depleted (CD34+CD38+) progenitor-enriched human CB cells, resulting in an enhanced proliferation of early CD34+CD38− cells in the absence or presence of serum-sustained MS-5 stroma and an impaired output of late committed CD34(+) cells on MS-5 stroma [258].

These studies suggest that in human hematopoietic progenitors, HOXB4 affects cell fate decisions (self-renewal, differentiation, or a differentiation block) in a concentration-dependent manner. Therefore, like HOXA9, the relative abundance of HOXB4 requires precise regulation. Molecular mechanisms and target genes responsible for HOXB4-induced HSC expansion remain to be elucidated. Overexpression of HOXB4 point mutations lacking the capacity to bind DNA (HOXB4(N^{51}->A) failed to enhance the proliferative activity of transduced BM populations whereas mutants that blocked the capacity of HOX to cooperate with the transcription factor PBX in DNA-binding (HOXB4 (W->G) conferred a pronounced proliferative advantage *in vitro* and *in vivo* to transduced BM populations [259]. This mutant was comparable to wild-type HOXB4 in that its elevated level promoted a comparable degree of HSC expansion. This was distinct from results obtained by knocking down the expression of PBX in HOXB4-overexpressing BM cells using lentivector transduction with a PBX antisense construct. In this system, the HSCs were > 20 times more competitive than HSCs overexpressing HOXB4 with PBX levels intact which were, in turn, 20−50 times more competitive that wild-type BM [260]. It appears that the likely explanation for these observations is that HOXB4 and PBX genes act on distinct pathways in the HSC, the former promoting self-renewal and the latter inhibiting it. A developmentally important mechanism for regulating HOX gene expression was identified by Davidson et al. [261]. Cdx4 belongs to the caudal family of homeobox genes that have been implicated in antero-posterior patterning of the axial skeleton and regulation of HOX gene expression. A Cxc4 mutation in zebrafish causes severe anemia with complete absence of Runx1 in blood cells [261]. Injection of mutants with HOXB7 and HOXA9 mRNA almost completely rescued Cdx4 mutants. Retroviral transduction of mouse ES-derived embryoid body cells with Cdx4 increased expression of HOXB4 (30×), HOXB3 (19×), HOXB8 (5×) and HOXA9 (4×) − all implicated in HSC or progenitor expansion. Cdx4 was more potent than HOXB4 in stimulating hematopoiesis and CFU-GEMM production [261].

The abundance of a given cellular protein is regulated by the interplay between its biosynthesis and degradation. During normal hematopoietic development, *HOXA9* is strongly expressed in the $CD34^+$ populations enriched in early myeloid progenitors, and is turned off when cells exit the $CD34^+$ compartment and undergo terminal differentiation. In conjunction with decreased biosynthesis, rapid turnover of HOXA9 would ensure low steady state levels, which is necessary for proper execution of differentiation into myeloid lineages. HOXB4 is also expressed at high levels in HSC/progenitor compartments, and is downregulated, but maintains low-level expression during differentiation. The studies of the biochemical mechanisms controlling the activities of HOXA9 and HOXB4 thus far have been focused primarily on transcriptional regulation. Little is known about the way that their cellular abundance is controlled at the post-translational level. Identification of proteins involved in the removal of HOXA9 and HOXB4 will be necessary for understanding the elaborate regulatory circuitry governing hematopoiesis.

Post-translational regulation of HOX protein levels has been linked to their ubiquitin-dependent proteolysis by the CUL-4A ubiquitin ligase [262]. Some transcription factors travel between cells because they contain protein domains that allow them to do so. This is the case for the HIV transcription factor TAT and for several homeoproteins, such as Engrailed, HOXA5, HOXB4, HOXC8, EMX1, OTX2 and PAX6. Direct paracrine homeoprotein activity has not been considered, yet in theory it would represent a way for neighboring hematopoietic cells to exchange proliferative and differentiative signals. Bone marrow stromal cells (murine MS-5 stroma) have been lentivector transduced with a vector expressing HOXB4 linked to an immunoglobulin kappa chain leader sequence (signal peptide) that is cleaved during protein secretion [192]. Co-culture of human CB CD34+ cells on this stroma resulted in a 2−3-fold greater expansion of cells and progenitors (CFC), a 4−10-fold greater expansion of LTC-IC, and a 2.5-fold expansion of NOD/SCID repopulating cells relative to controls over five weeks of culture [192]. A biologically active TAT-HOXB4 fusion protein has been produced and expressed as a recombinant protein that upon purification could be added to murine HSC cultures [12]. Since the half life of intracellular HOXB4 was approx. one hour, TAT-HOXB4 was added to cell culture every three hours for four days together with cytokines (KL, IL-6, IL-3) resulting in a 5−6-fold expansion of HSCs as measured by competitive repopulation. Ectopic overexpression of HOXB4 in murine BM produced HSCs that were ~40-fold more competitive than non-transduced cells in mouse repopulation assay, and by 3−5 months the HSC pool size of reconstituted mice equaled, but never exceeded, that of untreated control mice [12]. High-level ectopic expression of HOXB4 in human CB CD34+ cells had a selective growth advantage in NOD/SCID mice but with substantial impairment in myeloerythroid differentiation and B cell development. In non-human primate competitive repopulating transplantation models, HOXB4 overexpressing CD34+ cells had a 56-fold higher short-term, and 5-fold higher long-term (six months), engraftment than control cells [263]. HOXB4-transduced and expanded non-human primate CB cells resulted in superior engraftment of all hematopoietic lineages in pigtailed macaques over non-expanded controls [264].

HOXC4, a protein encoded by a HOXB4 paralog gene, has also been implicated in HSC expansion [265]. In co-cultures of HOXC4-producing stromal cells with human CB CD34+HSCs, the HOXC4 homeoprotein expanded immature hematopoietic cells by three to six times, significantly improving the level of *in vivo* engraftment. Comparative transcriptome analysis of CD34+ cells subjected to HOXB4 or HOXC4, or not, demonstrated that both homeoproteins regulate the same set of genes, some of which encode key hematopoietic factors and signaling molecules [265].

HOXA10 is a critical regulator of HSCs erythroid/megakaryocyte development and induced a 15-fold increase in the repopulating capacity of HSCs after 13 days of *in vitro* culture [266]. The HOXA10-mediated effects on hematopoietic cells were associated with altered expression of genes that govern stem cell self-renewal and lineage commitment such as HLF, Dickkopf-1 (Dkk-1), growth factor independent-1 (Gfi-1), and Gata-1.

NEGATIVE REGULATION OF HSC

Members of the transforming growth factor-beta (TGF-β) superfamily of growth factors have been shown to regulate the *in vitro* proliferation and maintenance of HSCs [267]. The TGF-β/Smad signaling pathway is active in dormant HSCs, and loss of the TGF-β type II receptor reduces long-term repopulating activity in HSCs. TGF-β maintains HSCs in a quiescent, or slowly cycling state and this cell cycle arrest was linked to upregulation of the cyclin-dependent kinase inhibitor p57KIP2 in primary human hematopoietic cells (Fig.47.2) [268]. Fortunel et al. [269] attributed the TGF-β affect in part to downmodulation of cell surface expression of tyrosine kinase receptors c-Kit, Flt3, IL-6R and the Tpo receptor Mpl. This negative regulatory role of TGFβ has been challenged by Larsson et al. [270], who showed that TGF-β type I receptor-deficient mice had normal *in vivo* hematopoiesis, a normal HSC cell cycle

distribution and did not differ in long-term HSC repopulating potential compared to wild-type animals. TGF-β type II receptor-deficient HSCs showed low-level Smad activation and impaired long-term repopulating activity, suggesting a critical role for TGF-β/Smad signaling in HSC maintenance [271].

TGF-β is produced in a latent form by a variety of cells, and so it is necessary to identify activator molecules for latent TGF-β. Non-myelinating Schwann (glial) cells maintain HSC dormancy in the bone marrow niche by activating latent TGF-β [271] (Fig. 47.4). These glial cells ensheath autonomic nerves, express HSC niche factor genes, and are in contact with a substantial proportion of HSCs [271]. Autonomic nerve denervation reduced the number of these active TGF-β-producing glial cells and led to rapid loss of HSCs from BM. The Smad signaling pathway, which operates downstream of the TGF-beta superfamily of ligands, regulates a diverse set of biological processes, including proliferation, differentiation and apoptosis, in many different organ systems. The self-renewal capacity of HSCs is promoted *in vivo* upon blocking of the entire Smad pathway. Working at a common level of convergence for all TGF-beta superfamily signals, Smad4 is key in orchestrating these effects and is critical for self-renewal of HSCs [267]. Enforced expression of Smad4 revealed a role for TGF-beta in human CB HSCs, suggesting that the Smad pathway regulates HSC self-renewal independently of differentiation, and demonstrating that the Smad signaling circuitry negatively regulates the regeneration capacity of human HSCs *in vivo* [271]. Systemic induction of Smad4 deletion was incompatible with survival four weeks after induction because of anemia and histopathological changes in the colonic mucosa [272]. Restriction of Smad4 deletion to the hematopoietic system via several transplantation approaches demonstrated a role for Smad4 in the maintenance of HSC self-renewal and reconstituting capacity, leaving homing potential, viability, and differentiation intact [272]. Furthermore, the observed downregulation of notch1 and c-myc in Smad4$^{(-/-)}$ primitive cells places Smad4 within a network of genes involved in the regulation HSC renewal [272].

HSCs overexpressing Smad7 have an unperturbed differentiation capacity, as evidenced by their normal contribution to both lymphoid and myeloid cell lineages, suggesting that the Smad pathway regulates self-renewal independently of differentiation (reviewed in reference [273]). The data reveal an important and previously unappreciated role for the Smad signaling pathway in the regulation of self-renewal of HSCs *in vivo* [273]. He et al. [274] showed that the ubiquitious nuclear protein Transcriptional Intermediary Factor 1gamma (TIF1gamma/TRIM33) selectively binds receptor-phosphorylated Smad2/3 in competition with Smad4. Rapid and robust binding of TIF1gamma to Smad2/3 occurs in hematopoietic, mesenchymal and epithelial cell types in response to TGF-beta. In human HSCs/HPCs, where TGF-beta inhibits proliferation and stimulates erythroid differentiation, TIF1gamma mediates the differentiation response while Smad4 mediates the antiproliferative response with Smad2/3 participating in both responses [274]. Thus, Smad2/3-TIF1gamma and Smad2/3-Smad4 function as complementary effector arms in the control of hematopoietic cell fate by the TGF-beta/Smad pathway. Distinct HSC subtypes are differentially regulated by TGF-beta1. Myeloid-biased HSCs (My-HSCs) and lymphoid-biased HSCs (Ly-HSCs) phenotypes are stable under natural (aging) or artificial (serial transplantation) stress but respond differently to TGF-β1, presenting a possible mechanism for differential regulation of HSC subtype activation [275].

Two cyclin-dependent kinase inhibitors, (CKI), p21 (Cip1/Waf1) and p27kip1, have been shown to govern the pool size of HSCs and progenitors, respectively [276]. Enforced expression of the HOXB4 transcription factor and downregulation of p21 (Cip1/Waf) can each independently increase murine HSC proliferation [256]. When p21 knockdown and HOXB4 overexpression were combined in HSC, long-term competitive repopulating cells expanded 100-fold in five days, compared to wild-type HSC, and three-fold greater than HOXB4 alone [256]. The transcription factor MEF (or EL4F) regulates quiescence of primitive

hematopoietic cells [278]. MEF null HSC display increased residence in G0 with reduced 5-bromodeoxyuridine incorporation *in vivo* and impaired cytokine proliferation *in vitro*. MEF null mice are consequently relatively resistant to the myelosupressive effects of chemotherapy or radiation.

Bone marrow adipocytes as negative regulators of the hematopoietic microenvironment

Adult BM contains adipocytes, the number of which correlates inversely with hematopoietic activity [277]. HSCs and short-term progenitors are reduced in frequency in the adipocyte-rich vertebrae of the mouse tail relative to the adipocyte-free vertebrae of the thorax [277]. In the mouse, the marrow of long (tubular) bones (made of compact cortical bone and medullary cavity) remains hematopoietically active for the animal's lifespan. In humans, in contrast, life-long hematopoiesis becomes restricted, in the third decade, to the axial skeleton and portions of long bone metaphyses, whereas it is reversibly lost in the rest of the marrow (reviewed in reference [279]). Adipose conversion is reversible, and so is the block of hematopoiesis in yellow marrow. Regional adipose conversion of the marrow would *per se* suggest that physiologically, the HSC niche might be a flexible entity in mammals. In lipoatrophic A-ZIP/F1 'fatless' mice, which are genetically incapable of forming adipocytes, and in mice treated with the peroxisome proliferator-activated receptor-gamma inhibitor bisphenol (a diglycidyl ether which inhibits adipogenesis), marrow engraftment after irradiation is accelerated relative to wild-type or untreated mice [277]. These data implicate adipocytes as predominantly negative regulators of the bone marrow microenvironment, and indicate that antagonizing marrow adipogenesis may enhance hematopoietic recovery in clinical bone marrow transplantation.

HEMATOPOIETIC STEM CELL NICHES

Niches in the BM provide specialized microenvironments for the regulation of HSCs. The strict control of HSCs by the niche coordinates the balance between the proliferation and the differentiation of HSCs required for maintaining the homeostasis of the blood system in steady states and during stress hematopoiesis. There are number of recent reviews on the BM niche(s) [179,279–284c]. Multiple cells in the BM, including osteoblasts, vascular endothelia, stromal cells (i.e., reticular cells, perivascular mesenchymal cells) are of mesenchymal lineage. An increased understanding of the role of HSC niche cells has been obtained from studies on the role of osteoprogenitor cells (mesenchymal stem cells, skeletal stem cells). MSCs, identified using Nestin expression, constitute an essential HSC niche component [285]. Nestin+ MSCs in BM can be identified by their ability to form fibroblast colonies (CFU-F) and can be propagated as non-adherent 'mesenspheres' that can self-renew and expand upon serial transplantations [285]. HSCs home near Nestin+ MSCs and adrenergic nerve fibers in the BM of lethally irradiated mice (Fig. 47.4). Nestin+ MSCs highly express HSC maintenance genes and others that trigger osteoblastic differentiation and these are selectively downregulated during enforced HSC mobilization or beta3 adrenoreceptor activation [285]. Nestin+ cell depletion of the BM *in vivo* rapidly reduces its HSC content and significantly reduces homing of HSCs. HSC dysregulation has been linked to the dysregulation of osteoprogenitors (reviewed in reference [279]). As preosteoblastic, periendothelial cells residing at the sinusoid wall, MSCs reconcile the notions of 'osteoblastic' and 'sinusoidal' niches with one another. In addition, MSCs have provided insight into the cross-regulation of skeletal and hematopoietic physiology mediated by the interplay of two stem cells (hematopoietic and skeletal) sharing a single niche. Skeletal progenitors have emerged as pivotal organizers of a complex, highly plastic niche and are direct regulators of hematopoietic space formation, sinusoid development, and hematopoietic function(s), as well as direct progenitors of positive and negative regulators of HSCs such as osteoblasts and adipocytes (reviewed in reference [279]). An intrinsic BM hematopoietic niche occupancy defect of HSC in scid mice has been reported

which facilitates exogenous HSC engraftment and provides a conducive BM niche environment for xenotransplantation [286].

Various types of lympho/myeloid cells can promote the retention of HSCs in the BM by modulating the abundance of CXCL12 and other adhesive molecules in the bone marrow niches [287–290]. Activated regulatory T cells can maintain the quiescence of HSCs in the BM niches by participate in creating a localized zone where HSCs reside and where T(reg) cells are necessary for allo-HSPC persistence. In addition to supporting HSC function, the niche provides a relative sanctuary from immune attack [290]. The BM has a reservoir of monocytes and macrophages that have a major role in host defense throughout the body and function cooperatively as niche cells to regulate HSCs (reviewed in reference [279]). BM macrophages play a critical role in maintaining osteoblasts, bone formation and the expression of CXCL12, Kit ligand, and angiopoietin-1 necessary to HSC maintenance [291]. In transgenic mice, in which expression of the G-CSFR is restricted to cells of the monocytic lineage, G-CSF-induced HSPC mobilization, osteoblast suppression, and inhibition of CXCL12 expression in the BM are intact [288]. This demonstrates that G-CSFR signals in monocytic cells are sufficient to induce HSC mobilization. G-CSF treatment of wild-type mice is associated with marked loss of monocytic cells in the bone marrow and G-CSFR signals in BM monocyte/macrophage cells inhibit the production of trophic factors required for osteoblast lineage cell maintenance, ultimately leading to HSC mobilizatio [288]. HSCs can be mobilized into the blood in mice by depleting macrophages with clodronate-loaded liposomes [289,291]. Two antagonistic, tightly balanced pathways regulating maintenance of HSCs/progenitors in the niche during homeostasis have been identified. In one, sympathetic nervous system (SNS) signals enhance HSC egress, while in the other, macrophages cross talk with the Nestin(+) niche stromal cells to promote HSC retention [287] (Fig. 47.4). Reductions in BM mononuclear phagocytes led to reduced BM CXCL12 levels and the selective downregulation of HSC retention genes in Nestin+ niche cells, and egress of HSCs/progenitors to the bloodstream. Specific depletion of CD169+ macrophages was sufficient to induce HSC/progenitor egress and enhance mobilization induced by a CXCR4 antagonist or G-CSF [287]. Ludin et al. [292] recently identified a previously unknown BM subset of activated monocyte/macrophages that expressed α-smooth muscle actin (α-SMA) and cyclooxygenase (COX-2). These myeloid cells were adjacent to HSC/HPCs and were radio-resistant and further upregulated COX-2 expression under stress conditions [292]. The upregulated COX-2 produced sufficient PGE2 to counteract HSC exhaustion during alarm situations by limiting the production of ROS via inhibition of the kinase Akt, and producing higher stromal cell expression of CXCL12, which is essential for stem cell quiescence [292].

Endosteal niche

Dexter et al. [188] were the first to demonstrate that *in vitro* hematopoietic cell cultures were able to sustain primitive cells only in the presence of a bone marrow stromal feeder layer. Studies in the 1970s using the CFU-S assay indicated that these stem-like cells are localized close to the endosteal bone surface, while differentiated cells move toward the central axis of the marrow [293]. Subsequently, the concept of a stem cell niche was first proposed by Schofield [295], who laid out the basic concepts of a stem cell niche as a defined anatomic site where HSCs could be sustained and reproduce and where differentiation was inhibited. A niche had a limited space that also limited the numbers of HSC and was a place where reversion to an HSC phenotype could be induced in a slightly more mature cell type. Only the latter observation remains controversial.

Long-term bromodeoxyuridine (BrdU)-label-retaining cells reside in the hypoxic areas distant from the endothelial-lined microvasculature and sinusoidal blood vessels of the bone marrow and closely attached to the non-endothelial extracellular matrix of the trabecular endosteum (Fig. 47.4). A similar spatial distribution of HSCs was observed near the endosteal region by 15

hours after bone marrow transplantation [296]. Subsequent studies using live microscopy techniques and genetic manipulations identified the endosteal region of the BM as a preferential site of residence for the most potent HSC with serial transplantation potential [297]. As discussed in the next section, opposite results were obtained by Kiel et al. [60]. Both CD105+Thy-1- and CD146+ mesenchymal cells have been shown to produce reticular and endosteal components of the endosteal reguion [138]. Osteoblasts and their progenitors lining the trabecular bone surfaces within the BM cavity can modulate the activity of HSCs and have been considered critical cellular elements of an 'endosteal' or 'osteoblastic' niches [297–297b]. Osteoblasts support the maintenance of long-term culture-initiating cells (LTC-IC) *in vitro* by produce factors that have the ability to regulate quiescence and maintenance of HSCs [298–298a].

Osteopontin is a niche component that negatively regulates stem cell pool size [299]. SDF1 (CXCL12) binds to its receptor CXCR4 expressed on HSCs and various progenitors, and has a multifaceted role in regulating HSC migration from, or homing to, BM niches. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and upregulating CXCR4 [177]. Low levels of calcium, detected by calcium-sensing membrane receptors in the parathyroid stimulate the release of parathyroid hormone (PTH) that increases the number and activity of osteoclasts to release calcium from bone, and thus stimulates bone resorption. Receptor activator of NF-kappa B ligand (RANKL)-activated osteoclasts, decreases the expression of CXCL-12, osteopontin and c-Kit ligand (KL, also known as stem cell factor -SCF) in the endosteum, and caused the egress of HSCs from the niche [178]. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency [300] whereas expression of constitutively active PTH/parathyroid hormone-related protein (PTHrP) receptor increased trabecular osteoblast numbers, which caused an expansion of LT-HSC [301].

Angiopoietin-1 (Angpt1) signaling via the Tie2 receptor regulates vascular and hematopoietic systems and regulates HSC quiescence in the bone marrow niche [302]. Enhanced Angpt1-Tie2 signaling affects the differentiation capacity of hematopoietic lineages during development and increases the repopulation ability of HSCs [303]. However, heterogeneous cell populations were found in endosteal regions that included mature bone lining cells, osteoblasts, and preosteoblasts (reviewed in reference [304]). Moreover, endosteal regions are highly vascularized [305] raising the possibility that vascular structures, in addition to osteoblasts, could also participate in the endosteal niche.

Vascular and perivascular niches

In addition to endosteal cells, other cell types likely participate in the regulation of HSCs, and more recent studies have pointed to the possibility of a vascular niche for HSCs. The observation that HSCs also reside at a distance from the endosteum triggered the concept of the vascular niche as alternative niche in BM [60,306]. Analyses of BM sections using SLAM family markers (CD150+CD244−CD48−CD41−) showed that about 60% of SLAM family HSCs were localized in the vicinity of sinusoidal endothelial cells (SECs) suggesting the presence of an additional niche in the vascular area [60,306] (Fig. 47.4). Interestingly, the majority (95%) of perisinusoidal HSCs was located within a five cell layer of the sinusoidal endothelium; in contrast only 8–20% of HSCs were located within a corresponding distance of the endosteum [60]. Additional evidence for the vascular niche was provided from a study using the GFP reporter in the CXCL12 locus [307]. The study identified a specific population of CXCL12-abundant reticular (CAR) cells that secreted high levels of CXCL12, that interacted with over 90% of the HSCs Xie. CAR cell constitute the subendothelial (adventitial) layer of sinusoidal walls projecting a reticular process that is in close contact with HSCs [308] (Fig. 47.4). CXCL12 release from BM stromal cells to the circulation emerges as a pivotal mechanism essential for steady state egress and rapid mobilization of HSC and hematopoietic progenitor cells, but not mature leukocytes [309]. Neutralizing antibodies against CXCR4 or CXCL12 inhibited both steady state and AMD3100-induced CXCL12 release and reduced

egress of murine HSC. Norepinephrine treatment, mimicking acute stress, rapidly increased CXCL12 release and HSC mobilization, whereas β2-adrenergic antagonist inhibited both steady state and AMD3100-induced CXCL12 release and HSC mobilization [309].

Previous data had already supported important interactions between the BM vascularity and hematopoietic progenitors. A number of studies have shown that endothelial cells are as capable as MSC stromal cells in supporting the long-term maintenance of HSCs *in vitro* [310−314a]. Endothelial disruption resulted in impaired thrombopoiesis under physiologic and stress conditions and Tpo is one of the critical regulators of HSC self-renewal [315]. Studies have also revealed that HSCs are localized differentially in the niches with respect to their distinct functional status, and that the biological activity of each niche is differentially influenced by extrinsic conditions (reviewed in reference [284]). Using a combination of confocal microscopy and video imaging, Lo Celso et al. [316] determined the spatial fate of transplanted hematopoietic cells and demonstrated that osteoblasts are surrounded by microvessels. They concluded that the endosteal niche is indeed perivascular (reviewed in references [317,318]). The above studies lend support to the concept of a vascular niche for HSCs. Hooper et al. [319] used a combination of immunohistochemistry on BM sections and polyvariate flow cytometry on bone marrow cells using VEGFR3 and Sca1 to distinguish the BM arterioles from SECs. They showed that SEC marker expression is unique and does not overlap with osteoblastic or hematopoietic cells. BM SECs are localized in close proximity to osteoblasts, suggesting that endosteal and vascular niches may not be distinct, as previously proposed models suggested.

Ding et al. [320] found perivascular cells throughout the BM expressed c-Kit ligand. HSC frequency and function were not affected when KL was conditionally deleted from hematopoietic cells, osteoblasts or Nestin-expressing stromal cells. However, HSCs were depleted from BM when KL was deleted from endothelial cells or leptin receptor (Lepr)-expressing perivascular stromal cells. Most HSCs were lost when KL was deleted from both endothelial and Lepr-expressing perivascular cells [320]. Within the BM, the c-Kit-expressing HSCs were positioned in proximity to Kit ligand (KL)-expressing niche cells [321−321a]. This c-Kit-mediated cellular adhesion was essential for long-term maintenance and expansion of HSCs. Conditional deletion of c-Kit resulted in hematopoietic failure and splenic atrophy both at steady state and after marrow ablation leading to the demise of the treated adult mice [321]. Thus, HSCs reside in a perivascular niche in which multiple cell types express factors that promote HSC maintenance.

Murine SLAM CD150-expressing HSC localize in various sites after *in vivo* injection, including endothelial niches provided by the sinusoids of spleen and BM, as well as endosteal niches [60,321b] (Fig. 47.4). The presence of the glycosaminoglycan hyaluronic acid on HSC appears critical for the spatial distribution of transplanted HSCs *in vivo* [298a]. Furthermore, binding of hyaluronic acid by a surrogate ligand results in marked inhibition of HSC proliferation and granulocyte differentiation. A unique feature of the bone niche for HSCs is the high concentration of calcium ions at the endosteal surface. HSCs express a calcium-sensing receptor (CaR), and mice deficient in CaR had normal numbers of HSCs in the fetal liver, but were highly deficient in HSCs localizing to the endosteal niche [322]. Further evidence for the importance of the osteoblast niche has been provided by studies in mice with genetic modifications leading to increased numbers of osteoblasts. Mice with constitutively activated parathyroid receptors have enhanced numbers of osteoblasts associated with a doubling of BM Sca-1+c-Kit+Lin− cells and increased LTC-IC and long-term repopulating HSCs [301]. Osteoblasts with activated receptors produced high levels of the Notch ligand Jagged 1 which may be responsible for HSC expansion, since HSC expansion was abrogated following treatment with a gamma secretase inhibitor that blocked Notch activation.

Mutant mice with conditional inactivation of the BMP receptor 1A had an increase in the number of spindle-shaped N-cadherin+, CD45- osteoblasts [262]. This was linked to a

2.4-fold increase in quiescent HSC and competitive repopulating cells in the marrow. β-catenin forms a complex with N-cadherin and this interact may play a role in retaining HSC in osteoblastic niches (Fig. 47.4). c-Myc was also reported to control the balance between HSC self-renewal and differentiation, possibly by regulating the interaction between HSCs and their niches (reviewed in references 323–325). Conditional elimination of c-Myc activity in BM resulted in severe cytopenia, and accumulation of self-renewing HSCs *in situ* with impaired differentiation. The c-Myc deficient HSC appear trapped in stem cell niches, possibly due to upregulation of N-cadherin and a number of adhesion molecules [324,325]. Enforced c-Myc expression in HSC repressed N-cadherin and integrins leading to loss of self-renewal at the expense of differentiation. The role of the cell adhesion molecule N-cadherin in HSC maintenance has been controversial. Some studies reported that N-cadherin was expressed in HSCs and played a critical role in the regulation of HSC engraftment (reviewed in reference [326]).

Overexpression of N-cadherin in HSCs was claimed to promote quiescence and preserved HSC activity during serial BM transplantation, and inhibition of N-cadherin by the transduction of N-cadherin shRNA was reported to reduce the lodgment of donor HSCs to the endosteal surface, resulting in a significant reduction in long-term engraftment. shN-cadherin-transduced cells were maintained in the spleen for six months after BM transplantation, indicating that N-cadherin expression in HSCs may be specifically required only in the BM (reviewed in reference [326]). However, the consensus is that HSCs do not depend on N-cadherin to regulate their maintenance [306,327–329]. Greenbaum et al. [327] conditionally deleted N-cadherin in osteoblasts, and showed that basal hematopoiesis, HSC number, cell cycle status, long-term repopulating activity and self-renewal capacity were normal. These mice responded normally to G-CSF and engraftment of wild-type cells into N-cadherin-deleted recipients was normal. Thus N-cadherin expression in osteoblast lineage cells is dispensable for HSC maintenance in mice. Bromberg et al. [328] also used a murine model in which deletion of N-cadherin had been targeted to cells of the osteoblastic lineage. This resulted in an age-dependent bone phenotype, ultimately characterized by decreased mineralized bone, but there was no difference in steady state HSC numbers or function at any time tested, and recovery from myeloablative injury was normal.

Gomes et al. [330] demonstrated that hypercholesterolemia in mice for 30 days induces dramatic alterations in hematopoiesis through CXCL12-mediated enhanced interaction of the hematopoietic cells with specialized BM sinusoidal endothelial cells. This results in thrombocytosis, lymphocytosis, and increased mobilization of HSC and progenitor cells to the peripheral circulation, possibly contributing to atherosclerosis.

The extracellular matrix (ECM) is a major anatomical component of the hematopoietic microenvironment of the BM niches and comprises fibrous proteins, such as types I and IV collagen and fibronectin (FN), and non-fibrous proteins, such as tenascin-C (TN-C). The expression of tenascin-C (TN-C) is dramatically upregulated in stromal cells and endothelial cells during hematopoietic recovery after myeloablation [331]; reviewed in reference [280]. TN-C expression is limited to the endosteal regions and functions by binding with high affinity to specific integrins such as integrin α9 or to other matrix proteins such as FN. TN-C stimulates proliferation of HSCs in an integrin α9-dependent manner and upregulates the expression of the cyclins (cyclinD1 and cyclinE1) and downregulates the expression of the cyclin-dependent kinase inhibitors [p57(Kip2), p21(Cip1), p16(Ink4a)]. Mice lacking TN-C showed normal steady state hematopoiesis; however, they failed to reconstitute hematopoiesis after BM ablation and showed high lethality [331].

A quiescent state is probably critical for the maintenance of HSCs (reviewed in reference [332]). HSCs expressing the Tie2 receptor are quiescent, anti-apoptotic and comprise a side population that adheres to osteoblasts in the BM niche [302]. The Tie-2 ligand, angiopoietin-1, induced HSC quiescence, their adherence to the endosteal surface, cobblestone formation and

retention of long-term repopulating activity. Disruption of gp130 in Tie-2 expressing cells resulted in dilatation of the vascular sinusoids and BM dysfunction [333]. This phenotype was reproduced when wild-type hematopoietic cells were transplanted into gp130$^{-/-}$ mice, indicating their microenvironmental origin in endothelial components [333].

The matrix glycoprotein, osteopontin (OPN), acts as a negative regulatory restraining factor on HSC within the osteoblast niche [299,334]. Osteoblasts at the endosteal bone surface produce varying amounts of osteopontin in response to stimulation and HSC specifically bind to OPN via β1 integrin. Exogenous OPN potently suppresses the proliferation of HSC *in vitro* and OPN-deficient mice have increased numbers of HSC with markedly enhanced cycling.

Using a combination of confocal microscopy and video imaging Lo Celso et al. [316] determined the spatial fate of transplanted hematopoietic cells, demonstrated that osteoblasts are surrounded by microvessels and concluded that the endosteal niche is indeed perivascular (reviewed in reference [317]). The above studies further support the concept of a vascular niche for HSCs. Hooper et al. [319] used a combination of immunohistochemistry on BM sections and polyvariate flow cytometry on bone marrow cells using VEGFR3 and Sca1 to distinguish the BM arterioles from SECs. This SEC phenotype was unique and did not overlap with that of the osteoblastic or hematopoietic cells [316]. Since the BM SECs are localized in close proximity to osteoblasts, it would suggest that endosteal and vascular niches are not as distinct as previously proposed models have suggested.

CONCLUSION

The current dogma recognizes that specific factors determine specific cell fates, ranging from stem cell (HSC) self-renewal through commitment to common myeloid (CMP) or common lymphoid progenitors (CLP) to lineage restricted progenitor differentiation. A contrary hypothesis to current dogma recognizes that HSC fate may instead be governed by (small) quantitative shifts in the relative activation of known signaling pathways [335]. Alterations in the relative levels of pathway activation may arise from dynamic shifts in the expression of signaling components (e.g., receptors, ligands). As extracellular conditions vary over small ranges, cells are induced to overcome signaling threshold barriers, and as a consequence, adopt new, stable cell fates. *Ex vivo* HSC culture systems can be manipulated:

1) Quantitatively, e.g., by varying the magnitude of signaling pathway activation influencing cell fate decisions;
2) Temporally, e.g., be defining windows of opportunity for stimulation;
3) Spatially, e.g., by fixed location-dependent signaling (niches).

While there has been remarkable progress over the last five years in increasing our understanding of the nature of HSCs, many questions still remain. The answers may come from in depth analysis of gene expression using RNA deep sequencing and mass Spec-based proteomic strategies that are able to extract data from small cell samples. These methods may be applied to HSC from multiple sources, highly enriched by improvements in multiparameter fluorescence activated cell sorting, and better *in vitro* HSC expansion strategies.

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CHAPTER 48

Blood Components from Pluripotent Stem Cells

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INTRODUCTION

For decades, supplies of transfusable blood components have failed to keep up with increasing demand. This continual shortfall has prompted efforts to develop safe and effective blood substitutes which can be produced from inexhaustible and non-immunoreactive sources and in limitless quantities. This chapter summarizes recent efforts to develop alternative sources for red blood cells, platelets, and various subtypes of white blood cells.

Given their potential to differentiate into any cell type in the body, human embryonic stem cells (hESCs) have been touted as the future of regenerative medicine. Unlike adult stem cells, hESCs are capable of expanding indefinitely in culture without losing their pluripotency, and this makes them an attractive cell source to be used for the large-scale production of a variety of therapeutic cell types [1]. Adding another dimension to the field of regenerative medicine, the advent of human induced pluripotent stem cells (iPSCs) may allow patient-specific therapies to be produced [2,3], thus circumventing potential issues with Human Leukocyte Antigen (HLA) mismatching and immuno-incompatibility. While each has its own advantages and disadvantages, hESCs and iPSCs represent two pluripotent stem cell sources with far-reaching clinical potential in treating neurologic disorders, repairing or replacing damaged tissues, and as detailed here, producing transfusable blood components.

Hematopoietic stem cells (HSCs) located within the bone marrow normally give rise to and are responsible for replenishing all mature cells within the adult blood system. Arising from HSCs, common myeloid progenitor (CMPs) are capable of differentiating into erythrocytes, megakaryocytes/platelets, monocytes, and granulocytes while common lymphoid progenitors (CLPs) eventually produce natural killer, T, and B cells (Fig. 48.1). By differentiating hESCs/iPSCs down particular hematopoietic lineages, researchers have already been able to obtain highly enriched populations of *in vitro* generated blood components. Each of the mature cell types within the blood system can be used for distinct clinical purposes and this chapter will focus on the ability of hESCs/iPSCs to serve as substitutes for primary cells in these endeavors.

RED BLOOD CELLS

Erythrocytes or red blood cells (RBC), the oxygen-carrying component of the blood, are the most plentiful cell type in the peripheral blood and comprise approximately 40–45% of total blood volume with a concentration of 5×10^{12} cells/liter (Fig. 48.1). Despite the body's

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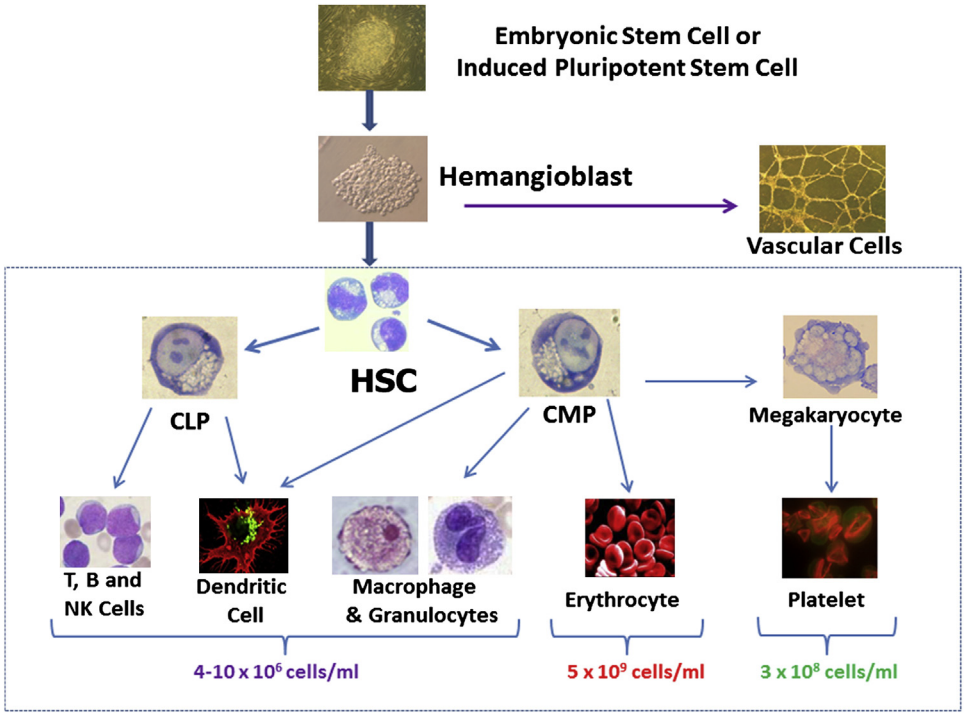

FIGURE 48.1
Simplified schematic of hematopoietic differentiation from human pluripotent stem cells. Human embryonic stem cells and induced pluripotent stem cells are able to recapitulate hematopoietic differentiation *in vitro* after initial differentiation to hemangioblasts, bipotential progenitors for both vascular and hematopoietic lineages. The boxed region shows hematopoietic differentiation as it is thought to occur *in vivo*. Hematopoietic stem cells (HSC) undergo successive stages of differentiation to give rise to progenitor cells in both the myeloid lineage and lymphoid lineage. These progenitors will undergo further differentiation to eventually give rise to mature cells within the peripheral blood. Numbers under each type of cellular blood components are normal cell numbers per milliliter of human peripheral blood.

seemingly abundant capacity to produce RBCs, they are transfused in over half of all anemic patients admitted to intensive care units in the United States [4–6] and it is estimated that nearly 5 million patients receive approximately 16 million units of RBCs per year in the United States alone [7]. Limitations in the supply of RBCs can have potentially life-threatening consequences for patients, specifically for those who have rare or unusual blood types with massive blood loss due to trauma or other emergency situations. Unfortunately, the supply of transfusable RBCs, especially 'universal' donor type (O)Rh$^-$, is often insufficient, particularly in the battlefield environment and/or major natural disasters due to the lack of blood type information and the limited time required for life saving transfusion. Moreover, the low prevalence of (O)Rh$^-$ blood type in the general population (<8% in Western countries and <0.3% in Asia) further intensifies the consequences of blood shortages for emergency situations where blood typing may not be possible. The derivation of (O)Rh$^-$ RBCs from hESCs/iPSCs clearly offers an attractive option for alleviating the constant shortage in donated RBCs.

Erythropoiesis

Erythropoiesis is a highly regulated, multistep process by which the body generates mature RBCs. During mammalian development, erythropoiesis consists of two major waves:

1) Primitive erythropoiesis, which is initiated in the yolk sac with the generation of large *nucleated* erythroblasts, and
2) Definitive erythropoiesis, which arises from the fetal liver with the development of smaller *enucleated* erythrocytes [8].

Definitive erythropoiesis in fetal liver features the production of enucleated RBCs that quickly become dominant in embryonic circulation. The switch of hemoglobin from embryonic ($\zeta_2\varepsilon_2$ and $\alpha_2\varepsilon_2$) to fetal types ($\alpha_2\gamma_2$) also occurs at the initiation of definitive erythropoiesis [9–11]. In the adult bone marrow (BM), definitive erythropoiesis begins when an HSC-derived CMP commits to the erythroid lineage. The appearance of the

pronormoblast (also called proerythroblast or rubriblast) marks the first stage of differentiation and is subsequently followed by early, intermediate, and late normoblast (erythroblast) stages, at which time the nucleus is expelled and the cell becomes a reticulocyte. Upon exiting the BM, reticulocytes enter the circulation to become fully mature RBCs, expressing adult forms of hemoglobin ($\alpha_2\beta_2$) and delivering oxygen to tissues of the body. They circulate for about 120 days before they are engulfed by macrophages and recycled [12].

RBCs from adult stem cells

RBCs can be derived from a variety of primary stem cell sources including umbilical cord blood (CB), peripheral blood (PB) and BM. $CD34^+$ cells from CB, PB and BM have been isolated and differentiated into erythrocytes with high purity after weeks of culture with a combination of cytokines including erythropoietin (EPO), stem cell factor (SCF), and interleukin-3 (IL-3) [13–15]. Co-culturing with mouse MS-5 stromal cells or human mesenchymal stem cells facilitates enucleation, which is the hallmark of mature RBCs, and is likely to impart flexibility and improve the ability to traverse through capillaries that are half the size of erythrocytes. The stem cell-derived erythrocytes have similar properties to normal RBCs, including membrane deformation capacity, intrinsic enzymatic activity, and balanced adult/fetal forms of hemoglobin that can bind and release oxygen. Additionally, these erythrocytes have been found to survive *in vivo* in NOD/SCID mice, being detectable three days after transplantation. Recently, Douay and his colleagues showed that autologous RBCs generated under good manufacturing practice (GMP) conditions from PB $CD34^+$ cells behaved the same as their natural counterparts do when re-infused into the autologous recipient [16]. This first preclinical study provided proof of principle for transfusion of *in vitro* generated RBCs and the necessary safety data that could support an investigational new drug application. Despite their potential utility and substantial *in vitro* expansion using bioreactors [17], however, these primary cells still represent donor-limited sources of blood substitutes.

RBCs from embryonic stem cells

Human embryonic stem cells (ESCs) represent an alternative stem cell source for generating blood components, one whose capacity for expansion far exceeds that of BM, PB, or even CB. Two different *in vitro* differentiation methods have been widely used to generate RBCs from hESCs:

1) Embryoid body (EB) formation whereby hESCs are initially allowed to cluster and form three-dimensional spheres prior to creating single cell suspensions; or
2) Co-culturing hESCs on top of animal stromal feeder cell layers.

While somewhat different in approach, both systems have encountered the same obstacles in generating fully mature adult RBCs. For example, Chang et al. generated erythroid cells from hESCs by isolating and expanding non-adherent cells of day-14 EBs for an additional 15 to 56 days [18]. The resulting cells co-expressed high levels of embryonic ε- and fetal γ-globins but little or no adult β-globin. In addition, the cells had not enucleated. In other studies, Olivier et al. [19] and Qiu et al. [20] used sequential stroma co-culture steps with FHB-hTERT human fetal liver stromal cells and mouse MS5 cells from 2 to 5 weeks to produce hESC-derived erythrocytes. Despite their careful multistep approach and relatively large yields (0.5 to 5×10^7 cells), the resulting cells had similar problems to those generated by the EB method; they mainly expressed embryonic (ε) and fetal (γ) globin isoforms, with only a limited amount of adult β-globin being detectable [19,20]. Despite these reports, other studies suggests that specific types of stroma can, in fact, facilitate the expression of adult β-globin in developing erythrocytes. Using immunostaining with globin chain specific monoclonal antibodies, Ma et al. showed that almost 100% of hESC-derived erythrocytes expressed the adult β-globin chain after co-culture with murine fetal liver derived stromal cells (mFLC) [21], however the β-globin content and identity was not verified by an accurate

physical method such as HPLC or mass spectrum. Yet, the majority of the cells still had not enucleated, a problem that does not seem to have an easily explainable mechanism or simple solution.

Not only must *in vitro* generated RBC substitutes be fully matured and enucleated to use in the clinic, but they must also be capable of large-scale production. Related to this issue, we recently developed a strategy that efficiently and reproducibly generates functional hemangioblasts (the common precursor cell to all hematopoietic and endothelial cell lineages) using a serum-free culture system and have been able to do so with high purity (>95%) and in a relatively large scale [22,23]. Using the hemangioblast system, we have generated functional RBCs (blood types A, B, O, and both RhD^+ and RhD^-) on a large scale from multiple hESC lines (Fig. 48.2), approximately 10^{10} to 10^{11} erythroid cells were generated from a single six-well plate of hESCs [24], which is over a thousand-fold more efficient than previously reported methods [19,20]. Oxygen equilibrium curves of hESC-derived erythroid cells were comparable to normal transfusible RBCs and responded to changes in pH and 2,3-diphosphoglyerate (Fig. 48.2). During the course of our studies, we found that extended *in vitro* culture facilitated further maturation of these erythroid cells, including a progressive decrease in size, increased expression of the erythrocyte cell surface marker, glycophorin A (CD235a), as well as chromatin and nuclear condensation. When the extended culture was performed on OP9 stromal cells, it resulted in the extrusion of the pycnotic nucleus in up to 65% of cells and the generation of enucleated erythrocytes with a diameter of approximately 6–8 μm, similar to their natural counterpart. At this stage, the erythrocyte population is nearly 100% positive for glycophorin A, has a very high content of hemoglobin, and expresses ABO antigen. Although the cells were found to express fetal and embryonic globin chains, globin chain specific-PCR and immunofluorescent analyses showed that after extended culture, expression of adult β-globin increased from 0% to 15%. Overall, these results show that it is feasible to differentiate and mature hESCs into functional oxygen-carrying erythrocytes on a large scale. The identification of a hESC line with a $(O)RhD^-$ genotype would permit the production of ABO and RhD compatible (and pathogen-free) 'universal donor' RBCs [24]. While considerable effort is still needed to bring hESC-derived RBCs to clinical trials, these efforts certainly provide a promising lead.

RBCs from induced pluripotent stem cells

The successful reprogramming of somatic cells into a pluripotent state has been achieved by ectopic expression of various combinations of transcription factors such as Oct4, Sox2, Klf4, c-Myc, LIN28 and Nanog cells [2,3]. The derivation of these iPSCs is less controversial than that of hESCs and thus, they open up an exciting new route to obtain pluripotent stem cells. Moreover, the fact that iPSCs can be produced in a patient-specific manner will eliminate the issue of immuno-rejection in cell, tissue, or organ replacement therapies in the future. A number of groups including our own have published methods for generating RBCs from iPSCs [25–34]. These erythrocytes possess classic morphology, express glycophorin A, and have abundant hemoglobin content. As observed in the hESC differentiation systems, regardless of their origin, iPSCs generate RBCs expressing mostly fetal hemoglobin (hemoglobin F). RBCs expressing hemoglobin F are slightly less efficient than those expressing adult hemoglobin for oxygen delivery. However, patients who retain expression of hemoglobin F in adult life are not anemic. Thus, the fetal phenotype of RBCs derived from iPSCs derived from patients with hemoglobinopathies is not necessarily a barrier for autologous transfusion. The majority of the cells derived from iPSCs consisted of ortho-chromatic erythroblasts, enucleated RBCs accounted for less than 10% of the culture. Thus, there is a long way to go in terms of achieving 100% enucleation *in vitro*.

However, our study revealed some intrinsic molecular and cellular abnormalities in the iPSC derivatives, such as increased apoptosis, limited CFU capability and limited expansion [34].

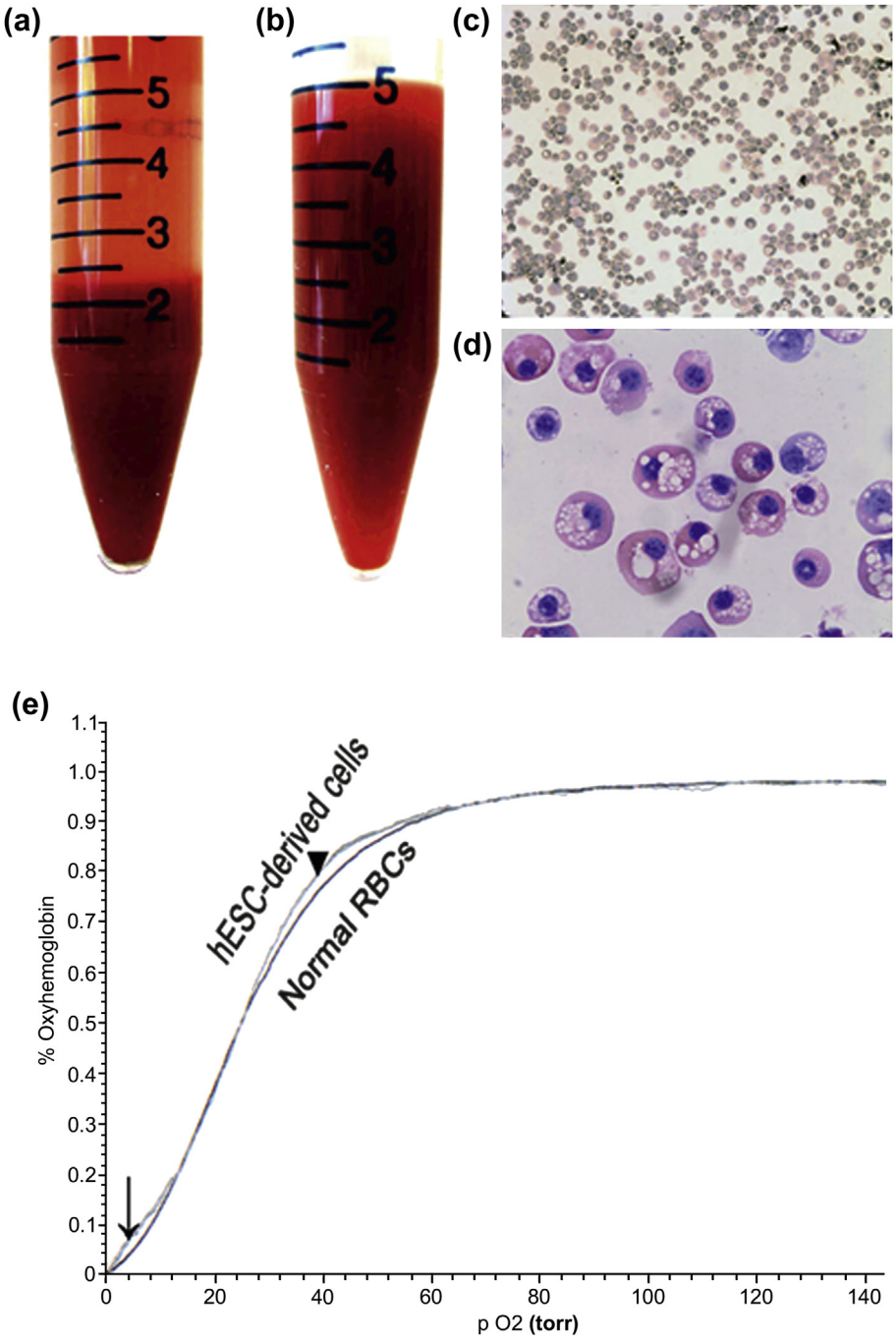

FIGURE 48.2
Large-scale production of erythroid cells from hESCs. (a) Erythroid cells (pellet) derived from 2×10^6 hESCs; (b) Erythroid cells from panel a were resuspended in equivalent hematocrit of human whole blood; (c,d) Morphology of erythroid cells derived from human ESCs (c, ×200; d, ×1000); (e) Functional characterization of hESC-derived erythroid cells. Oxygen equilibrium curves of normal human erythrocytes and human ESC-derived erythroid cells. The two curves are virtually indistinguishable at their midpoints, whereas the curve of human ESC-derived erythroid cells is leftward shifted at low (→) and high (▼) oxygen saturation percentages. *(This research was originally published in Blood. Lu et al. Biologic properties and enucleation of red blood cells from human embryonic stem cells, Blood 2008; 112:4475—4484. © the American Society of Hematology.)*

The exact cause(s) of these abnormalities is unclear at this time but may be due to alterations caused by the modified genome of virally reprogrammed cells. In spite of transgene silencing after reprogramming, Dias et al. [28] reported a background of transgene expression in differentiated cells obtained from transgenic hiPSCs and showed that it can affect their properties. However, these authors observed no difference in erythroid differentiation between transgene-free and transgenic hiPSC lines. Our recent data showed that viral-free hiPSCs have much improved hemangioblast and erythroid lineage capability than hiPSCs carrying viral DNA. The hemangioblast colonies derived from viral-free iPSCs displayed healthy morphology, large colony size comparable to hESC-derived hemangioblasts, and solid further expansion capability. Upon further erythroid differentiation, a relative large quantity of RBCs

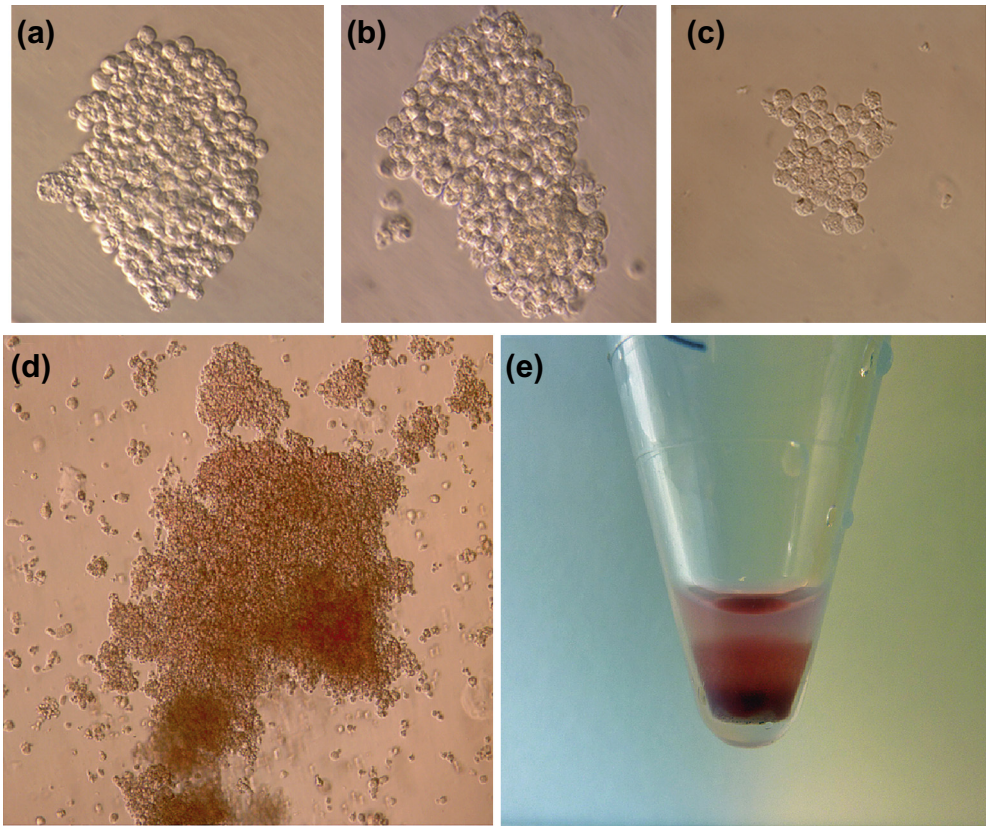

FIGURE 48.3

Transgene-free iPSCs show comparable hematopoietic differentiation capability as hESCs. (a) Hemangioblast colony derived from hESCs (×400); (b) hemangioblast colony derived from transgene-free iPSCs (×400); (c) hemangioblast colony derived from transgenic (viral-integrated) iPSCs (×400); (d) CFU-E from transgene-free iPSCs; (e). Erythrocytes from transgene-free iPSCs.

was obtained from these viral-free iPSCs (Fig. 48.3). More research will be needed to figure out the mechanism underlying the disparate observations.

Another new approach for RBC generation involves the direct differentiation of human fibroblasts to multipotent hematopoietic progenitors, thus bypassing the need for pluripotent stem cells like hESCs or iPSCs altogether [35]. In this study, Szabo et al. used ectopic Oct4 to transdifferentiate fibroblasts directly to $CD45^{+}$ hematopoietic progenitors, and by exposing them to EPO, were able to produce erythroid lineage cells that expressed high levels of adult β-globin, low levels of fetal γ-globin, and were capable of enucleation [35]. Further investigation will be required to determine which starting cell source, hESCs, iPSCs, or Oct4-transduced fibroblasts, will be the most useful for the development of *in vitro* generated RBC substitutes.

The manufacture of safe and effective red blood cell substitutes will help alleviate many of the risks, complications, and hardships associated with donor dependent RBC sources. As summarized here, significant progress has been made towards this end by manipulating the differentiation potential of hESCs and iPSCs and driving them towards erythrocyte development. *In vitro* differentiation systems that can be scaled-up for mass production of RBC substitutes have already been developed and the hemangioblast methodology described in this chapter represents one such possibility. Despite many exciting advances with *in vitro* culture systems, problems associated with the final stages of erythrocyte maturation, namely enucleation and globin switching will still need to be fully resolved before hESC/iPSC-derived RBCs can be produced in a stroma-free manner, scaled-up for mass production,

and brought to the clinic. Another obstacle for application of these methods is that they require very high cost to produce sufficient number of RBCs that are applicable in the clinic.

MEGAKARYOCYTES AND PLATELETS

Platelets (thrombocytes), anucleate discoid-shaped cell fragments, are generated from large (50 to 100 μm in diameter) multinucleated (up to 128 N) megakaryocytes (MK). Given their central role in hemostasis (the stoppage of blood loss at sites of vascular injury) and vascular repair, it is no surprise that platelets are incredibly abundant in the peripheral blood. With a concentration of $\sim 3 \times 10^{11}$/liter, their levels are second only to those of RBCs (Fig. 48.1). However, compared to RBCs, platelets have a rather short life span, lasting only 7–9 days in the circulation. Given their high rate of turnover, a serious condition called thrombocytopenia (platelet counts are $<50 \times 10^3$/μl) can occur if platelet production is somehow defective as in patients with liver failure or leukemia, or if platelets are being destroyed, as in patients undergoing chemotherapy. Platelet transfusions can be given to those suffering from life-threatening thrombocytopenia but, due to high demand and limited shelf life, transfusable platelets are often in short supply [36]. Furthermore, refractoriness occurs in approximately one out of every three patients who require repeated platelet transfusions [37,38] and while both immunological and non-immunological complications may be to blame, HLA alloimmunization is the primary cause of refractoriness [7,38]. Over the past few decades, a steady increase in demand for platelets in combination with their limited shelf life has presented a constant challenge for blood centers and donor dependent programmes. Scientists and clinicians have become increasingly interested in developing alternative sources, like pluripotent stem cells, for functional transfusable platelets.

Megakaryopoiesis and thrombopoiesis

Megakaryocytes, the precursor of platelets, provide a constant source of platelets to the blood system and are themselves produced through a process called megakaryopoiesis. As with RBCs, MKs are generated through the initial differentiation of HSCs into CMPs. Progressive commitment of CMPs to the megakaryocyte lineage is principally regulated by thrombopoietin (TPO). The maturation of megakaryocyte involves an increase in expression of the cell surface markers, GPIIb/IIIa (also known as CD41 or αIIb/βIII integrin receptor) and GPIb/GPIX/GPV receptors and a substantial increase in cell mass (50 to 100μm in diameter), which results in cytosolic accumulation of α granules, dense bodies, and platelet-associated proteins like von Willebrand factor (vWF) and platelet factor-4 [39,40]. Several rounds of endomitosis lead nuclear polyploidization and cells with up to 128N in DNA content [41,42]. Once polyploid MKs are produced, cellular processes on the MK body called 'proplatelets' begin to appear, with their eventual fragmentation and release resulting in the generation of platelets. On a molecular level, thrombopoiesis is a highly coordinate process, with sophisticated reorganization of membrane and microtubules and precise distributions of granules and organelles [43]. Despite the mechanism of platelet generation from MKs is not completely understood, it appears to be extremely efficient *in vivo*, with 2,000–10,000 platelets being produced per MK [44,45].

Megakaryocytes/platelets from adult stem cells

In theory, megakaryopoiesis and thrombopoiesis can be performed *in vitro*, the first evidence of which was reported in 1995 using CD34+ HSCs [46]. Matsunaga et al. [47] used an *in vitro* culture system to demonstrate the feasibility of producing functional platelets for clinical use. A 3D culture system described by Sullenbarger et al. [48] provides additional evidence that it is possible to produce platelets in scalable quantities. Platelets generated from these studies demonstrated aggregation capacity when stimulated with either ADP or thrombin, the physiological agonists for normal blood platelets. Subsequent studies have shown that

HSCs from PB, BM, and CB are also capable of producing megakaryocytes and functional platelets [49–51]. Yet, the limited *in vitro* expansion capabilities of primary $CD34^+$ cells make these cells unable to replace donation as a source of platelets. For this reason, hESCs may be a better starting cell population for large-scale *in vitro* production.

Megakaryocytes/platelets from embryonic and induced stem cells

The first study to report the *in vitro* production of MKs from hESCs was published in 2006 using an OP9 co-culture method [52], yet the MKs rarely generated any proplatelet-like structures. Since then, one group has reported the successful generation of both MKs and functional platelets from hESCs and, more recently, from iPSCs [53,54]. In these studies, Takayama et al. co-cultured hESCs or iPSCs on C3H10T1/2 stromal cells for 14–15 days, then handpicked sac-like structures containing hematopoietic progenitors and replated single cell suspensions onto fresh stroma in medium containing TPO, stem cell factor (SCF), and heparin for 9–23 days. During this time, polyploid, CD41a/CD42b double positive MKs emerged and produced platelets containing characteristic morphology, as assessed by electron micrography [53]. A variety of *in vitro* tests confirmed platelet functionality, while in their most recent study, a laser-induced vascular injury model was used to show that their iPSC-derived platelets readily incorporate into newly developing thrombi *in vivo* [54].

The use of both serum and animal feeder layers throughout the above mentioned studies hinders the ability of these methods to be adapted for clinical use. Additionally, as hand-picking of sac-like structures is both time- and labor-intensive, alternative methods will likely have to be developed for large-scale production. Towards this end, we have been able to use the hemangioblast system described above for RBC, as an alternative, serum- and feeder-free method for the generation of MKs [55]. Yet, similar to Takayama's studies, we also found that efficient platelet generation from MKs still requires conventional stroma co-culture. Differential-interference contrast and electron microscopy analyses showed ultrastructural and morphological features of hESC-derived platelets were indistinguishable from those of normal blood platelets. In functional assays, hESC-derived platelets adhered to and spread on fibrinogen, vWF, and type I collagen-coated surfaces in respond to thrombin stimulation, formed micro-aggregates, and facilitated clot formation/retraction when stimulated with physiological agonists. Live cell microscopy demonstrated that hESC-platelets formed lamellipodia and filopodia in response to thrombin activation, and tethered to each other as observed in normal blood. Importantly, fluorescence intravital microscopy studies demonstrated that hESC-derived platelets incorporated into developing mouse platelet thrombi at sites of laser-induced arteriolar wall injury in a manner and degree similar to that observed for normal human blood platelets, providing the first evidence for *in vivo* functionality of hESC-derived platelets [55] (Fig. 48.4).

The studies described above provide an important proof of principle for the *in vitro* manufacturing of functional platelets from hESCs/iPSCs, yet the efficiency of platelet production will need to be significantly improved in order to achieve clinically relevant yields. Using animal stromal cells for *in vitro* platelet generation from hESCs/iPSCs is another major obstacle for clinical translation. Our recent studies demonstrated that MKs generated from hESCs or iPSCs can efficiently produce functional platelets without animal stromal cells, which should be beneficial for future applications. Considering the optimal *in vivo* capacity of megakaryocyte development from HSCs and platelet production from megakaryocytes, both the initial hematopoietic amplification stage and downstream platelet biogenesis could stand to be optimized. Strategies to increase the efficiency of megakaryocyte/platelet production include the development of novel culture systems that mimic the *in vivo* BM microenvironment as well as the optimization of media formulations and concentrations of cytokines, small molecule mimetics, and nutrients. For instance, TPO receptor agonists such as YM477, AMG531 have been found to enhance megakaryopoiesis and thrombopoiesis [56,57].

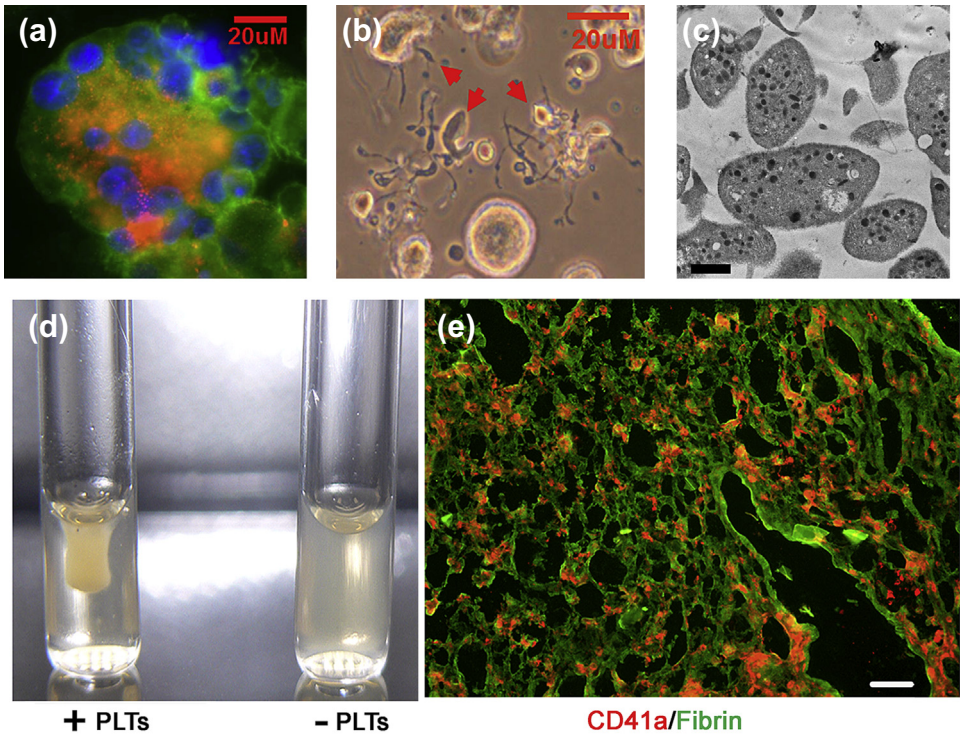

FIGURE 48.4

Generation and characterization of megakaryocytes (MKs) and platelets derived from hESCs. (a) Immunofluorescence of von Willebrand factor (vWF, red) and CD41 (green) proteins in MKs derived from hESCs. vWF is localized in the cytoplasm in a punctate pattern, which is typical for MKs. CD41 is expressed on the surface. DAPI (blue) stain shows polynuclei (polyploidy); (b) Phase contrast image of proplatelet forming MKs derived from hESCs; (c) Thin-section transmission electron microscopy of platelets generated from hESCs, Bar = 1 μm; (d) *In vitro* functional characterization of platelets generated from hESCs. Platelet-depleted human plasma was added with (+PLTs) or without (−PLTs) hESC-platelets (1.5×10^7/ml), and thrombin (2 U/ml) and $CaCl_2$ (10 mM) were then added to the suspensions to induce clot formation/retraction. No clot formation/retraction was observed without addition of hESC-platelets (−PLTs); (e), Clot cryo-sections were stained with anti-human CD41 (red) and anti-human fibrin (green) antibodies. Images were taken under a fluorescence microscope. Bar = 50 μm. *(This research was originally published in Cell Research. Lu et al, Platelets generated from human embryonic stem cells are functional* in vitro *and in the microcirculation of living mice, Cell Res. 21:530–545, 2011. © IBCB, SIBS, CAS.)*

In addition, physiological parameters such as pH, media viscosity, and oxygen levels may all be optimized for increased platelet biogenesis. Lastly, *in vivo* observations which helped formulate the proplatelet model of platelet biogenesis suggest that shear force could play an important role in platelet release [43,58]. Adaptation of such mechanical force in culture systems may also significantly promote proplatelet growth and platelet release.

WHITE BLOOD CELLS

Dendritic cells

Even though white blood cells (WBCs or leukocytes) only represent about 1% of the cells within the peripheral blood (Fig. 48.1), they play extremely important roles in protecting the body against viruses, bacteria, and the outgrowth of cancer cells. Straddling the interface between innate and adaptive immunity, dendritic cells (DCs) are one of the body's three main types of professional antigen-presenting cells (APCs). Clinically, DCs may be used in the development of vaccine-based therapies in order to stimulate specific T cell responses against a variety of disease-associated antigens [59,60]. Originating from HSCs, human DCs can develop through both myeloid and lymphoid lineage differentiation pathways. Myeloid (m) DCs arise from monocytes, secrete IL12 in response to activating stimuli, and express toll-like

receptors TLR2 and TLR4 (Fig. 48.1). Lymphoid lineage-derived DCs (plasmacytoid (p) DCs) have similar functional characteristics to mDCs, but secrete IFNα, and express TLR7 and 9 [61]. Once in contact with a suitable antigen, immature DCs become activated and undergo the process of maturation, which involves proteolyzing an antigen and presenting its fragments on the DC surface using MHC class I or II molecules for $CD8^+$ or $CD4^+$ T cells to recognize, respectively.

Innovative work performed in the late 1990s provided the proof of concept for clinical use of DCs as studies showed that *ex vivo* generated DCs (from allogeneic or autologous BM or PB sources) could be loaded with melanoma-specific antigens and stimulate anti-tumor immune responses once injected into patients [62,63]. Since then, other studies have shown that DCs exposed to whole killed tumor cells can also elicit specific cytotoxic CD8+ T cell responses [64]. Currently, over 200 clinical trials are underway to explore the safety and efficacy of DC-based vaccines for diseases such as melanoma, multiple myeloma, type I diabetes, HIV, and hepatits C viral infections (www.clinicaltrials.gov). In April 2010, Provenge (Silpuleucil T, developed by Dendreon) became the first DC-based vaccine therapy to gain full Food and Drug Administration (FDA) approval and is a treatment option for patients with metastatic castration-resistant prostate cancer [65]. Given the high cost of tailor-made autologous or allogeneic DC-based vaccines like Provenge, hESCs may serve as a cost-effective alternative cell source for the derivation and large-scale manufacture of antigen-primed DCs.

By applying methods established in the mouse ESC system, Slukvin et al. [66] were the first group to produce functional DCs from hESCs. They used a three-step differentiation method involving an initial OP9 stroma co-culture followed by two successive liquid culture steps and a purification procedure. A few other studies have since reported the generation of hESC-derived myeloid-lineage DCs using EB formation and have done so in a serum-free or serum- and feeder-free manner [67–69]. These hESC-derived DCs had characteristic large eccentric nuclei, spiny dendritic processes, and expressed DC surface markers CD11c, CD40, CD45, CD86, HLA class I, and HLA class II to varying degrees. Reported yields ranged from 2 DCs per hESC in one study [67] to 3–5 DCs per hESCs in a more recent study [68]. Our laboratory has similarly been able to produce both hESC-and iPSC-derived DCs with characteristic morphological features (Fig. 48.5). Despite subtle differences compared to monocyte-derived DCs, hESC-derived DCs produced in these studies and in our own lab appear to be functional upon maturation in assays measuring IL12p70 secretion, chemotaxis, antigen-uptake and proteolysis, induction of T cell proliferation, and stimulation of antigen-specific cytotoxic CD8+ T cell responses [67,68] (and unpublished data). Importantly, rigorous preclinical animal studies will still be needed before hESC-DCs can be further developed for clinical use.

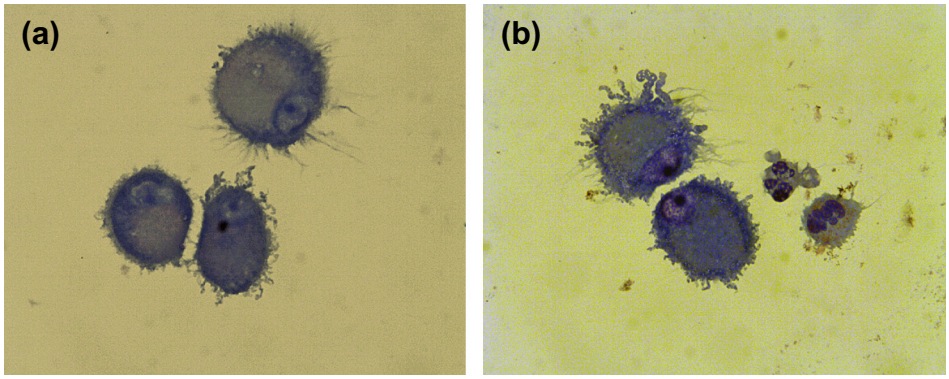

FIGURE 48.5
Dendritic Cells generated from human ESCs and iPSCs. Wright-Giemsa stains of dendritic cells from hESCs (a, x1000) and iPSCs (b, ×1000).

Natural killer cells

Arising from HSC-derived CLPs, human natural killer (NK) cells comprise approximately 1−2% of peripheral blood lymphocytes, or 0.01−0.02% of all cells in the PB and circulate for about 2−4 weeks [70]. Belonging to the innate immune system, they provide rapid, non-specific responses against various microbial infections and contribute to tumor cell detection and elimination [70]. NK cells mount a protective response if they encounter a cell with insufficient MHC I expression, yet, to prevent inappropriate cell killing, the process for surveying MHC I expression is rather complex. In brief, if a cell lacks sufficient self MHC I molecules, interplay between various activating and inhibitory signals help NK cells mount an appropriate protective response, being either cytokine release, natural cytotoxicity, or antibody-dependent cellular cytotoxicity (ADCC). $CD56^{bright}$, $CD16^{low}$, KIR^{low}, CD94- immature NK cells are the principal cytokine secretors while $CD56^{dim}$, $CD16^{high}$, KIR^{high}, $CD94^{+}$ mature NK cells are largely responsible for cytotoxicity responses [71].

Groundbreaking work in the 1980s by Rosenberg and colleagues stimulated considerable interest in and a variety of approaches for harnessing the cytotoxic capabilities of NK cells [72,73]. While the details of these studies are reviewed elsewhere [74], *ex vivo* stimulation and infusion of autologous or allogenic NK cells have now been used in experimental therapies for many different types of cancers with variable efficiency. With many improvements being made to clinical protocols over the years, over 200 clinical trials are currently being performed to evaluate the safety and efficacy of NK cell-based immunotherapy for leukemia, lymphoma, melanoma, glioma, renal cell carcinoma, and cancers of the breast, pancreas, lungs, head and neck (www.clinicaltrials.gov). The biggest hindrance to these adoptive transfer approaches thus far appears to be the difficulty in obtaining sufficient numbers of NK cells from peripheral blood mononuclear or lymphokine activated killer cell collections.

The use of hESCs for *in vitro* generation of NK cells may provide larger pools of suitable effector cells and thus may be able to overcome this hurdle. However, only one group, led by Dan Kaufman at the University of Minnesota has been able to successfully and reproducibly derive functional NK cells from hESCs [75,76]. Their optimized two-step differentiation procedure begins by co-culturing undifferentiated hESCs on M210-B4 stroma for 17−20 days, at which time, CD34+/CD45+ double positive cells, which represent <5% of all cells, are isolated from the culture and transferred onto AFT024 stroma in medium containing SCF, FL, IL7, and IL15. After 30−35 days total, a highly enriched homogenous population of $CD45^{+}CD56^{+}CD94^{+}$ NK cells emerge. *In vitro* assays showed they are capable of secreting IFNγ in response to IL12/IL18 stimulation, and also display potent natural cytotoxicity against K562 erythroleukemia cells and ADCC against Raji cells [75]. These hESC-NK cells were subsequently shown to harbor natural cytotoxicity against other types of cancer cells and displayed *in vivo* anti-tumor activity in a xenograft mouse model [76]. More recently, this same group has been able to successfully produce functional NK cells from iPSCs and shown that they harbor anti-HIV activity [77]. Despite these exciting findings, the requirement for two different types of stroma co-culture as well as the need to isolate rare CD34/CD45 double positive cells limits the utility of this approach for large-scale, cost-effective, clinical grade production of hESC/iPSC-generated NK cells. Further optimization will need to be performed before such hESC/iPSC-derived NK therapies can move into clinical trials.

T cells

As part of the adaptive immune system, T cells develop in the thymus and can be stimulated to mount antigen-specific immune responses against a variety of pathogens and cancer cells. They are present at a concentration of $\sim 1 \times 10^{9}$ cells/liter of peripheral blood, thus representing ~10% of WBCs or 0.1% of all circulating cells (Fig. 48.1). While a detailed background on T cell biology is beyond the scope of this article, T cells can generally be divided into six main subtypes based on function and cell surface marker expression: helper $CD4^{+}$ T

cells; cytotoxic $CD8^+$ T cells; long-lived $CD4^+$ or $CD8^+$ memory T cells; immunosuppressive regulatory T cells (Tregs); skin, gut, or lung-resident γδ T cells; and rare, CD1d-restricted NK T cells.

Clinical interest in T cells as therapeutic agents largely revolves around the isolation and *ex vivo* expansion of specific antigen-responsive helper and/or cytotoxic T cell subsets in order to generate a highly-specific immune response once infused into a patient. $CD4^+$ helper T cells will secrete particular cytokines in response to MHC II-presented antigens while $CD8^+$ cytotoxic T cells will respond to MHC I-presented antigens and unload cytotoxic enzymes to induce apoptosis in antigen-expressing target cells. Adoptive T cell therapy (ACT) was first described in 1988 as a treatment option for melanoma [78] and since then, many improvements have been and are still being made to increase the utility, safety, and efficacy of ACT protocols [79–81]. The risks and complexities involved in exploiting the adaptive immune system make T cell-based therapies incredibly expensive and still largely experimental. However, the power of ACT is large enough to warrant efforts that might be able to streamline or make the therapy more cost-effective. Towards this end, several groups have devised protocols to differentiate T cells from pluripotent human cell sources.

In 2006, the first report that hESCs could be differentiated into T cells was published [82]. In it, Galic et al. [82] cultured H1 hESCs on regular OP9 cells for 7–14 days whereupon, CD34+ cells were isolated and injected into a Thy/Liv implant within SCID or $RAG2^{-/-}$ mice. Within the thymus-like environment of the implant, the hESC-derived cells were found to differentiate into T cells [82]. Three years later, Galic et al. [83] switched to an EB-based method and noted improvements in the Thy/Liv generated hESC-derived T cells. By four weeks, CD4/CD8 double positive cells began to appear and by eight weeks, CD4 single positive and CD8 single positive cells that had undergone TCR rearrangements also emerged [83]. Another study published in 2009 was able to achieve mature T cells using a completely *in vitro* culture system [84]. Timmermans et al. [84] began their T cell differentiation protocol by co-culturing H1 hESCs on OP9 cells in αMEM plus 20% fetal calf serum. Similar in nature to the ES sacs described for megakaryocyte generation, they described the appearance of 'hematopoietic zones' in the cultures. After 12 days, they isolated $CD34^{hi}CD43^{lo}$ cells and replated them onto delta 1 (DL1)-expressing OP9 cells for five to seven weeks in the presence of FL, SCF, and IL7. After 14 days, they observed the emergence of CD4/CD8 double positive cells within a larger $CD3e^+$ $CD5^+$ population and after 30 days, noted the presence of $CD3^+$ T cells that had undergone TCR rearrangements. These hESC-derived T cells were found to be functional in assays examining their PHA-induced proliferation and IFNγ production [84]. In total, these hESC-based differentiation protocols for T cell generation show great potential to be further developed for use with iPSCs and one day, will hopefully be applicable to ACT protocols for human immunotherapy.

Granulocytes

Other leukocyte populations, including granulocytes (neutrophils, eosinophils, basophils) may have utility as hESC-based therapies, however interest in and/or development of these cell populations has not been as great as for other cell types. For granulocytes, the expense and/or difficulty in bringing hESC-based therapies to the clinic may not be warranted. For example, neutrophils are chemotactic phagocytes that migrate to sites of infection and provide protection against bacteria. Neutropenia (neutrophil counts less than 5×10^8 cells/liter) can cause an afflicted individual to be at higher risk for developing infections. While allogeneic neutrophil transfusions were shown to alleviate the risk of infections over thirty years ago, the use of antibiotic, antiviral, and/or antifungal therapies have largely replaced them in the clinic.

Nonetheless, hESCs can be induced to differentiate into neutrophils, as shown by two studies published in 2009 [85,86]. Both studies used KhES-C hESCs in an EB-based method to

generate $CD11b^+$ neutrophils that expressed varying levels of other neutrophil cell surface markers. While hESC-derived neutrophils were noted to be slightly larger than those in the peripheral blood [86], they were found to be functional in three *in vitro* assays assessing chemotaxis, phagocytosis, and production of reactive oxygen species [85,86]. One study also showed *in vivo* chemotaxis of hESC-derived neutrophils in response to IL1β expressed in an air-pouch inflammatory mouse model [85]. It remains to be determined whether hESC-neutrophils (or other types of granulocytes) will ever be developed for use as transfusion reagents, but *in vitro* differentiation systems for their generation may be useful for delineating cytokine requirements for hematopoietic differentiation, drug screening efforts, or elucidating molecular details of certain inherited diseases.

PERSPECTIVES

As reviewed here, peripheral blood components have many different therapeutic applications and hESCs/iPSCs have garnered a lot of interest as a renewable cell source that can be used for their generation. From RBCs and platelets being used in transfusions to treat cytopenias to DCs, NK cells, and T cells being used in immunotherapies to treat cancer and HIV, hESCs/iPSCs may be useful for generating these mature cell types in abundant supplies and in cost-effective ways. Although CB, BM, and PB have been investigated as sources of progenitors for the generation of large-scale transfusable RBCs and platelets [13–15,47,48], it is clear that even after expansion and differentiation, these progenitors represent donor-limited sources. Pluripotent stem cells, especially transgene-free human iPSCs [87–90], represent a new source of stem cells that can be propagated and expanded *in vitro* indefinitely, providing a potentially inexhaustible and donorless source of blood components for therapy. For hESCs, the ability to create banks of cell lines with matched or reduced incompatibility could potentially decrease or eliminate the need for immunosuppressive drugs and/or immunomodulatory protocols (e.g., $(O)RhD^-$ lines for the generation of universal RBCs). Inasmuch as iPSCs could potentially be produced from a patient's own cells, they carry enormous potential as another alternative source of pluripotent stem cells for treating human diseases related to many different organ systems, and could eliminate tissue incompatibility issues altogether.

While the field of regenerative medicine is still in its infancy, some hESC-based therapies are starting to be tested in clinical trials. In early 2012, researchers from Advanced Cell Technology and UCLA reported their preliminary results on treatment of patients with Stargardt's macular dystrophy and dry age-related macular degeneration using retinal pigment epithelium (RPE) cells derived from hESCs [91]. They have not noted any abnormal proliferation, teratoma formation, graft rejection, or other untoward pathological reactions or safety signals. Functional visual improvements were observed in both patients [91]. This is the first clinical trial of using hESCs or iPSCs therapeutically in human beings, and the safety and efficacy of it will likely have a significant impact on the development of other types of hESC/iPSCs-based therapies as well as the policies of the FDA towards the use of any hESC/iPSCs-derivatives to treat human diseases.

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CHAPTER 49

Red Blood Cell Substitutes

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INTRODUCTION

In 1957, the author initiated the first research on artificial cells including modified hemoglobin [1,2]. Artificial cells (Fig. 49.1) retain biologically active materials like hemoglobin, enzymes, cells, adsorbent and other materials [2–5]. Their dimensions can vary in the macro, micro, nano and molecular ranges (Fig. 49.2). The membrane of artificial cells retains larger molecules and suspensions, but allows permeable molecules like oxygen and substrates to enter and products, peptides and other material to leave. In this way the enclosed materials are protected from immunological rejection and other materials from the external environments. Until 1989, most of the research concentrated on artificial cells containing enzymes, cells, microorganisms, adsorbent, peptides and others.

Concentrated research and development on modified hemoglobin only started after 1987 because of public concerns about HIV in donor blood. After extraction from red blood cells and before modification, hemoglobin can be sterilized by pasteurization, ultrafiltration and chemical means. These procedures can remove microorganisms including those responsible for AIDS, hepatitis and others. However, as described below, hemoglobin has to be modified before it can be used. There are many situations where modified hemoglobin can potentially be used to substitute for red blood cells [4–12]. These include trauma surgery, cardiopulmonary bypass surgery, cancer surgery, other elective surgery, and cardioplegia. Another area is in dealing with severe traumatic injuries like traffic accidents and other accidents that result in

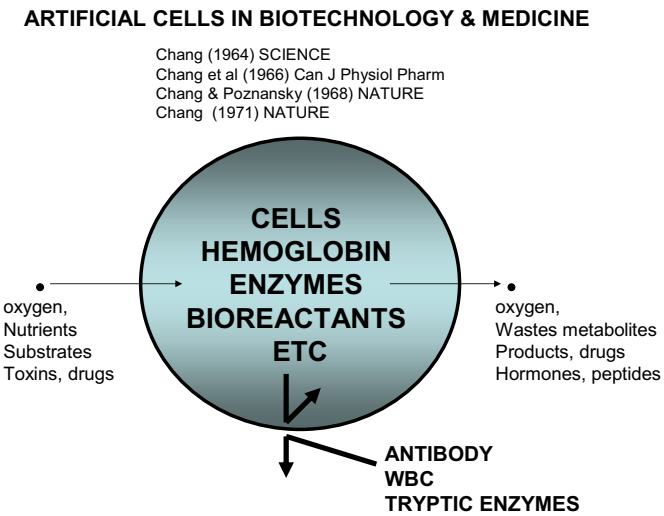

FIGURE 49.1
Basic principle of artificial cells. *(From: Chang, Art Cell Blood Sub Biot, 2004. With copyright permission from Marcel Dekker Publisher)*

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00049-5

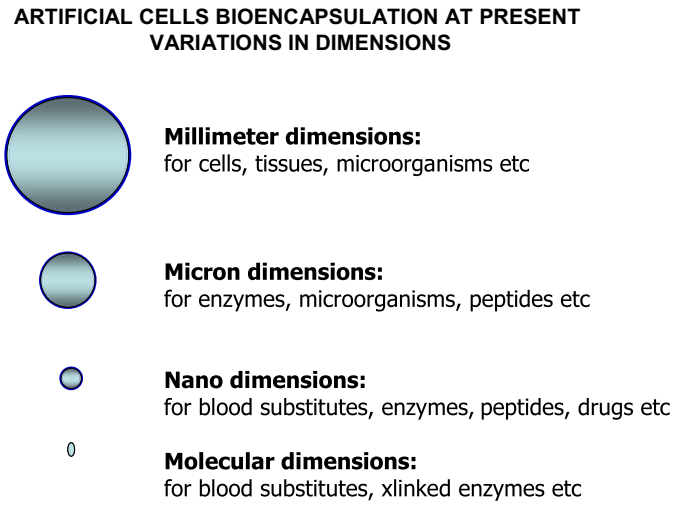

FIGURE 49.2
Artificial cells in the macro, micro, nano and molecular dimensions. *(From: Chang, Art Cell Blood Sub Biot, 2004. With copyright permission from Marcel Dekker Publisher)*

severe bleeding and hemorrhagic shock. The number of traumatic injuries requiring blood substitutes in civilian use is small when compared to the requirements in major disasters or wars. Modified hemoglobin is especially useful in emergency situations such as these. Since modified hemoglobin does not contain red blood cell membrane and therefore no blood group antigens, it can be used without the need for cross-matching or typing. This would save much time and facilities and would permit on-the-spot transfusion as required, similar to giving intravenous solution. This is further facilitated by the fact that modified hemoglobins can be stored as a solution at room temperature for more than two years. They can also be lyophilized and stored as a stable, dried powder, which can be reconstituted with the appropriate salt solution just before use. Another area of use is where the patients' religious belief do not allow them to use donor blood for transfusion.

MODIFIED HEMOGLOBIN

Why do we have to modify hemoglobin?

Hemoglobin in the red blood cell is responsible for carrying oxygen from the lung and delivering it to the tissues. It is a tetramer of four subunits: two alpha subunits and two beta subunits [13] (Fig. 49.3). Hemoglobin is in the 'oxy', relaxed, or 'R' state when it is carrying oxygen. To release oxygen, the hemoglobin molecule undergoes conformational change with a 15° rotation. The molecule is then in the 'deoxy', tensed, or 'T' state. A red blood cell contains a cofactor, 2,3-DPG, which facilitates this conformational change. Thus, hemoglobin inside red blood cells has a high P_{50} which allows it to readily release oxygen to the tissue at physiological oxygen tensions.

Hemoglobin can be extracted from red blood cells by removing the cell membranes to form stroma-free hemoglobin. Attempts in clinical trials in patients to use stroma-free hemoglobin as blood substitute have not been successful, because of renal toxicity and other adverse effects [14]. When infused into the circulation, each four subunit hemoglobin molecule (tetramer) is rapidly broken down into two subunits (dimers). These toxic dimers are the major causes of the adverse effects.

Modified hemoglobin

Biotechnological approaches can be used to either crosslink or encapsulate the hemoglobin molecules to prevent it from dissociating into dimers, and also to allow it to have an acceptable P_{50} [1–3,15,16]. Many groups have since contributed to progress in this area, especially since 1989 [1–79].

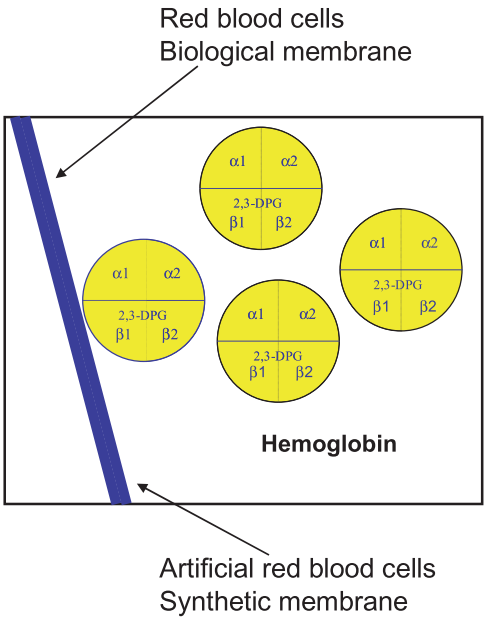

FIGURE 49.3

Inside the red blood cell, each hemoglobin molecule stays as a tetramer of four subunits: two alpha subunits and two beta subunits. Cofactors, 2,3-DPG, retained in the red blood cell facilitate the hemoglobin to release oxygen as required by the tissues. Artificial red blood cell has the same content as red blood cells but the membrane is made of synthetic material. *(Modified From Chang, Biomaterials, Artificial Cells and Immobilization Biotechnology, an International Journal, 20:154-174, 1992 with permission of Marcel Dekker Publisher)*

Microencapsulation of hemoglobin to prepare a complete artificial red blood cell [1,2] is a rather ambitious concept (Fig. 49.3). Although this attempt to mimic red blood cells has resulted in a complete red blood cell substitute, it is rather complicated. Further research is needed and this approach is now considered as a third generation modified hemoglobin and will be discussed later. Simpler crosslinked modified hemoglobin has been developed as first generation modified hemoglobin for more immediate clinical applications, as is discussed below.

FIRST GENERATION MODIFIED HEMOGLOBIN

Polyhemoglobin based on nanobiotechnology

Hemoglobin contains many amino groups, many of which are on the surface of the hemoglobin molecule. This author first reported the use of nanobiotechnology using a bifunctional agent (diacid) to crosslink and assemble hemoglobin molecules into polyhemoglobin [2,3] (Fig. 49.4). This was first used to form ultrathin polyhemoglobin membranes for artificial red blood cells. It was also found that with decreasing size of artificial cells all the hemoglobin molecules are crosslinked into polyhemoglobin. Crosslinking prevents the breakdown of hemoglobin tetramers into dimers. The reaction is as follows:

$$\underset{\text{Diacid}}{\text{Cl}-\text{CO}-(\text{CH}_2)_8-\text{CO}-\text{Cl}} + \underset{\text{Hemoglobin}}{\text{HB}-\text{NH}_2} = \underset{\text{Polyhemoglobin}}{\text{HB}-\text{NH} = \text{CO}-(\text{CH}_2)_8-\text{CO}-\text{NH}-\text{HB}}$$

Glutaraldehyde is another bifunctional agent first used by this author in a similar nanobiotechnological approach to prepare soluble nanodimension polyhemoglobin [15]:

$$\underset{\text{Glutaraldehyde}}{\text{H}-\text{CO}-(\text{CH}_2)_3-\text{CO}-\text{H}} + \underset{\text{Hemoglobin}}{\text{HB}-\text{NH}_2} = \underset{\text{Polyhemoglobin}}{\text{HB}-\text{NH}-\text{CO}-(\text{CH}_2)_3-\text{CO}-\text{NH}-\text{HB}}$$

Since then, a number of other groups have independently carried out extensive studies using this principle of glutaraldehyde crosslinked polyhemoglobin. The two most successful ones are glutaraldehyde crosslinked human polyhemoglobin [17–19] and glutaraldehyde crosslinked bovine polyhemoglobin [20–22,42].

Other sources of hemoglobin for forming glutaraldehyde crosslinked polyhemoglobin include hemoglobin from procine red blood cells [25] and hemoglobin from red blood cells of placentas discarded after birth [26]. Other cross-linkers have also been developed.

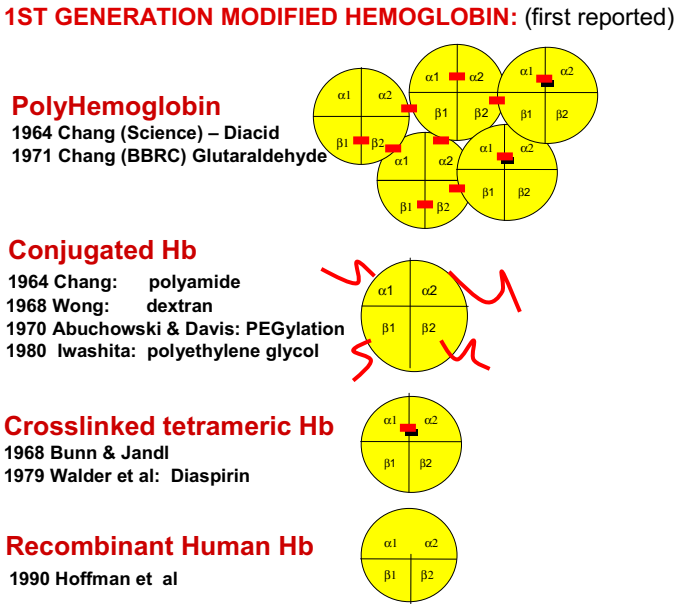

FIGURE 49.4
Molecular dimension red blood cell substitutes in the form of polyhemoglobin, conjugated hemoglobin, intramolecularly crosslink hemoglobin and recombinant hemoglobin. *(Modified from: Chang, Art Cell Blood Sub Biot, 2004. With copyright permission from Marcel Dekker Publisher)*

Intramolecularly crosslinked hemoglobin

Studies have been carried out to specifically crosslink hemoglobin molecules intramolecularly to form crosslinked tetrameric hemoglobin (Fig. 49.4). For example, a bifunctional agent, 2-Nor-2-formylpyridoxal 5-phosphate which is also a 2,3-DPG analogue can intramolecularly crosslink the two beta subunits of the hemoglobin molecules [16]. Another 2,3-DPG pocket modifier, bis(3,5-dibromosalicyl) fumarate (DBBF) intramolecularly cross-links the two alpha subunits of the hemoglobin molecule [27–29].

Conjugated Hemoglobin

Conjugated hemoglobin is the crosslinking of hemoglobin into polymers [2,3]. The use of soluble polymers resulted in soluble conjugated hemoglobin with good circulation time [30,31]. Two new nano dimension conjugated hemoglobin based the use of polyethylene glycol are being tested in ongoing clinical trials [12,32–34].

Sources of hemoglobin

Where do we obtain all the hemoglobin needed for preparing modified hemoglobin? Red blood cells from outdated donor blood are one major source of human hemoglobin. Another source is human hemoglobin from red blood cells of discarded placentas after the delivery of babies [26]. In addition to hemoglobin from human sources another source is bovine hemoglobin [20] and hemoglobin from porcine red blood cells [25]. Another promising source is human hemoglobin produced by recombinant technology in microorganisms [35–37]. Another potential source that has been developed is synthetic heme that is linked to recombinant human albumin [10,38].

Efficacy and safety of modified hemoglobin blood substitutes

Modified hemoglobin no longer has problems related to the breakdown of hemoglobin into toxic dimers. However, how safe is modified hemoglobin when injected into animals and humans? Animal studies using properly prepared modified hemoglobin have not shown adverse effects on coagulation factors, leucocytes, platelets or complement activation [4–6,39,40], [8–12]. Very stringent, sensitive immunological studies have been carried out for polyhemoglobin, conjugated hemoglobin and microencapsulated hemoglobin [6,41]. Results show that modified homologous polyhemoglobins (e.g., rat polyhemoglobin injected into

rats) are not immunogenic even with repeated injection. Heterologous polyhemoglobin (e.g., non-rat hemoglobin injected into rats) is not immunogenic initially but is immunogenic after repeated injections with Freud's adjuvant. Conjugation and microencapsulation markedly decreased the antigenicity of heterologous polyhemoglobin. Other studies show that without the use of Freund's adjuvant, repeated subcutaneous and intravenous injections of heterologous cross linked hemoglobin are much less immunogenic [28]. Clinical trials using a bovine polyhemoglobin did not show adverse immunological effects [23,24,42]. Another important area of safety study is the distribution of modified hemoglobin after infusion and also its effect on the reticuloendothelial system. Another factor is that safety response in animals is not necessarily the same as in humans, especially in the case of immunological and hypersensitivity reactions. We have worked out a simple *in vitro* screening test [6] which consists of adding 0.1 ml of modified hemoglobin to a test tube containing 0.4 ml of human plasma or blood, and then analyzing for complement activation. This have been used to detect trace contamination of blood group antigens, antibody-antigen complexes, endotoxins, trace fragments of microorganisms, impurities in polymers, some emulsifiers and others. Detailed animal studies show that modified hemoglobins are effective in short-term applications like hemorrhagic shock, exchange transfusion, hemodilution in surgery and some other conditions. However, despite all the detailed *in vitro* and *in vivo* safety and efficacy studies and screening tests described above, extensive clinical trials in the last 20 years show that some of the blood substitutes have problems related to increased vasoconstriction as discussed below.

Vasoactivity and nitric oxide

Not all modified hemoglobins have been successful in clinical trials, since vasopressor effects have been observed in some of the first generation modified hemoglobin blood substitutes. For example, intramolecularly crosslinked hemoglobin and first generation recombinant human hemoglobin blood substitutes contain 100% of molecular dimension modified hemoglobin. Infusion causes vasopressor effects and also increased smooth muscle contractions. With another type of polyhemoglobin that contained more than 30% molecular dimension modified hemoglobin, significant vasoactivity and increased smooth muscle contractions were also observed when using larger volumes. On the other hand, the use of nanodimension polyhemoglobin with $<1\%$ molecular dimension modified hemoglobin did not show vasopressor effects even when large volumes, of up to 10 liters, were infused. This has led to the proposal that the intercellular junctions of the endothelial lining of vascular wall allow molecular dimension hemoglobin to enter into the interstitial space [6] (Fig. 49.5). There, hemoglobin acts as a sink for binding and removing the nitric oxide needed for maintaining the normal tone of smooth muscles [6,43]. This results in the constriction of blood vessels and other smooth muscles, especially those of the esophagus and the GI tract. On the other hand, nano dimension polyhemoglobin would not cross the intercellular junction and therefore does not result in vasoconstriction (Fig. 49.5). Others argue that one cannot compare the different types of modified hemoglobin since there are major differences in the chemistry involved and in the oxygen affinity. We have therefore prepared laboratory versions of nano dimension polyhemoglobin, each containing different percentages of molecular dimension hemoglobin using the same glutaraldehyde crosslinking, and characterized them to ensure that they all have the same oxygen affinity [5,44]. The results show that samples with a very low percentage of molecular dimension modified hemoglobin do not cause vasoconstriction or changes in electrocardiograms. With increasing percentage of molecular dimensions modified hemoglobin, there was increasing degree of vasoconstriction and elevation of the ST segment of the electrocardiogram. ST elevation could be due to vasoconstriction, resulting in a decreased supply of oxygen to the heart, and this may explain the observation of small myocardial lesions in some primates and swine after they were infused with one type of molecular dimension modified hemoglobin [45]. This theory is further supported by the lack of vasoconstriction using non-extravasating hemoglobin polymer [46]. Furthermore, a second

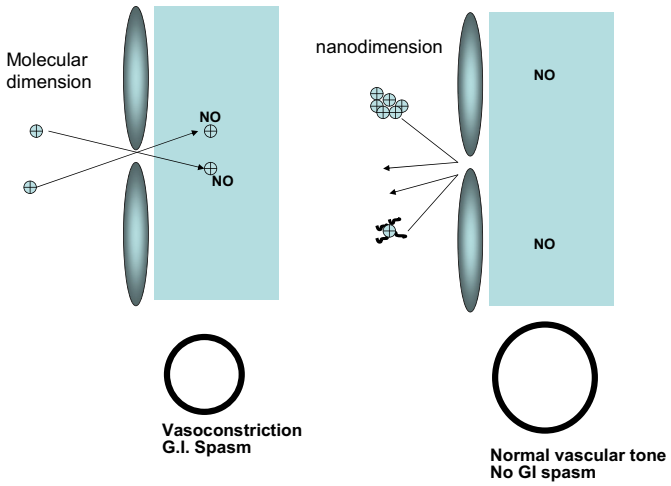

FIGURE 49.5

Molecular dimension modified hemoglobin can transverse the intercellular junction of the endothelial cells of the vascular wall. It will then bind with nitric oxide thus removing it resulting in increase vasoactivity since removal of nitric oxide results in contraction of smooth muscles of the vascular wall and other smooth muscles. Nanodimension modified hemoglobin that do not contain any significant free molecular dimension modified hemoglobin. They cannot transverse the intercellular junction and therefore do not cause vasoconstriction. *(From Blood Substitutes. 1997 monograph by TMS Chang with permission of copy right holder.)*

generation molecular dimension recombinant human hemoglobin that did not bind nitric oxide, did not cause vasoconstriction [43]. Since recombinant hemoglobins crosses the intercellular junction and are removed quickly, their circulation time can be increased by crosslinking to form nanodimension polyhemoglobin [35]. Another approach in ongoing clinical trial is to prepare polyethylene glycol (PEG) conjugated hemoglobin that with its water of hydration would result in a nano dimension modified hemoglobin, and this does not result in vasopressor effects [12]. A more recent observation in mice with endothelial dysfunction showed that unlike normal mice, polyhemoglobin with <1% tetratmer can still result in vasoconstriction and this can be prevented by nitrite oxide inhalation [47]. A number of other approaches are being investigated to solve this problem for instance Kluger's group [62] and Hsia's group [49].

Present clinical status of first generation modified hemoglobin

Two of the most successful ones are based on glutaraldehyde crosslinked polyhemoglobin. These have been developed independently by two groups based on Chang's basic principle of glutaraldehyde crosslinked polyhemoglobin [15]. One is pyridoxalated glutaraldehyde human polyhemoglobin [17,18]. They show in Phase III clinical trial that this can successfully replace extensive blood loss in trauma surgery by maintaining the hemoglobin level with no reported side effects [18]. They have infused up to 10 liters into individual trauma surgery patients. In 2009 this group reported their clinical trial results in trauma hemorrhagic shock ambulance patients [19]. The control group was given the standard saline solution in the ambulance. Since polyhemoglobin does not have blood group antigens, it can be given in the ambulance before reaching the hospital. The control group needs red blood cell transfusion on arrival at the hospital. On the other hand, when given up to a total of six units, the patients did not need red blood cell transfusion for an average of 12 hours. Although repeated infusion can be given to prolong this time, this was not part of the clinical trial protocol. Side effects observed were non-fatal myocardial infarctions (0.6% in the control group and 3% in the polyhemoglobin group). Another one is glutaraldehyde crosslinked bovine polyhemoglobin that has been extensively tested in

	DONOR RBC	BLOOD SUBSTITUTES (PolyHemoglobin)
INFECTION	POSSIBLE	STERILIZED
SOURCE	LIMITED	"UNLIMITED ?"
BLOOD GROUPS	YES	NONE
USAGE	Xmatch, typing	Immediately
STORAGE	42 DAYS	>1YEAR
FUNCTIONS	RBC	OXYGEN CARRIER

FIGURE 49.6

Comparison of polyhemoglobin with donor red blood cells. Polyhemoglobin has many advantages over red blood cells and is useful for use during surgery. However, it cannot be used in a number of other clinical conditions. This is because unlike red blood cells (RBC), polyhemoglobin is only an oxygen carrier. It does not have RBC enzymes needed for many functions including the removal of oxygen radicals. Furthermore, its circulation time is much shorter than that of RBC. *(Modified from: Chang, Art Cell Blood Sub Biot, 2004. With copyright permission from Marcel Dekker Publisher)*

clinical trials for surgical patients [20,22,42]. This bovine polyhemoglobin has been approved for routine patient use in South Africa and more recently in Russia [50]. The above two polyhemoglobins have been approved for compassionate uses in humans in North America. Conjugated hemoglobin of sufficient nanodimension is in earlier stages of clinical trials [12,33].

Nanodimension polyhemoglobin and conjugated hemoglobin have a number of advantages when compared to donor red blood cells (Fig. 49.6). Unlike red blood cells, there is no blood group and thus they can be given immediately, on the spot, without the need for time-consuming typing and cross-matching. They can be sterilized and are free from infectious agents. Donor blood has to be stored at 4°C, and is only good for 42 days. Polyhemoglobin can be stored at room temperature for more than one year. Thus, they have important roles in a number of clinical conditions. In some other conditions related to severe and sustained ischemia we may have to add antioxidant enzymes to oxygen carriers in order to avoid the potential for ischemia reperfusion injury and other potential problems [51,52,76] (Fig. 49.7).

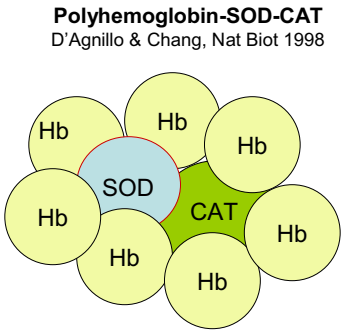

FIGURE 49.7

Crosslinking of hemoglobin with two RBC enzymes to form polyhemoglobin-catalase-superoxide dismutase (PolyHb-CAT-SOD). Unlike polyhemoglobin, this has RBC enzymes that can remove oxygen radicals. *(Modified from: Chang, Art Cell Blood Sub Biot, 2004. With copyright permission from Marcel Dekker Publisher)*

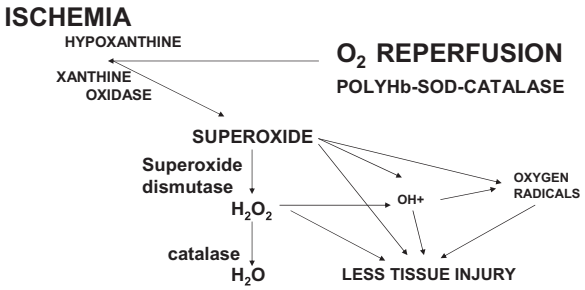

FIGURE 49.8
In conditions like severe sustained hemorrhagic shock, stroke, myocardial infarction and organ transplantation, reperfusion with polyhemoglobin can sometimes result in oxygen radicals that causes tissue injury. PolyHb-CAT-SOD can supply oxygen and at the same time significantly lower any oxygen radicals formed. *(From: Chang, Monograph on Blood Substitutes, 1997. With copyright permission from copyright holder TMSC)*

NEW GENERATIONS OF MODIFIED HEMOGLOBIN

Polyhemoglobin crosslinked with RBC antioxidant enzymes

In sustained severe hemorrhagic shock or other ischemic conditions, oxygen carriers alone may result in ischemia reperfusion injuries due to the production of oxygen radicals [51,52] (Fig. 49.8). We are studying the use of a crosslinked polyhemoglobin-superoxide dismutase-catalase (PolyHb-SOD-CAT) (Fig. 49.7) [52,53]. The intestine is one of the organs that is most likely to be injured in this type of ischemia reperfusion injury. We found that unlike PolyHb, PolyHb-SOD-CAT did not cause a significant increase in oxygen radicals when it is used to reperfuse ischemic rat intestine [6]. Polyhemoglobin exists in solution, and is therefore more likely to be able to perfuse partially obstructed vessels in stroke, myocardial infarction and other conditions than red blood cells. In a rat stroke model (Fig. 49.9), we found that after 60 minutes of ischemia, reperfusion with polyHb resulted in significant increase in the breakdown of blood-brain barrier and increase in brain edema [54]. On the other hand, polyHb-SOD-CAT did not result in these adverse changes [54]. Supported by another group in Korean kidney liver transplant [40]. These results have been supported by research using this approach in kidney and liver transplantation [40].

Polyhemoglobin crosslinked with RBC antioxidant enzymes and carbonic anhydrase

Even though erythrocytes transport both oxygen and carbon dioxide, research on blood substitutes has concentrated only on the transport of oxygen and its vasoactivity and oxidative effects. Recent study in a hemorrhagic shock animal model shows that the degree of tissue

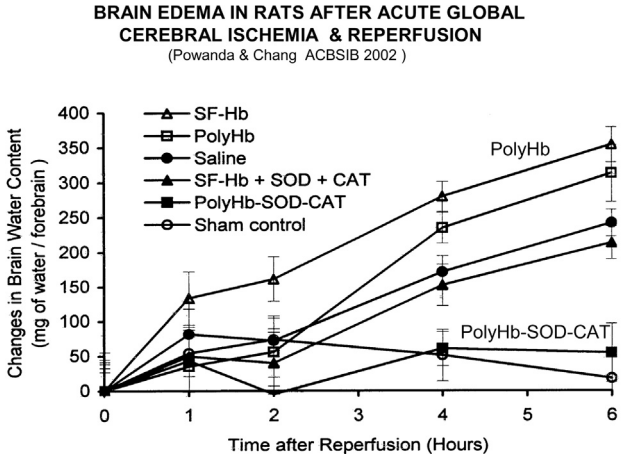

FIGURE 49.9
This is a rat model of acute global cerebral ischemia followed by reperfusion with different oxygen carrying solutions. Unlike polyhemoglobin, polyHb-CAT-SOD does not cause brain edema when used in this situation. *(From: Powanda and Chang, Art Cell Blood Sub Biot, 2002. With copyright permission from Marcel Dekker Publisher)*

PCO_2 elevation is directly related to mortality rates [55]. Therefore we have designed a novel nanobiotechnological preparation with antioxidant properties that can transport both O_2 and CO_2 [56]. This is in the form of polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase. We have studied its effect on elevated tissue PCO_2 in a hemorrhagic shock rat model and found that it is more efficient than red blood cells in lowering elevated tissue PCO_2 levels [57].

Polyhemoglobin-fibrinogen: an oxygen carrier with platelet properties

Polyhemoglobin, like red blood cells, does not contain clotting factors or platelets to aid in coagulation when needed. Thus with extensive replacement of whole blood with polyhemoglobin or red blood cells, there is a need to replace clotting factors and platelets. We have prepared a novel blood substitute that is an oxygen carrier with platelet-like activity [75]. It is formed by crosslinking fibrinogen to hemoglobin to form polyhemoglobin-fibrinogen (polyHb-Fg). This was studied and compared to polyHb for its effect on coagulation both *in vitro* and *in vivo*. In the *in vitro* experiments, PolyHb-Fg showed similar clotting times to whole blood whereas polyHb showed significantly higher clotting times. This result was confirmed in *in vivo* experiments using an exchange transfusion rat model. Using PolyHb, exchange transfusion of 80% or more increased the normal clotting time (1−2 mins) to >10 mins. Partial clots formed with PolyHb did not adhere to the tubing wall. With PolyHb-Fg, a normal clotting time is maintained even with 98% exchange transfusion.

Polyhemoglobin crosslinked with tyrosinase

Tumors are not well perfused with blood, but radiation therapy and chemotherapy works better with better tissue oxygenation. Since polyhemoglobin is in solution, it can perfuse tumors more effectively than red blood cells, and thus has been shown to increase the sensitivity of tumors to radiation therapy and chemotherapy [20,34,58]. We have recently crosslinked tyrosinase with hemoglobin to form a soluble polyhemoglobin-tyrosinase complex [59]. This has the potential dual function of supplying the needed oxygen for radiation therapy and also lowers the systemic tyrosine needed for the growth of melanoma. This preparation inhibits the growth of murine B16F10 melanoma culture. In mice, it significantly delays the growth of B16F10 melanoma compared to the control group, without causing adverse effects or changes in the growth of the treated animals [59]. We are now investigating the nanoencapsulation of polyhemoglobin-tyrosinase in polyethyleneglycol-polylactide (PEG-PLA) or polylactide (PLA) nanocapsules [60,77].

Artificial red blood cells

The first artificial red blood cells (Figs. 49.1, 49.3) have oxygen dissociation curves similar to red blood cells, since 2-3-DPG is retained inside [1]. Hemoglobin also stays inside as a tetramer, and red blood cell enzymes like carbonic anhydrase and catalase retain their activities [2,3]. These artificial red blood cells do not have blood group antigens on the membrane and therefore do not aggregate in the presence of blood group antibodies [3]. However, the single major problem is the rapid removal of these artificial cells from the circulation. Much of the studies since the initial research aim to improve survival in the circulation by decreasing their uptake by the reticuloendothelial system. Since removal of sialic acid from biological red blood cells results in their rapid removal from the circulation, we started by modifying the surface properties of the artificial red blood cells [3]. This included the use of synthetic polymers, negatively charge polymers, crosslinked protein, lipid-protein, lipid-polymers and others. Since that time many researchers around the world have started to carry out research on artificial red blood cells [5].

One major step forward is the preparation of submicron lipid membrane artificial red blood cells, resulting in very significant improvements in circulation time [61]. Due to the addition

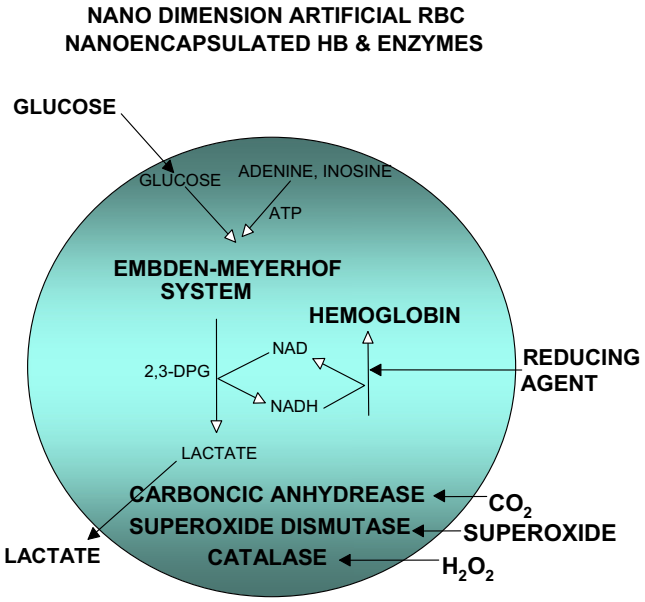

FIGURE 49.10
Nanodimension artificial red blood cells with polyethylene-glyco-polylactide membrane. In addition to hemoglobin, this contains the same enzymes that are normally present in red blood cells. Thus, it has the complete function of the red blood cells. *(From: Chang et al, Art Cell Blood Sub Biot, 2003. With copyright permission from Marcel Dekker Publisher)*

of polyethylene glycol to the lipid membrane, the circulation time has been increased to a half time of about 36 hours in rats [9,10,79]. These advances make it now possible to scale-up for detailed preclinical studies towards a clinical trial [10,11]. The uptake is mainly by the reticuloendothelial system. It is possible to replace 90% of the red blood cells in rats with these artificial red blood cells. The animals with this percentage of exchange transfusion still remain viable. Studies also reported effectiveness in hemorrhagic shock. There are no changes in the histology of the brain, heart, kidneys and lungs of rats treated in this way.

Using a modification of this author's earlier method of micron dimension biodegradable polymeric membrane artificial cells [63] we have recently prepared nano dimension artificial red blood cells [6,39,64,65]. These nano artificial RBC, sized 80 to 150 nanometers, contain all the red blood cell enzymes and can convert methemoglobin to hemoglobin [5,39] (Fig. 49.10). Our studies show that using a polyethylene-glycol-polylactide copolymer membrane we are able to increase the circulation time of these nano artificial red blood cells to double that of polyhemoglobin (Fig. 49.11) [39]. Further studies show that one infusion with a volume of one third of the total blood volume did not result in long-term adverse effects on the biochemistry and histology of the kidney [64] or liver [48]. Our most recent study was carried out using PEG-PLA membrane nano artificial cells containing polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase in a hemorrhagic shock rat model with two thirds of the blood removed, resulting in a mean arterial blood pressure of 30mm Hg. After one hour of hemorrhagic shock at 30mmHg, infusion of this preparation effectively resuscitated the animal and lowered the elevated tissue PCO_2 [78].

A CHEMICAL APPROACH BASED ON PERFLUOROCHEMICALS

There is another type of blood substitute based on a chemical approach that was first demonstrated by Clark and then Geyer using perfluorochemicals (PFCs) [66]. This approach was developed by a number of investigators including Chen, Clark, Geyer, Faithful, Keipert, Lowe, Mitsuno, Naito, Nose, Ohyanagi, Reiss, Sloviter, Yokoyama and many others [67,68]. Perfluorochemicals are synthetic fluids in which oxygen can dissolve and that can be made into fine emulsions for use as oxygen carriers. Their greatest advantage is that they can be produced in large amounts. Furthermore, their purity can be more easily controlled. However, PFCs have a lower capacity for carrying oxygen than Hb. Improved perfluorochemicals have allowed a higher concentration of PFC to be used and a blood substitute based on perfluoro-octyl

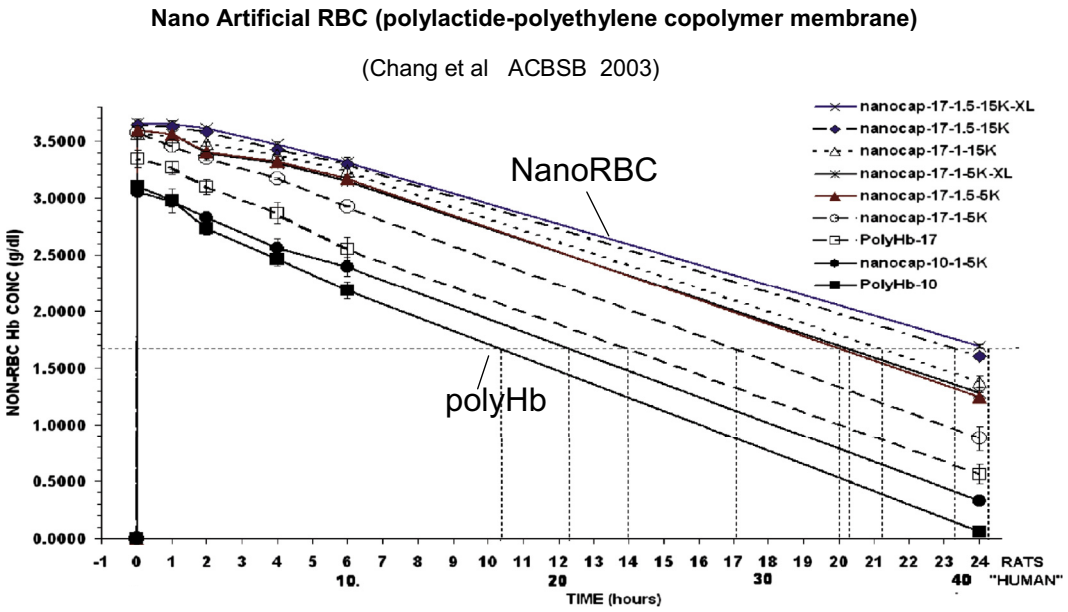

FIGURE 49.11

Nanodimension artificial red blood cells with polyethylene-glyco-polylactide membrane. The circulation time is double that of polyhemoglobin. The circulation half time of polyhemoglobin in human is about 24 hours. This means that the nanodimension artificial RBC may have a circulation time of about 48 hours in human. *(From: Chang et al, Art Cell Blood Sub Biot, 2003. With copyright permission from Marcel Dekker Publisher)*

bromide ($C_8F_{17}Br$) with egg-yolk lecithin as the surfactant has been tested in clinical trials. The results are being carefully analyzed.

CONCLUSIONS

Blood substitutes have encountered two major barriers. First, it was mistakenly thought that a blood substitute would be simple to produce, would not need much basic research and hence could be easily developed for clinical applications when needed. Thus, the crisis of HIV contaminated donor blood was followed by catch up and short cut efforts to quickly produce some type of blood substitute. The urgency of the HIV crisis resulted in a rush into clinical trials resulting in a number of blood substitutes with rather severe adverse side effects. Eventually, two types of polyhemoglobin were manufactured and tested clinically with promising results [19,42]. Unfortunately, Natanson et al. [76] carried out a meta-analysis that included all the results of all hemoglobin based blood substitutes, including those that had failed earlier. This has led to a second barrier with the mistaken conclusion that all blood substitutes are toxic and not effective. South Africa and Russia have analyzed the risk/benefit according to their own needs and have approved the routine clinical use of one type of polyhemoglobin [50].

We now know that a blood substitute is a very complicated material that cannot be produced for clinical use without the required basic knowledge. We have to approach a blood substitute as a highly interdisciplinary type of basic and applied research and development, involving polymer chemistry, cell physiology, molecular biology, biotechnology, clinical medicine and other areas. The belated but ongoing emphasis on this interdisciplinary approach will accelerate the clinical realization of blood substitutes. This brief overview cannot include the numerous ongoing studies and research in this area. More details are available in many detailed publications, books and symposium volumes in this area [4–6,8–12,18,33,42,68–74].

LINK TO WEBSITES

www.artcell.mcgill.ca or www.artificialcell.info is the website of the International Network for Artificial Cells, Blood Substitutes and Biotechnology. It contains review articles and books (including Chang's 1972 ,1997 and 2007 monographs [3,5,6] (accessed June 2, 2013)) which can be accessed without cost. This website also links to other websites in the area of blood substitutes and artificial cells.

Acknowledgments

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CHAPTER 50

Lymphoid Cells

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INTRODUCTION

For researchers interested in cell-based therapy, it is a great challenge to learn how to expand lymphoid cells and their precursor cells *ex vivo* under defined conditions. Lymphocytes are defined by their cell surface receptor — BCR (B Cell Receptor, or immunoglobulin, Ig) for B cells and TCR (T Cell Receptor) for T cells. Precursor B cells bear a pre-B receptor, which contains lambda 5 in mice ('physi' in humans), and precursor T cells bear a pre-TCR-alpha. No biochemically and genetically defined surface receptors for the progenitors of lymphoid precursors have been reported. It is not entirely clear how committed stem cells differentiate into lymphoid cells, which then mature into effector cells. Many theories have been postulated. Differentiation and maturation involve both antigen-independent and antigen-dependent processes. For the first process, I will explore the sequential commitment model proposed in references [1,2]. For my treatment of the second process, I am indebted to many colleagues who helped me to summarize current views.

Ligand-receptor interactions must play an important role in causing lymphoid cells to proliferate and differentiate. The term ligand means all external signals, including those provided by stroma cells and cytokines during the antigen-independent stage and those provided by Ag (antigens) and APCs (antigen-presenting cells), or accessory cells during the antigen-dependent stage. The process of turning on and turning off transcription factors at each stage of differentiation as a consequence of ligand-receptor interaction is one of the key elements controlling both the phenotype and the function of cells. Transcription factors known to be important at various stages of lymphopoiesis, due to the notion that the growth of lymphoid (precursor) cells can be manipulated at will by using genetic tools, have been discussed in the last three editions. There are examples of expansion of normal lymphoid (precursor) cells in culture. All are based on the support of stroma cell lines and cytokines. On removal of these elements, cells either differentiate toward terminal effector cells or die. Recently, the post transcriptional regulations of various pathways are shown to be conducted by the miRNAs (micro RNAs), which are the regulators of networks.

I have arbitrarily defined seven stages of lymphopoiesis to discuss in detail. For each one, I discuss the available cellular and molecular markers, the current understanding of cellular phenotype, the potential of these cells to expand *ex vivo*, and if possible, what could be done in the future.

Both somatic and genetic manipulations are possible in animal models. By combining modern cellular and molecular technologies and available mutant mice, one should be able to grow normal lymphoid (precursor) cells at every stage of lymphopoiesis.

Despite the efforts of many, human stroma cell lines that can reproducibly support the growth of different stages of lymphoid (precursor) cells are still under development. The inducible

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regulation of stage-specific and lymphoid-specific transcription and post-transcriptional factors in controlling the growth of normal lymphoid cells seems to be the key element.

The aim of this chapter is to help the reader to understand lymphocytes and their precursors. The intention of research is to learn how these cells might be manipulated to make them useful in cell-based, somatic gene/molecular therapy. I attempt to address the possibility of growing normal lymphoid cells and their stem cells – of mouse and human origin – in a controllable manner. That is, cells should proliferate *ex vivo* without becoming malignantly transformed and with little or no differentiation.

Three main types of stem cells can be distinguished: totipotent, pluripotent, and multipotent. Totipotent and pluripotent stem cells usually divide symmetrically to give rise to two daughter stem cells, with the same properties and identical phenotype to their parent. Under appropriate conditions, totipotent and pluripotent stem cells can differentiate into any other form of stem cell. The known stem cell lines that seem to be close to totipotent are ESCs (embryonic stem cells) and, more recently, iPCs (induced pluripotent cells). Multipotent stem cells, which exist for the lifetime of an organism, undergo primarily asymmetric divisions. One daughter cell is another stem cell like its parent, whereas the other daughter cell is a more highly differentiated cell that performs a tissue-specific function. Due to this unique property, multipotent stem cells are ideal vehicles for cell-based, somatic, gene/molecular therapy. They will carry the transgene for the lifetime of the organism, and they will maintain expression of the transgene in the differentiated cells that they spawn.

Both lymphoid and lymphoid precursor cells are the progeny of HSCs (hematopoietic stem cells). One of the main tasks here is to review whether lymphoid cells and their progenitors possess stem cell-like properties. Are they self-renewing? And with available culture conditions and technology, can they expand *ex vivo* under controllable growth conditions? Unlimited growth of lymphoid cells and their progenitors is well documented. Lymphoid leukemia cells develop either spontaneously or after infection with viruses. These cell lines are useful for studying lymphoid cells but not for cell-based therapy, because they develop tumors when reintroduced into the organism. Thus, this chapter is devoted to exploring the possibility of growing normal lymphoid cells that can be engineered in culture and re-implanted into syngeneic or autologous hosts for therapeutic purposes. Other mouse normal cell types, expandable at will using genetic approaches, are available: tet-on (tetracycline-inducible) glial stem cells, mesenchymal stem cells, tet-off (tetracycline-regulated) ectodermal progenitor cells, lung stem cells, as well as tet-on and tet-off pancreatic beta islet cells, summarized in Chen [3].

PROPERTIES OF LYMPHOCYTES

Lymphoid precursor cells are bipotential progenitors of pre-T and pre-B cells. The development of lymphoid precursor cells is independent of the presence of Ag (Antigen). It is controversial whether lymphoid precursor cells divide asymmetrically with self-renewal. Lymphocytes and their precursors are similar to cells of other lineages, in that they proliferate, differentiate, migrate, communicate with other cells, age, and die. However, lymphocytes also possess the following unique properties that distinguish them from other cells:

1) Formation of receptor genes by VDJ recombination,
2) Requirement for a thymus-like environment to generate mature CD4+/8+ T cells,
3) Requirement for somatic education and selection by Ag,
4) Somatic hypermutation to generate more diversity in B cells,
5) Immunologic memory,
6) Ig heavy-chain CSR (Class Switch Recombination) in B cells,
7) Kappa light-chain editing somatically to generate new specificities of B cell receptor,

8) Secretion of various cytokines by CD4+T helper cells, Treg (T regulatory) cells, in communicating and regulating other lymphoid cell populations [4].

Development of techniques to expand *ex vivo*, to manipulate genetically, and to re-implant lymphocytes and their precursors into host animals requires lymphocyte engineers.

LYMPHOCYTE ENGINEERING: REALITY AND POTENTIAL

Two models describe the sequence of cell commitment during lymphopoiesis: stochastic and inductive. In this section, I use an inductive model in an attempt to explain lymphocyte commitment during development of the organism and in the adult.

Inductive model of sequential cell commitment of hematopoiesis

This model is divided into two parts. The first part deals with the Ag-independent stage, starting with the differentiation from the null cells (fertilized egg to ESCs, or iPS cells) to mesoderm and then to lymphoid precursor cells. A significant portion of the first part of this model was expanded from Bailey's hypothesis of developmental progress [4a] and modified according to Brown et al. [1,2].

ANTIGEN-INDEPENDENT STAGE OF LYMPHOPOIESIS — PART 1

Because multipotent stem cells are committed to differentiate, it is hypothesized that the differentiation sequence is genetically determined. Cells within the sequence are pre-committed in how they are determined. Cells within the sequence are pre-committed in their ability to respond to various inducers of differentiation; and on encountering appropriate factors or a suitable microenvironment, they proliferate and mature along a particular pathway. It is proposed that multipotent stem cells that do not receive a signal to differentiate into mature end cells progress to the next stage in the sequence of development. Alternatively, cells that do not receive a signal die. Only cells that receive proper signals will differentiate further toward mature end cells. Because commitment is gradual, there is a continuous spectrum of multipotent stem cells. Thus, some of them may be able to respond to inducers of one sort or another. In terms of self-renewal, multipotent stem cell populations continually occupy each potential for proliferation at any given time and therefore respond to the requirement for each cell type. Some daughter cells will differentiate into the next stage. The continual development of stem cells may not be diverted entirely toward one cell type, even in the presence of differentiation factors for that type. Because stem cells divide as they respond, some cells are still able to progress to the next stage of commitment.

ANTIGEN-DEPENDENT STAGE OF LYMPHOPOIESIS — PART 2

The second part of this model deals with the Ag-dependent stage, after receptors are expressed on the surface of lymphocytes. There are two main lineages of lymphocytes: T cells and B cells. T cells can mature into CD8+ cytotoxic T cells and CD4+ helper/regulatory T cells. Based on their function and the spectrum of cytokines produced, CD4+ helper T cells can be divided into Th1, Th2 and more recently, Th17 subpopulations. Treg cells are defined by the expression of Foxp3 and CD25 markers, and the regulatory function. The main functions of CD4+ T cells are to recognize cell-bound antigens, and to communicate with and help cytotoxic T and B cells to perform their duties as effector cells. Both lineages generate memory cells. How and when lymphoid cells become committed to differentiate into effector cells or into memory cells remains a mystery.

Diagrams to explain this model

Fig. 50.1 is a simple diagram to explain this model. HSCs are multipotent stem cells derived from the mesoderm. Originally they are descended from common stem cells (null, or 0, cells),

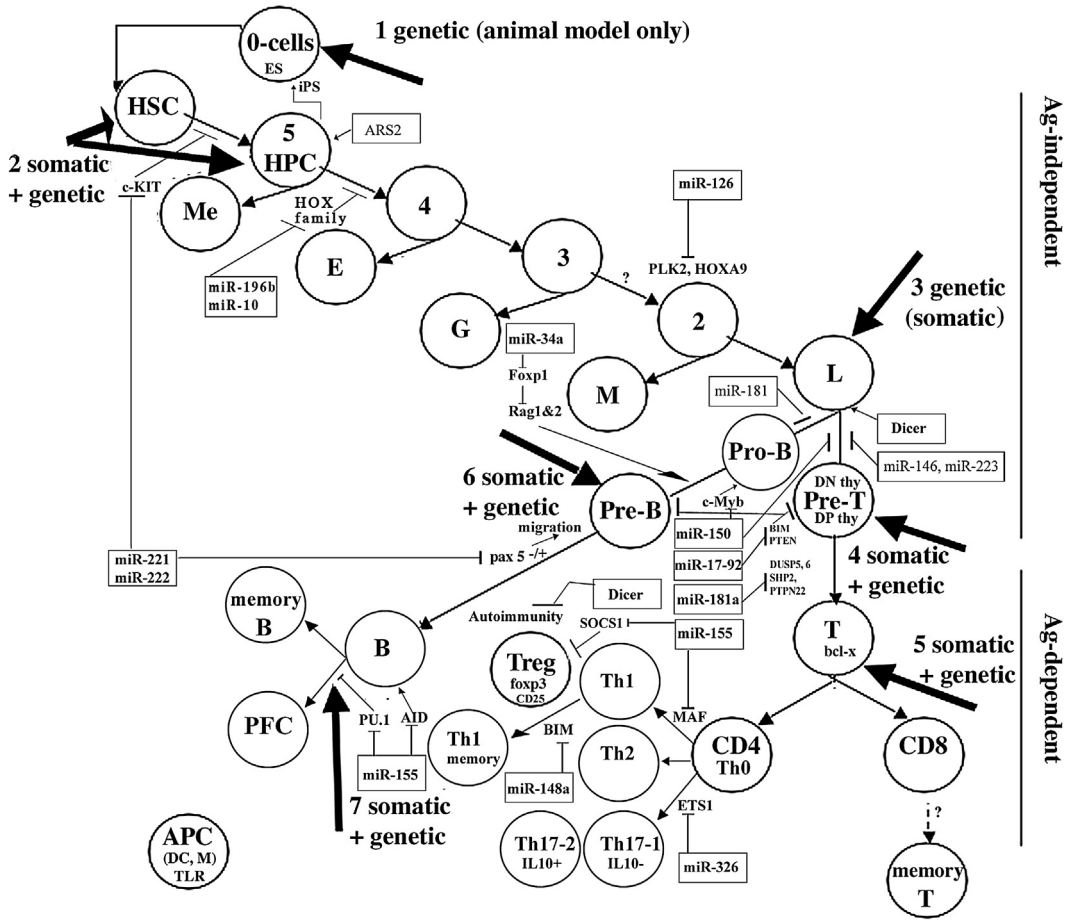

FIGURE 50.1

Proposed sequence of cell commitment during lymphopoiesis and stages of hematopoiesis and lymphopoiesis at which somatic and/or genetic engineering of lymphocytes and their precursor cells might be feasible. Solid block arrows point to the cell stages that might be targeted. The first part of the theory is the sequential commitment model of hematopoiesis of Brown et al. [1,2], modified to include the pattern of expression of transcription factors. The cells depicted diagonally to the right represent HSCs (hematopoietic stem cells) committed to multipotent precursor cells or HPCs (hematopoietic progenitor cells), which gradually lose pluripotency, as expressed by the numbers 5, 4, 3, and 2 inside the stem cells. Number 5, for example, means that the HPCs at this stage have the potential to differentiate into five different types of precursor cells. The committed monopotent precursor cells can then differentiate toward mature end cells, such as Me (megakaryocytes), E (erythrocytes), G (neutrophils/granulocytes), M (monocytes), and finally L (lymphocytes). HSCs are derived from very primitive stem cells (null cells, or 0 cells), such as fertilized eggs and/or ESCs, ES (embryonic stem cells), and/or iPSCs, iPS (induced pluripotent stem cells). In the second part of the theory, lymphoid precursor cells differentiate into mono-potential precursor B-cells (B) and monopotent precursor T cells (T). After the expression of cell surface receptors and on encountering Ag (antigen), B cells can differentiate to PFCs (plaque-forming cells) or memory B cells. T cells can become CD8+ or CD4+ cells after education and maturation in the thymus. CD8+ memory T cells can be generated at a certain stage of differentiation. CD4+Th1+memory T cells are also described. Foxp3+ (Forkhead box P3), CD25+ Treg (T regulatory)-cells are rediscovered and APCs (antigen processing cells), including dendritic cells and macrophage, seem to play a role in directing peptide-antigen recognition and activation of Th1, vs Th2, vs Th17+ cells. Th17+ cells can be further distinguished into two populations: IL10-, Th17−1 cells and IL10+, Th17−2 cells. Lipid antigen is directed to TCR via the CD1 molecule pathway. The transcription factors are expressed and are known to affect differentiation. The data come mostly from phenotypic expression in loss/gain of function approaches. The known transcription factors are cited in Chen 2007 and not repeated here. The microRNA (miRNA) pathway, which involves ARS2 (arsenate resistance protein 2), affects engraftment or reconstitution potential of HSC. Individual miRNA's, which shown to repress the expression of HSC relevant genes and to affect the differentiation of lymphoid cells and other lineages from stem cells, are indicated: miR-221 / miR-222 regulate c-KIT expression, which is thought to influence stem cell homeostasis. MiR-221 / miR-222 are also suggested to repress pax5−/+ expression, implied in the migration destination of PreB-cells/B-cells to peripheral lymphoid tissues (Melchers, personal communication, unpublished). MiR-196b is expressed in HPC and regulates mRNAs encoding the homeobox (HOX) family, possibly in cooperation with another, not yet well defined,

such as fertilized eggs, and from ESCs or iPS. The cells diagonally to the right (Fig. 50.1) represent cells committed to hematopoietic lineages, which gradually lose their multipotentiality during embryogenesis. This concept is expressed with numbers (5, 4, 3, and 2) inside the progenitor cells on the diagonal. For example, HSCs (non-cycling) enter the proliferating stage to become the hematopoietic progenitor cells (HPCs, cycling). The number 5 on a cell, for instance, means that the HPCs at this stage have the potential to differentiate into five different types of precursor cells.

The committed, monopotent precursor cells then differentiate toward mature end cells, such as Me (megakaryocytes), E (erythrocytes), G (neutrophils/granulocytes), M (monocytes), or L (lymphocytes). Lymphoid precursor cells then differentiate into monopotential precursors for B and T cells. After expression of Ag receptors on the cell surface and on encountering Ag, B cells differentiate into plasma cells, i.e., PFCs (plaque-forming cells), or memory B cells. T cells become CD8+ or CD4+ after thymic education, maturation, and negative and positive selection. Memory T cells are generated later during differentiation. Fig. 50.2 shows the markers and events that occur during the development of pre-T cells – the process of maturation, education, and apoptosis of mature CD4+ or CD8+ T cells from CD4+8+ T cells. Genes encoding transcription factors and other important genes known to be expressed specifically in lymphopoiesis and that affect function, i.e., loss of function (knockout, knock-down) or gain-of-function (ectrophic expression by transducing with retroviral vectors containing transgene of interests) are cited in the chapter of Chen [4]. Recently post-transcriptional events, especially the miRNA pathways, have been shown to play important roles in targeting the mRNAs encoding for genes relevant to the differentiation and function of the precursor/mature cells of the immune system. Some are described in the Fig. 50.1.

Some comments on this model

Very little is known about how totipotent and pluripotent stem cells differentiate into ectoderm, mesoderm, and endoderm or how mesoderm differentiates into hematopoietic lineages. These are the central issues of embryogenesis, and this part of the model is intentionally very sketchy. Why and when multipotent, lineage-specific stem cells divide asymmetrically or progress to the next stage of commitment are unknown. The underlying rules may be stochastic or inductive. But the progression of the stage of commitment is more likely due to the turning on and off of genes encoding transcription factors, and the post-

miR-10 family members. MiR-126 represses the mRNAs encoding polo-like kinase 2 (PLK2) and HOXA9, promotes the expansion of lymphoid-myeloid committed progenitor cells [16]. The production of miRNAs by Dicer is required for efficient T cell, as well as B cell development. miR-146, and miR-223 target the developmental stage from lymphoid committed stage to T cell development, and miR-181 repress the development from lymphoid committed stage to pro-B stage [58]. During the T cell development in the thymus, the miR-17–92 cluster is shown to target mRNAs encoding for PTEN (phosphatase and TENsin homologue) and BIM (BCL-2-Interacting Mediator of cell death). The repression of mRNAs coding for BIM by miR-17–92 cluster also affects the differentiation of pre-B to B cells. MiR-181a is shown to target mRNAs encoding for phosphatases such as DUSP5 (DUal-specificity protein phosphatase 5), DUSP6, SHP2 (SH2-domain-containing protein tyrosine phosphatase 2) and PTPN22 (protein tyrosine phosphatase, non-receptor type 22). In the periphery, T cell differentiation is modulated by several miRNAs: miR-155 is shown to have dual roles in T cells: to repress mRNAs of MAF (Macrophage-Activating Factor) which leads to in favor of the differentiation of CD4+Th0 cells towards Th1 cells; to repress mRNAs of SOCS1 (suppressor of cytokine signaling 1) which is implicated in the survival of Treg cells. MiR-148a is shown to repress mRNAs encoding for BIM, affecting the commitment of Th1 memory (Haftmann and Radbruch, personal communication, unpublished). MiR-326 is shown to target mRNAs encoding ETS1 and resulting in promoting the differentiation of CD4+Th0 cells toward Th17 cells. miRNAs are implied to maintain immune tolerance of Treg cells to self- tissues, thereby preventing autoimmunity. During the B cell development, besides the role of miR-17–92 cluster on BIM (above), miR-150 is shown to repress mRNAs encoding for MYB, affecting the development of ProB cells. In the periphery B cell development, Beside affecting two steps of T cells development (above), miR-155 targets mRNAs encoding for AID (activation-induced cytidine deaminase) and PU.1 which promotes class switch and antibody production [16].

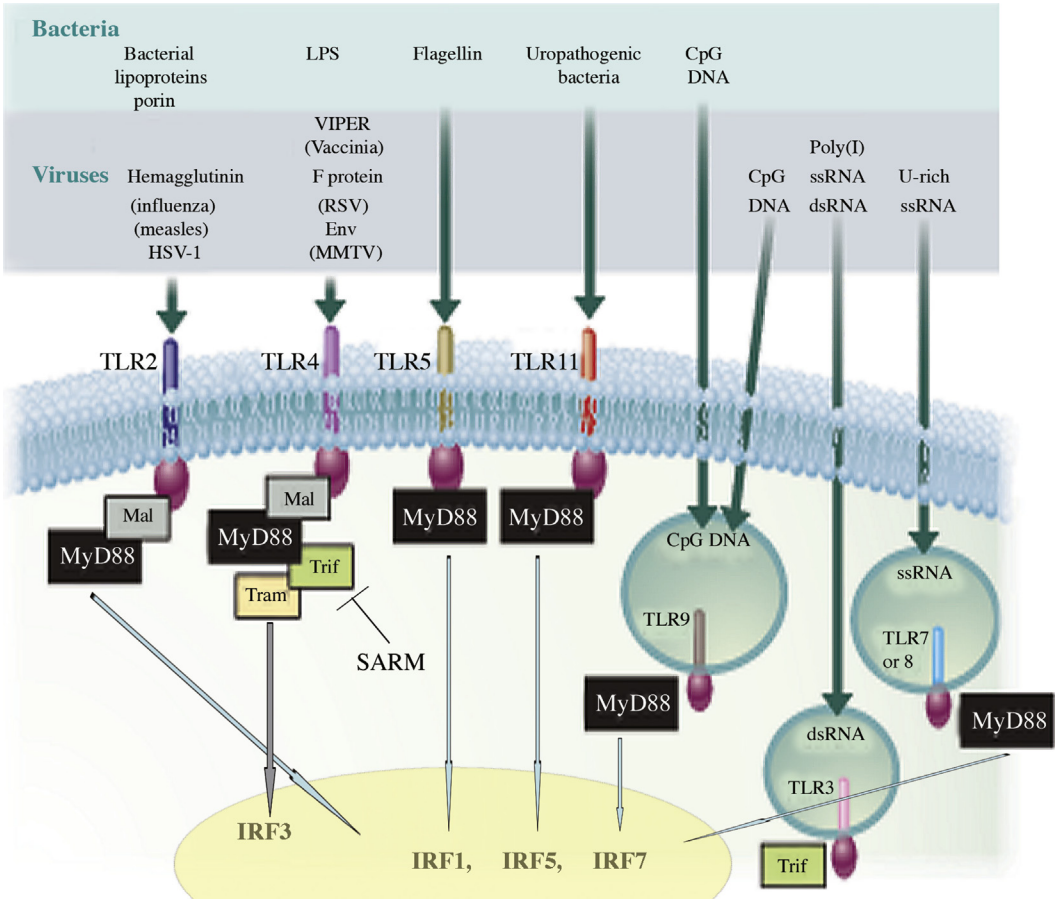

FIGURE 50.2

Going down the Toll mine. Toll-like receptors (TLRs) provide a repertoire for sensing pathogen-derived molecules during the innate immune response. TLRs in endosomal membranes detect bacterial and viral nucleic acids. The relative contribution of each TLR to the innate immune response is not yet known because pathogens contain multiple ligands specific for several different TLRs. The signaling pathways associated with each TLR are different, although they share common components. Specific signals may emanate from the adapter proteins recruited by each TLR (MyD88, Mal, Trif, and Tram). One important question concerns how the immune response is tailored to each pathogen according to the activation of specific signaling pathways triggered by different pathogen products (O'Neill et al., 2003). Besides dsRNA, TLR3 was shown to be able to sense ssRNA such as poly(I) (polyinosinic adid) [105]. HSV-1, herpes simplex virus 1; LPS, lipopolysaccharide; VIPER (Viral inhibitor peptide of TLR4) from A46 protein of vaccinia virus [104], RSV, respiratory syncytial virus (corrected following the erratum, Science, postdate 4 June 2004); MMTV, mouse mammary tumor virus; Porin (bacterium, not influenza). *(From O'Neill [62] with permission. The figure has been modified following the erratum in Science, postdate 4 June 2004), i.e., Porin is a bacterial component and not viral and is shifted from the grey region upward to the green region. I have also included the IRF family transcriptional factors as targets for TLR 5 members signaling [64]).*

transcriptional events regulating the epigenesis, and expression, degradation of mRNAs and the translation machinery.

Brown and coworkers originally formulated their model based on data from the differentiation pattern of several myeloid cell lines. Their scheme of the development of lymphoid lineages was purely hypothetical in the original model, and the development of mature lymphoid cells (in Part 2, Ag-dependent step) was not included. It was proposed that precursor B cells become committed before precursor T cells. I have modified this step to the pre-commitment to lymphoid precursor cells. Also, I have changed the commitment of lymphoid precursor cells to occur after the myeloid precursor cells. However, the committed lymphoid precursor cells should still be viewed as hypothetical, because there are many examples supporting the common lineage development of B cells and myeloid cells and not of precursor T cells. Committed lymphoid precursor cells are not clearly identified and isolated (details in Stage 3, later).

There is ambiguity in the original model; for example, the issue of the timing of each point of decision was not addressed. The original hypothesis implied that stem cells that do not receive the appropriate signals would proceed 'spontaneously' to the next stage in the differentiation sequence. Based on published data and on observations on the abortive development of lymphoid cells from blood islands derived from EBs (embryoid bodies) [4,5] and on the essential requirement of cytokines to maintain and to propagate stem cells derived from transgenic fetal liver, I tend not to be in favor of spontaneous progression; appropriate inductive signals for the transcriptional activation of lineage-committed genes are stringently required. Moreover, because programmed cell death plays an important role in the fate of cells during embryogenesis, I have included apoptosis as another important factor. This model has gained support from the phenotypes of mice in which genes encoding transcription factors have been deleted by knockout, knock-down, the so-called loss-of-function approach, and from the phenotype of cells in which transgenes are transduced using retroviral vectors, the so-called gain-of-function approach. Transcription factors controlling the sequential commitment of HSCs have been summarized [4]. In this chapter, I shall include the individual/clusters of miRNAs, which are reported to play critical roles in regulating the differentiation pathways and some functions of lymphoid (precursor) cells.

CRITERIA FOR ENGINEERING DEVELOPMENTAL STAGES OF LYMPHOPOIESIS

I attempt to explore the feasibility of engineering lymphoid cells and their stem cells at several defined stages of differentiation. The criteria for deciding on feasibility are:

1) Intrinsic self-renewal of HSCs, HPCs, and more differentiated cells,
2) The availability of cell surface markers for identification and purification,
3) The supply of recombinant growth factors and appropriate stroma cells,
4) The existence of favorable cell culture conditions that promote growth instead of differentiation,
5) The possibility of educating lymphocytes to become antigen-specific memory cells,
6) The possibility of introducing, either genetically or somatically, genes of interest along with inducible promoter and regulatory sequences,
7) The possibility to introduce siRNAs / miRNAs of interests into the cells in culture or by genetic manipulation of mice to show the phenotype of targeting the mRNAs encoding the gene products.

Technical advances make the engineering of HSCs, HPCs, and lymphoid cells attractive for further manipulation, such as introducing new genes or reprogramming cells to iPS or manipulating the gene products and function post-transcriptionally via miRNAs.

STAGES OF LYMPHOPOIESIS FOR ENGINEERING

Fig. 50.1 shows a schematic diagram of the stages at which lymphoid cells and lymphoid precursor cells might be engineered. These stages are now discussed individually.

Stage 1: 0 cells

Null (0) cells include the fertilized eggs, which are totipotent, the blastocyst-derived Embryonic Stem Cell (ESC) lines, and somatic cells reprogrammed into induced Pluripotent Stem Cells (iPS), both of which are pluripotent. Mouse ES cell lines of different strain origin have been available for a long time, and they possess the property of differentiating into different somatic lineages *in vitro* and *in vivo*, homologous recombination, and germline transmitted through the crossing and breeding of progeny. ES cells from many other animal species are also available. Establishment of human ES-like cell lines has been reported since 1998, and many

In Vitro Fertilization (IVF) units can derive such cell lines. Cellular reprogramming of mouse somatic cells to ES-like, or iPS was reported, when embryonic fibroblasts were infected with retroviral vectors containing four transcription factors: Oct4, Sox2, Klf4 and c-Myc (mOSKM) [6,7]. Using similar strategy, human iPS cells (h-iPS) were derived using human OSKM transcription factors [8] or when Klf4 and c-Myc were replaced with transcriptional factors, Nanog and Lin28 [9].

Other technologies and delivery methods have been rapidly applied to this field; integrating viral vector system in combination with Dox-inducible expression, transient or excisable methods have been reported. More encouraging approaches include non-integrated RNA viral vectors (such as the Sendai virus [10], or more recently temperature-sensitive Sendai virus (Yamada, et al., personal communication, unpublished)), recombinant proteins incorporating cell-penetrating peptide moieties [11,12], improved episomal plasmid vectors [13], modified synthetic mRNAs of hOSKM [14,14a], are shown to be able to reprogram human skin fibroblasts and other cell types to become h-iPS cells. Some strategies are labor intense, but have reasonable efficiency and no genomic alternation which allow potential clinical application. More strategies to derive iPSs are developing.

Through differentiation *in vitro*, several lineage-committed cells, such as those from bone and cartilage, have been shown to be obtainable from hES cells and h-iPS. There are conflicting reports about the differentiation potential of hES/h-iPS cells into lymphoid lineages *in vitro* (see later).

Due to its potential to be an unlimited source for 'personalized' transplantation or autotransplantation for treating blood diseases, many groups are investigating whether iPSs could differentiate into lymphoid lineages *in vitro*. Some investigators suggested that iPSs might retain epigenetic memory; i.e., they are prone to differentiate preferentially into the original somatic cell types from which they are derived. This might distinguish iPSs from ES cells.

Strictly speaking, the only way to prove that an hES/iPS cell line is truly pluripotent is to demonstrate germline transmission in human beings − a task that should not even be considered. Similarly, genetic and cellular engineering at this level should be limited to studies *in vitro*. ES/iPS cells are stem cell lines that divide symmetrically. Unlike lineage-committed stem cells, which are rare, ES/iPS cells are almost an exception, being readily available in abundance. mES cells can proliferate without differentiation if feeder cells and/or cytokines such as LIF (leukemia inhibitory factor) are provided, whereas the hES/h-iPS cell lines established so far are feeder-cell-dependent, and LIF has no supportive effect. They can be induced to differentiate by removing the feeder cells (and cytokines). A few surface molecules are available for characterizing ES/iPS cells. Antibodies against various components that have been used to characterize such cells, such as those against stage- specific embryonic antigen-l (SSEA-l), enable one to distinguish undifferentiated from differentiated mES cells. mES/m iPS cells are SSEA1+, SSEA3−, SSEA4−, TRA-1−60−, TRA-1−81−, Oct4−, Oct6+, AP+ (alkaline phosphatase), telomerase+; hES/h-iPS cells are SSEA1−, SSEA3+, SSEA4+, TRA1−60+, TRA1−81+, Oct4+, AP+, telomerase+.

Stem cell genomics, or the systems biology approach to stem cells, is being used. It considers the complex nature of cells and aims at a comprehensive understanding of stem cell mechanisms. The gene expression profiles of different stem cell types are being determined using DNA microarrays. These molecular signatures are stored and available in the stem cell database and allow a comparison of different stem cell types. On the basis of the obtained molecular signature, candidate genes, such as transcription factors, are subjected to the loss-of-function approach by knocked-out, knocked-in, or knocked-down by RNAi/siRNA, and the effects on the expression of downstream genes are determined with DNA microarrays. In this way, gene regulatory networks can be rapidly identified. More miRNAs pathways identified to regulate networks are characterized.

This approach has been used to identify gene regulatory networks implicated in the self-renewal and differentiation of mES cells. Based on a microarray time course study of retinoic acid-induced ES cell differentiation, 169 genes that are downregulated in the course of differentiation were found, and 40 of them were investigated further. ES cell cultures infected with lentiviral vectors containing the EGFP gene in addition to one of these 40 genes (RNAi/siRNA knock-down or overexpression) were investigated in a competition assay, where respective infected cells were mixed with not infected cells. In this way, higher or lower proliferation rates of the infected cells could be determined based on measured EGFP fluorescence. Changes in ES cell number could be due to a function of the respective gene in cell cycle progression, cell survival, or prevention of lineage-specific differentiation. Downstream effects of changes of gene expression were determined with DNA microarrays. Such an approach also applies to the study of other cells, such as hES cells, hEC cells, h-iPS cells, HSCs, pre-B cells of mouse and, human origins, as well as mouse pre-T cells [15], and Treg cells (ref. cited in [4]).

In addition to microarray studies for stem cells, the extent of post-transcriptional modifications within the stem cell transcription has been reported. Thousands of genes expressed in HSCs and ES cells undergo alternative splicing. Using combined computational and experimental analyses, the frequency of alternative splicing has been found to be especially high in tissue-specific genes, as compared to ubiquitous genes. Negative regulation of constitutively active splicing sites can be a prevalent mode for generation of splicing variants, and alternative splicing is generally not conserved between orthologous genes in human and mouse (ref. cited in [4]).

The study of miRNAs has become a major subject in recent years. miRNAs are highly conserved, small, single-stranded non-coding RNAs, encoded by genomic DNA and are most commonly transcribed by RNA polymerase II. They function by directly binding to the 3′ untranslated regions (UTRs) of specific target mRNAs, leading to the repression of protein expression and the promotion of target mRNA degradation. The initial discovery was made in plants and worms, and later they were found in mammals. At least 700 different miRNAs have been identified in the human genome, and $>$ 100 different miRNAs are expressed by immune cells. miRNAs have the potential to influence the molecular pathways that control the development and function of the immune system. The pathways of miRNAs are shown to be the regulators of networks. In addition, several post-transcriptional regulatory mechanisms that affect miRNA processing have been identified. The processes of miRNA biogenesis, target mRNA repression and function have been reviewed [16–18] and are beyond scope of this chapter.

Many genes encoding lymphoid-specific functions have been knocked out, miRNA/siRNA knock-down technology has been applied, and more studies are under way to develop specific deletions or replacements of genes. Vectors with inducible promoters are being used to introduce genes in a controllable, tissue-specific manner. Fig. 50.1 includes various individual or clusters of miRNAs, which have been shown to target the mRNAs encoded by genes critical for the regulation of differentiation to lymphoid lineages from HSCs.

Growth requirement for differentiation of ES/iPS cells to lymphoid cells

Under appropriate conditions, ES/iPS cells can differentiate *in vitro*. Many different culture systems are available for studying the development of lineage commitment. Because ES/iPS cells are pluripotent, optimal culture conditions would enable the formation of mature cells and tissues and even of organs in well-organized three-dimensional structures *in vitro*. That is, one should be able to generate an artificial fetus in culture without any external cells or other factors. In fact, organogenesis from ES/iPS cell culture is still in its infancy. Nevertheless, with the culture conditions and system that we have developed, mature and embryonic cells in

some form of tissue-like organization, though never become the functional and true organs, of certain orderly structures (gut-like, epidermal-like, and thymus-like structures), can be observed.

Many genes have been identified that play important roles in the differentiation of ES/iPS cell to mesoderm and then to derivatives such as HSCs (see figure legends [4]).

Many researchers have attempted but failed to study the differentiation of lymphopoietic precursor cells obtained from mES cells. Many have observed the development of yolk sac blood islands in culture; these presumably contain HSCs. But only myeloid cells could be produced in the culture systems reported. Only a few systems allow the generation of lymphoid precursor cells, and these were mostly B cells, but also T and B cells, in a quantity sufficient for further manipulation [4,5].

The reasons for these variations are unclear. It is partially due to the origin and pluri-potentiality, hence the fragility and variability, of ES cells but partially due to experimental manipulations, different culture conditions [5,18a], and the transient expression of genes relevant to the developmental stage of hemato-lymphopoiesis from mesoderm equivalent at the yolk sac stage. Homeodomain transcription factors, HoxB4 (see later) is an example of such genes. Even in the hands of a single researcher, the potential of lymphopoiesis from the same ES cell lines varies greatly among different experiments, and why this is so remains a mystery. The culture conditions vary, depending on which lineages are chosen for study. For example, methylcellulose culture is preferred for myeloid lineages but is inferior for lymphoid lineages. Several culture conditions that favor the differentiation of ES / iPS cells to lymphoid lineages are available.

Through the work of many groups, it has become clear that lymphoid precursor cells can be obtained from mouse ESCs in culture. However, the development of mature B plasma cells and mature T cells requires a two-step procedure — a two-step culture procedure for plasma cells, and culture and animal implantation for mature B and T cells. When chimeric bone marrow cells from embryoid body-implanted nude mice, which contain cells of ESC origin, were injected into the host mice, the bone marrow cells were shown to repopulate the primary and secondary lymphoid organs, i.e., bone marrow, spleen, and lymph nodes. The data indicate that HSCs and HPCs derived from ESC can be transferred. However, when injected into the host animal, such as lethally irradiated adult mice instead of un-irradiated nude mice, dissociated cells from blood island of the EB, instead of the whole EB, killed the mice when intravenial injection was performed, and failed to repopulate the host immune system or to achieve a stable engraftment when intra-peritoneal injection was performed [5].

The work on the expression of (transcriptional) molecules such as HoxB4, Cdx4, and especially, the transduction of transgenic expression under the inducible tet-promoter using retroviral vector, has been shown to understand the mechanism and pathway. HoxB4 is a homeodomain-containing transcription factor with diverse roles in embryonic development and the regulation of adult stem cells. This gene has a double life and can act in opposite ways when expressed by different cells, promoting the proliferation of stem cells while activating the apoptotic pathway in some embryonic structures. It is implicated in the self-renewal of definitive HSCs, being transiently expressed in blood developmental stages somewhere between the yolk sac stage and the fetal body stage and also being expressed in human cervical carcinoma. Persistent overexpression of HoxB4, however, inhibits the development of blood lineage.

It was examined to show its effect on hemopoiesis using a variety of tools, including retroviral transgenic expression. Expression of Homeodomain transcription factors, HoxB4 in primitive progenitors, and when combined with culture on stromal cells, induces a switch to the definitive HSC phenotype. These progenitors engraft lethally irradiated adult mice and contribute to hematopoiesis in primary and secondary hosts. Thus, HoxB4 expression

promotes the transition from primitive cells to become HSCs. Overexpression a high level of expression, of HoxB4 is shown to perturb the differentiation and to predispose the manipulated cells to leukemogenesis. HoxB4 may affect cell growth in a dose-dependent manner and may disturb the differentiation into lymphopoiesis.

The manipulation of essential genes has partially overcome the problems of variability in the development of hematopoietic cells from mesoderm-derived cells, such as yolk sac cells and EBs, and increased engraftment into irradiated adult mice. Combining HoxB4 gain-of-function and caudal gene family, Cdx4 gain-of-function has been shown to increase the multilineage haematopoietic, including lymphoid, engraftment of lethally irradiated adult mice.

Others that have been tried are: mMix, Stat 5, a signal transducer under similar inducible conditions to express in mES-derived HSC and to grow similarly on OP9 stroma cells, shown to increase the induction of preferable myeloid, rather than lymphoid, differentiation. BCR/ABL, a chronic myeloid leukemia-associated oncoprotein, is shown to transform a subset of HSC between erythryo- and lymphoid-myeloid lineages (ref. cited in reference [4]).

The combined *in vitro* and *in vivo* culture system described earlier using nude mice as the hosts can also be employed to study thymic stroma as well as well-organized, highly differentiated cells of many other lineages, including gut epithelia, skin with hair follicles, bone, muscle, neurons, and glia. One essential requirement for successfully applying this combined system is that the cells should first differentiate in culture, until the EB developed before the subcutaneous implantation. Otherwise, tumors may grow, due to the expression of the acylated dimeric iLRP (immature 32—44 kDa Laminin Receptor Protein) and other, still-to-be-identified OFA (OncoFetal antigen) positive in early to mid-gestation [19]. The development of endogenic thymic epithelial cells as well as stroma cells inside the ES-embryonic bodies also explain partially the findings of an efficient development of T cells in the absence of exogenous stroma cells.

Stroma cell line RP.0.10 is able to support both T- and B-lymphoid precursor cells derived from ESCs in some labs. Stroma cell lines OP9 and S17 were originally reported to support B precursor cells and myeloid but not T precursor cells derived from ESCs. Many other stroma cell lines do not support the differentiation of ESCs to lymphoid precursor cells. The S17 stroma cell line was compared to many stroma cell lines for the production of cytokines and stroma function, i.e., the stem cell support function (ref. cited in reference [4]) (see also later, Stage 2; there were no correlations in the assays. Thus a yet-to-be-identified extracellular matrix molecule of S17 cell lineage may play a role in supporting the function of HPCs.

With the insertion of genes involved in Notch-Delta signaling pathways, it was shown that the OP9-derived cell line, OP9-DL1 (Delta-like 1), increases efficient T cells, which appeared from sources such as fetal liver [20], human cord blood [21], ES cells [22] and recently ES / iPS (see later). The study using switch cultures shows that while necessary to induce and sustain T cell development, Notch/Delta signaling is not sufficient for T-lineage specification and commitment but, instead, can be permissive for the maintenance and proliferation of uncommitted progenitors that are omitted in binary-choice models. Moreover, it was demonstrated that a three-dimensional structure to allow the interaction of precursor-supporting cells plays an essential role for it to function well (see also Stage 4).

Other candidate molecules and cells that may support the lymphopoiesis include the following: dlk, an epidermal growth factor-like molecule of 66 kDa in stroma cell lines was reported to influence the requirement for IL (interleukin)-7 in supporting mouse pre-B lymphopoiesis, and dlk is suggested to play an important role in the bone marrow HPC microenvironment. Another high-molecular-weight CD166 (HCA/ALCAM) glycoprotein was shown to express in human HPCs as well as stroma cells. This molecule is involved in adhesive interaction between HPCs and stroma cells in the most primary blood-forming organs.

Rethinking the seemingly conflicting results and the inconsistent report of differentiation of ES/iPS cells to lymphocytes, I wonder if culturing ES/iPS-derived lymphoid progenitor cells on stroma cell lines derived from the most primitive stroma environment from the fetus (such as paraaortic cells) may be a better choice for obtaining consistent results, rather than using the existing stroma cell lines. Indeed, stroma cell lines from such a source have been derived.

The field of hESC/iPS (human ESCs/induced pluripotent) cells represents a theoretically inexhaustible source of precursor cells that could be differentiated into any cell type to treat degenerative, malignant, or genetic diseases or injury due to inflammation, infection, and trauma. This pluripotent, endlessly dividing cell has been hailed as a possible means of treating various diseases. hESCs/iPSs are also an invaluable research tool to study development, both normal and abnormal, and can serve as a platform to develop and test new therapies.

They are also a potential source of HSCs for therapeutic transplantation and can provide a model for human hematopoiesis. The first stable hES cell line was reported in 1998 [23]. Now many centers can establish hES cell lines, and clinical trials for treating spinal injury and Macular Dystrophy are proceeding in the US and Europe. The first human iPS cell line was reported in 2007 [8], and many other labs could derive such somatic reprogrammed stem cell lines using better strategies. The current goal is to establish clinical grade hES/iPS cell lines for potential therapeutic purposes. Many clinical grade hES cell lines are available. However, so-called clinical grade, personalized, stem cell lines — the human iPS cell lines — still need to be derived in the clean air condition. Hopefully in due time, the limiting steps such as the sources for establishing Good Manufacturing Practices (GMP) grade non-integrating vectors/synthetic RNAs, other essential technical tools, raw materials required for culturing of transfected or transduced somatic cells, and the isolators instead of GMP facility, will become widely available and affordable.

More attempts to differentiate hESCs to HSCs *in vitro* have been reported recently [24–29b]. From such studies, hES cells have been shown to differentiate into HSCs, using various protocols and different supportive stroma cell lines, with or without cytokines. It seems that the differentiation into mesoderm-derived lineages and then to MHE (mesodermal-hematoendothelial) lineage can occur, resembling human yolk sac development. The cellular and molecular kinetics of the stepwise differentiation of hESCs to primitive and definitive erythromyelopoiesis from hEBs in serum-free clonogenic assays have been demonstrated [27]. Hematopoiesis initiates from CD45+ hEB cells, with the emergence of MHE colonies. A first wave of hematopoiesis follows MHE colony emergence and is predominated by primitive erythropoiesis. A second wave of a definitive type of colonies of erythroid, Granulocyte-Macrophage (GM), and multi- lineage Colony Forming Cells (CFCs) follows. These stages of hematopoiesis proceed spontaneously from hEB-derived cells without requirement for supplemental growth factors during hEB differentiation. Gene expression analysis revealed that initiation of hematopoiesis correlated with increased levels of SCL/TAL1, GATA1, GATA2, CD34, CD31, and Cdx4. These data indicate that the earliest events of embryonic and definitive hematopoiesis can be differentiated from hESCs *in vitro*.

However, differentiating hEBs further into HSCs, and lymphohematopoietic lineages is variable and unpredictable.

Vodyanik et al. [25] reported that hES cells could differentiate to CD34+ cells using mouse stroma OP9 (better than S17 and MS-5) co-culture system. However, when cultured on stroma MS-5 (but not OP9, S17) in the presence of stem cell factor, Flt3-L, IL7, and IL3, such CD34+ cells could differentiate into natural killer cells and pre-B-lymphoid cells which expressed mRNAs for VpreB and Ig-alpha (CD79a or mb-1) components of pre-B cell receptor complex, as well as myeloid lineages, but not T lymphoid cells. In another report, the hES-derived CD34+ cells were implanted into fetal sheep intraperitoneally and differentiated in primary

and secondary hosts, the cells could differentiate into many cell types but no lymphoid cells could be detected [29a]. Martin et al. [109] claimed that natural killer (NK) cells could be derived, but not T or B lymphoid cells under the culture condition and protocol used.

The expression of transcription factor HoxB4 appears to play a role in causing cells to become lymphoid cells in the mouse system and would explain the occasional determination of precursor cells to commit to lymphoid lineages. It implies right away that can apply to study the lymphohemopoiesis in the human system. Indeed, Bowles et al., (2006) have reported such an attempt. Culture of hES cells on mouse feeder cells or in cell-free culture conditions result in low levels of differentiated HPCs. Transgenic stable HoxB4-hESC clones were generated by the lipofectamin transfection method. *In vitro* differentiation of hESCs, as EBs, in serum-containing media without cytokines led to the sequential expansion of erythroid, myeloid, and monocytic progenitors from day 10 of culture. These cells retained the capacity to develop into blood elements during culture. Overexpression of HoxB4 considerably augments the development of three myeloid lineages of hESCs; however, no lymphoid progeny were reported.

Theoretically, lentiviral vector introducing HoxB4 under a tet-on system should be a comparable approach for further study. However, another report does not support this notion to show a functional role of HoxB4 in HSCs in their combined *in vitro* and *in vivo* system, using Server Combined ImmunoDeficiency (SCID) mice [26]. It is interesting that hES-derived HSCs failed to reconstitute i.v. transplanted host because of cellular aggregation causing fatal emboli formation, which is similar to our attempt using mEB-derived cells. Direct femoral injection allowed host survival and resulted in multilineage hematopoietic repopulation. However, hES-derived HSCs had limited proliferative and migratory capacity compared with somatic HSCs, which correlated with a distinct gene expression pattern of hESC-derived HSCs that included HoxA and HoxB gene clusters. Transduction of HoxB4 had no effect on the repopulating capacity of hES-derived cells. A yet-to-be-identified molecular program is postulated to contribute to the atypical behavior of hES-derived cells *in vivo*.

The following more recent reports contribute to the positive aspects of the debate. Using sequential *in vitro* co-culture of EGFP-expression hESC on stroma OP9 for 10 days, and engraftment into the kidney capsule of SCID mice, or RAG2−/− mice, containing fragments of human thymic- and fetal liver tissues, the so-called SCID-hu(Thy/Liv) / RAG2-hu(Thy/Liv) mice, Galic et al. [28] demonstrated that T lymphoid lineage with the phenotype of EGFP-expressing cells could be found in various stages of thymocyte maturation, including immature CD4+CD8+, and mature CD4+CD8−, and CD8+CD4− cells. When such thymocytes were harvested and cultured with plate-bound anti-CD3 plus soluble anti-CD28 stimulating conditions, the cells were shown to be responsive to the stimulation by increasing CD25 expression. The reporter EGFP transgene continued to express at high frequency in the hosts throughout thymopoiesis in 3−5 weeks of experimental time.

Timmermans et al. (2009) used four steps of differentiation protocols by co-culturing hESC with stroma OP9, and OP9-DL1. After 7−9 weeks of differentiation, they could find T lymphoid cells, with the phenotype of CD4+CD8beta+ and TCRgamma-delta+ cells. CD3+ T cells was stimulated with PHA, and shown to be able to proliferate and to produce IFNγ.

Thus, conflicting reports continue to surface, giving the impression that the differentiation into lymphohemopoiesis seems to be, if not more rare, as rare as variable as the *in vitro* differentiation system using mES cells. The question of the variability among different hES cell lines and clones, similar to the observation using mES cells, are also reported. Derived, somatic, tissue-committed, stem, precursor cells remain rare. The study of individual gene expression using hES cells and its relationship with the group of genes and the final outcome of the cell fate is a challenge and will require more research in the future. Combining multiple genes, such as mMix, Cdx4, and HoxB4, with a gain-of-function approach might be a possible way of increasing the lymphohematopoiesis derived from hES cells.

In addition to hES cells, many groups are investigating whether iPSs could differentiate into lymphoid lineages *in vitro*, using the iPSs derived from blood cells as well as fibroblasts.

Below are some selected examples of potentially promising experiments. Wada et al. [110] used a three step system for m-iPS differentiation *in vitro*: EB formed after 5 days differentiation: in suspension culture, single cells were harvested and co-cultured with Flt3L (Flt3 Ligand) and stroma OP9 for eight days, then harvested every six days and co-cultured with Flt3L, IL7, and stroma OP9 for B cells, or stroma OP9-DL1 for T cells. The results showed that mouse B cells and embryonic fibroblast derived iPS could differentiate into T cells by showing TCRVbeta rearranged, CD4−CD8+ were generated, Treg could be detected upon TGFbeta stimulation, but no B cells could be detected. The data challenged the suggestion that iPS tend to retain epigenetic memory.

Carpenter et al. [111] reported differentiation into pre-B cells by co-culturing human dermal fibroblast derived iPS cells with stroma OP9 for 10 days. CD34+ cells were harvested, then co-cultured with stroma MS-5 for a further 21 days. The differentiated cells had the phenotype of CD45+ CD19+ CD10+; and expressed mRNAs encoding for Pax5, IL7R-like, and VpreB receptor; exhibited partial genomic D fragment-J fragment of Heavy chain (D-JH) rearrangements, but no full Variable region of Heavy chain -J fragment of Heavy chain (VHJH) rearrangement. Such cells were negative for surface IgM and CD5 expression.

For the purpose of treating patients infected with HIV, Ye et al. (personal communication, unpublished) developed HIV resistant h-iPS by preparing iPS cells from patients CD34+ cells and peripheral blood mononuclear monocytes using Sendai viral vector. iPS cells were knocked out both wild-type CCR5 alleles or replaced them with the CCR5δ32 mutation using BAC based homologous recombination and differentiate them into HSC by the spin Embryoid Body (EB) method without any stroma cells in the culture. The HSCs are ∼50% CD34+ and ∼25% CD34+/CD43+ HSC, HIV resistant, and could be used for further characterization, differentiation into mature blood cells including monocytes and lymphocytes, and for autologous transplantation.

In order to completely remove the residual Sendai viral vector containing the MOSK which was shown to interfere with the differentiation potential of iPS *in vitro*, Yamada et al. (personal communication, unpublished) derived h-iPS cells from blood T cells or fibroblasts, which were reprogramd using temperature sensitive (ts)-Sendai viral vector containing tsA58 (SV40Tag)MOSK. Such iPS could differentiate in culture to HSC by co-culturing with stroma OP9, and further cultured at the presence of stroma OP9-DL1 plus SCF, IL7, Flt3L, T lymphoid lineages could be detected. Tumor antigen-specific CD3+, CD4+ or CD8+ lymphocytes from precursor T cells could be generated and expanded. Antigen-specific T cells derived from iPS were also obtained by Nishimura et al. (personal communication).

Vizcardo et al. (personal communication) could establish Mart1-T-iPS cells from Mart1 (Melanoma antigen recognized by T cells 1)-specific CTL cells. These iPS cells were differentiated *in vitro* into CD4+CD8+Mart-1+ precursor T cells. In the mouse system they could expand proT cells with solid phase DL-1 and IL7. The h-Mart1-T-iPS was shown to differentiate to proT-cell stage *in vitro* and to expand using a set of human cytokines. They are trying to induce mature T cells by transplantation into NSG (NOD-SCID common gamma−/−) mice and *in vitro*.

Stage 2: HSCs and HPCs

The terms hematopoietic stem cells, progenitor cells, and precursor cells are used loosely in the literature. Hematopoietic stem cells and HPCs are defined as two populations of stem cells sharing similar surface markers. HSCs are rather quiescent, non-cycling stem cells, whereas HPCs are cycling cells. In mice, c-kit+, thy-1low, lin−, and scal+ bone marrow cells are defined as HSCs or HPCs. HSCs and HPCs in bone marrow are heterogeneous in size and in self-renewal capacity. Moreover, they have finite life spans. In human bone marrow,

CD34+HLA-lin-thy-1low rhodamine123low stem cells are generally defined as HSCs and HPCs. However, CD34 is also expressed in other lineages, such as vascular endothelial cells and muscle precursor cells. It is well established that mouse HSCs and HPCs can be isolated, cultured, and transduced by retroviruses and used to repopulate animal hosts. In addition to bone marrow, they can be isolated from fetal liver, cord blood, fetal blood, yolk sac, and para-aortic spanchnopleura.

DO HSCS AND HPCS SELF-RENEW?

The self-renewal of HSCs and HPCs was carefully examined (ref. cited in reference [4]). By transferring bone marrow stem cells from one host to the other, it was found that the pool of donor bone marrow stem cells was smaller on transfer, and it was concluded that self-renewal is limited for somatic bone marrow stem cells. However, the results could be due to a dilution effect of the donor stem cells, if a certain absolute number of stem cells were required for the successful implantation of donor stem cells in the bone marrow. Using fluorescence *in situ* hybridization (FISH) and telomerase assays, it was shown that telomere DNA length predicts the age and replication capacity of human fibroblasts. DNA telomere length has been correlated with the replication potential of HSCs and HPCs. In human CD34+, CD71low, and CD45RAlow bone marrow stem cells, the length of telomeric DNA in HSCs and HPCs is correlated with the age of the donor. Stem cells in adults have shorter telomeres than those found in fetal cells, but both have the same telomerase activity. When the HPCs were expanded with cytokines without the support of stroma cells, there was a loss of telomeric DNA in culture. The loss of telomeric DNA in HSCs and HPCs from older people and cultured stem cells was interpreted as meaning that their replication potential is finite. An alternative interpretation would be that the culture conditions that allow stem cells to grow in the presence of cytokines may not favor the maintenance of telomerase activity and hence not the self-renewal of HSCs and HPCs.

Work on global miRNA expression profiling of human CD34+ HSC / HPC from Bone Marrow (BM), mobilized Peripheral Blood Cells (PBC) harvests, and umbilical cord blood have helped to identify several miRNAs expressed by the stem cell population. Genetic manipulation of the mice also helped to identify the miRNAs pathway related factors. Arsenate-resistance protein 2 (ARS2) is one of the factors identified to be involved in post-transcriptional regulatory mechanisms that affect miRNA processing. ARS2 expressed by HPC and is a component of the RNA cap-binding complex that promotes processing of pri-miRNA transcripts [30]. Mice deficient in ARS2 have bone marrow failure, possibly owing to defective HSC function, such the engraftment or reconstitution potential of HSC. Individual miRNAs, which shown to repress the expression of HSC relevant genes and to affect the differentiation of lymphoid cells and other lineages from stem cells are indicated in Fig. 50.1: miR-221 and miR-222 were shown to inhibit c-Kit expression in HSC and HPC, leading to impaired cell proliferation and engraftment potential of human CD34+ cells in NOD-SCID mice [31]. miR-221 and miR-222 are also suggested to play a role influencing cell migration at the Pre-B cell stage.

miR-196 and miR-10 were found to be located in the HOX loci; directly repress HOX family expression, thus regulating HSC homeostasis. Particularly, miR-196b was found to express in mouse HPC, regulated by transcription factor Mll (Mixed lineage leukemia), and to modulate HSC homeostasis and lineage commitment by repressing HOX family [32]. miR-126 is thought to act on suppressor PlK2 (polo-like kinase 2), shown to increase colony formation of bone marrow M-L progeny *in vitro* [33]. More work is needed to study the role of miRNAs in the long-term engraftment potential of HSC *in vivo*.

Many culture systems for studying the biology of mouse HSCs and HPCs are available. Many mouse stroma cell lines support the growth of HPCs. Studies on human stem cells use mouse stroma cell lines, such as S17 and AFTO24 among many others, or mixed primary stroma populations, called MSCs (mesenchymal stem cells), isolated from human bone marrow or

other sources, to support purified HSCs and HPCs or unseparated bone marrow cells. The establishment of heterogeneous human stroma cell lines with the help of a plasmid-containing SV40 T antigen under the control of an inducible metallothionein promoter has been reported, but their ability to support lymphopoiesis could not be demonstrated. Mixed human stroma cells, or MSCs, and cell lines that support human long-term culture-initiating cells also exist. However, these human stroma cell lines could not be shown to support the development of human lymphocytes from bone marrow stem cells.

MSCs have become a promising field, and many clinical trials are under way. The expansion of human MSCs in culture presents a challenge. They proliferate poorly in media supplemented with human serum, and they require the presence of selective lots of Fetal Calf Serum (FCS). Human MSCs are not immunogenic in an allogenic system *in vitro*, nor are they rejected *in vivo*. This makes them candidates for allogenic cellular therapy. MSCs have immunomodulatory effects: they inhibit T cell proliferation in mixed lymphocyte cultures, prolong skin allografts survival in baboons and decrease graft versus host disease (GvHD) when co-transplanted with hematopoietic cells. MSC induce their immunosuppressive effect, at least in part via a soluble factor and in part due to the activation of CD4+CD25hFoxp3+ Treg cells with suppressor activity through production of prostaglandin E2, transforming growth factor beta 1 and by direct cell contact [34]. Interestingly, MSCs have been shown to express Foxp3 [35]. A report indicated that prolonging the culture of MSCs runs the risk of them becoming tumor cells [36]. However, others have claimed that over several years of study in treating GvHD [37], no tumor has been observed (Le Blanc, personal communication, unpublished). Clinical trials which use MSCs to treat familial hemophagocytic lymphohistiocytosis [38], autoimmune disease such as scleroderma, multiple sclerosis, Crohn's disease and other diseases are ongoing [39].

Major problems in growing HSCs and HPCs are the variability of culture conditions, the efficiency of differentiation of the cultured cells to lymphoid lineages, and the poor reproducibility in the hands of different investigators. Also contributing to problems is the failure to report specific reagents and ingredients used as well as undescribed procedures, stroma cell conditions, batches of serum, and growth factors required in each system. Of course, the intrinsic multipotentiality of HSCs and HPCs also contributes significantly to variability. Many have claimed that a cocktail of cytokines alone could promote the differentiation of myeloid lineages from HSCs and HPCs in mouse and human systems (ref. cited in reference [4]).

A few have claimed the development of B cells at various stages of maturation, but no mature T cells could be found in such culture conditions, however, they are not reproducible in other's laboratories. The maintenance of long term cultures of self-renewing stem cells with potency for lymphopoiesis, especially T lymphopoiesis, requires stroma cells, additional cytokines, a three-dimensional structure to support the precursor cell-stroma cell interaction (also see Stage 3), and other culture conditions yet to be defined. Recently, several groups reported that solid phase-Delta-L-1, either fixed on tissue culture dish surface, or integrated into mouse stroma OP9 can mimic the thymic stroma requirement for growing human and mouse T cells from early progenitor cells including iPS derived HSC (see stage 1, above). Several culture systems seem to be promising for expansion *ex vivo* and potential differentiation of human and mouse HSCs. Human-based supporting cell lines, such as an endothelial-like cell line, ECV304/T24, were used to generate split-function amphotropic packaging cell lines. This manipulated cell line, APEX, was used for transduction and for support of the growth of HSPs [40]. The development of supporting human cell lines for lymphopoiesis is essential for the production of large quantities of cultured cells for manipulation and re-implantation.

Ruedl et al. [41] reported that transduction of mouse HPS with retroviral vector containing gene coding for NUP (NUcleoPorin) 98 and HOXB4 fusion can expand bone marrow derived HPCs for several weeks in culture, with the expansion of cells of ca.1016 fold. The expanded cells can differentiate into all lineages including T and B cells, mimic the self-renewal property

of primary HSC-HPS. An additional important finding is that such immortalized cells do not produce tumor in transplanted mice. This study opens interesting questions, such as whether this viral vector containing NUP98-HOXB4 fusion protein can immortalize the HSCs of embryoid bodies differentiated from mES cells in culture, and show similar potential to differentiate into all lympho-hemapoietic lineages. Another question is whether this viral vector can immortalize human HSCs-HPCs, and if not, whether the counterpart of the NUP98-HOXB4 fusion protein exists and exercises similar effects in human HSCs.

SELECTED EXAMPLES OF THE CLINICAL APPLICATION OF HUMAN HSCS AND HPCS

The use of human HSCs and HPCs as the cell base for gene therapy is a complicated issue. In most clinical protocols, HPCs are transduced *ex vivo* with retroviral vectors and re-implanted into patients. One example which has undergone phase I clinical trial for decades is the treatment of ADA (Adenosine DeaminAse) deficiency. ADA deficiency exists in all cells examined. However, T cells of these patients are selectively missing, causing a SCID symptom. Despite decades of study, the mechanism by which the ADA defect causes this specific deficiency of T cells remains unknown. Possible mechanisms have been postulated, such as the apoptotic pathway of CD95 (Fas/apo-1)-induced cell death.

An ongoing, improved ADA gene therapy protocol has been described. This is a modified protocol running with more than 20 patients, using a combination of low-dose chemotherapy with busulfan/fludarabine and a single transplantation with ADA-retroviral-transduced HPC. No ADA enzyme replacement was included in this modified protocol to treat the patients. The low dose (one quarter of the full dose) chemotherapy is to condition or to make space in the bone marrow for the transplanted, gene corrected HPC. No side effect has been reported of the gene-transferred patients, they live normally, and more important, no patient died so far. This improved treatment was conducted first in Milan [42], then in several centers in UK and USA [43].

CD34+ HPCs and progeny PBL cells were collected from five ADA-SCID children before transplantation and in their myeloid and lymphoid progenies up to 47 months after transplantation and a genome-wide analysis of RISs (retroviral vector integration sites) were performed [44,45]. The data revealed similar patterns of integration into the human genome before and after transplantation, with a preference for gene-dense regions, promoters, and transcriptionally active genes. The occurrence of insertion sites proximal to proto-oncogenes or genes controlling cell growth and self- renewal, including LMO2, was not associated with clonal selection or expansion *in vivo*. Clonal analysis of cell progeny revealed highly polyclonal T cell populations and shared RISs among multiple lineages, indicating that they were derived from the engrafted HPCs.

Besides ADA-deficient SCID, several clinical trials of gene correction therapy using CD34+ stem cells have been conducted, trying to restore immune cells in patients with SCID-Xl (X-linked severe combined immune deficiency), CGD (chronic granulomatous disease), WAS (Wiskott-Aldrich Syndrome).

The study of the SCID-Xl gene therapy clinical trials has been reported in Paris, in which HPCs are the target of MLV (murine leukemia virus)-derived retroviral vectors containing IL (interleuken) receptor gamma common chains. In this trial, no chemotherapy was administered, with the expectation that the progeny lymphoid cells would have a very high selective survival in the patients, and marrow conditioning to facilitate higher engraftment of HPC would not be needed.

Immune function has been restored in nie of 10 treated children with the transduction of gamma common gene transfer in CD34+ cells. The distribution of both TCR V beta family

usage and TCR V beta CDR3 (complementarity-determining region 3) length revealed a broadly diversified T cell repertoire. Further retroviral integration site analysis showed that insertion sites were shared by progenic T and B cells, granulocytes, monocytes, and the transduced CD34+. This finding demonstrates the initial transduction of very primitive multipotent progenitor cells with self-renewal capacity. These results provide evidence in the setting of a clinical trial that CD34+ cells maintain both lympho-myeloid potential as well as self-renewal capacity after *ex vivo* manipulation [108]. A trial in the US followed using similar techniques achieved immune restoration in another 10 patients [46].

However, due to the developed leukemia-like T lympho-proliferative disorder of a total of five of the 20 treated children in both trials, the trials were on hold [47–50]. Patients who had been clinically stable with good immune function developed a relatively abrupt onset of escalating levels of circulating T lymphocytes, with thymic mass and organomegaly. They were treated with chemotherapy and four have remained in complete remission with continued restored immunity, but one died from the leukemia. The research now focuses on the basic biology of the oncogenicity of the vector-related insertion site LMO2 of the affected T cells and of using the Lmo2-TLL transgenic mice model. The integrations are found to reside within FRA11E, a common fragile site known to correlate with chromosomal break points in tumors. The fragile sites attract a non-random number of MLV integrations. This explains the mechanism of four of the leukemia cases. The other leukemia is attributed to the transplanted IL2RG gene [48].

The reasons why this leukemic complication occurred in five of 20 XSCID gene-corrected patients, but none of more than 20 ADA-deficient SCID gene treated patients are not clear. The reasons possibly involve the role of the common gamma gene product (a trans-membrane protein capable of providing intracellular signaling), the nature of the HPCs of the XSCID patients, effects of the common gamma-deficiency on the susceptibility to transformation and the rapidity of immune reconstitution in the XSCID patients [43].

New approaches using safer integrating mouse SIN (Self INactivate) retroviral vectors, HIV-based lentiviral vector, or direct correction of the defective gene underlying the immune deficiency diseases are being developed, which might lead to safer and effective gene therapy.

Stage 3: Lymphoid precursor cells

The characterization of lymphoid lineages – committed precursor cells defined in terms of cell surface markers and functional assays of precursors and progenies – is still a challenge. One described cell type that is closest to the definition of lymphoid precursor cells is the CD4low precursor population isolated from adult thymus (ref. cited in reference [4]). Because this population of cells is restricted in its potential to differentiate into myeloid cells, it is preferentially committed to differentiate into T and B cells. These cells can also differentiate into thymic dendritic cells. The molecular markers of this population have not been well characterized. It would be interesting to see whether this population of cells can differentiate into myogenic cells, because it is possible to obtain differentiated myogenic cells from the adult thymus. The thymus contains lymphoid and MSCs, which can develop into other lineages, such as bone, cartilage and lung.

Little is known about the culture conditions for growing this population of cells. It is possible that stroma cell lines such as S17 could be used to expand the CD4low population of cells in culture, because it has been possible to use S17 and cytokines to maintain para-aortic splanchnopleural-derived precursor cells possessing T cell lineage potency in culture (in reference [4]).

A lymphoid lineage-preferable transcriptional factor, Ikaros, was thought to be an important molecular marker of lymphoid precursor cells at this specific stage of development. Ikaros was subsequently shown to have multiple effects on HSCs, lymphoid cells, T precursor cells, and others.

Using the transgenic approach, it is possible to trace lymphopoiesis between common lymphoid precursor and alpha-beta T cells. A transgenic mouse system is applied using pTalpha (pre-T cell receptor alpha) promoter to drive the human CD25 (hCD25) surface marker as a reporter. It marked intra- and extra-thymic lymphoid precursors but not myeloid cells. The extrathymic precursors were characterized as a common lymphoid precursor population (no. 1) expressing CD19-B220+Thy1+CD4+ cells using clonogenic assays. The earliest intrathymic precursors were CD4lowCD8−CD25−CD44+c-Kit+ cells and Notch-1 mRNA+.

By using the regulatory sequences from the gene encoding pre-T cell receptor alpha to drive hCD25 reporter and to produce transgenic mice, another common lymphoid precursor population (no. 2) was identified, which was B220+c-Kit−. In short term culture, population no. 2 cells could be derived from the no. 1 subset and contained cells that in clonogenic assays were characterized as bipotent T and B precursors. Mature alpha-beta T cells were produced when transgenic bone marrow cells were injected i.v.; thymocytes were cultured using a thymic organ culture system. The no. 2 subset may represent the most differentiated population with T cell potential before commitment to the B cell lineage.

The human counterpart of the mouse CD4low population is unknown. The expansion of CD34+, CD31+ human bone marrow B-progenitor cells and partial differentiation to B-precursors in serum-free culture medium in the presence of mixed primary human stroma cells and IL7 for a limited duration has been shown to be possible (ref. cited in reference [4]). A few such CD34+ precursor cells could differentiate into NK cells and T precursors when subjected to a secondary culture condition, with the thymic environment provided. The three-dimensional structure and the expression of Notch/Delta signaling of the thymic environment have been shown to play critical roles for supporting the differentiation of cells to the T cell lineages (also, in Stage 2) [51−54].

Stage 4: Precursor T cells

Mouse precursor T cells are identified to be thy-l+, CD117low, CD3−, pT+ (ref. cited in reference [4]). They can be derived from fetal and adult blood. Using markers such as the CD25, CD44, and TIS 21 (TPA-inducible sequence 21), pre-T cells can be classified into four subpopulations. They are the quiescent CD44+CD25−TIS21+ early cells and CD44−CD25+TIS21+ cells prior to TCR-beta selection. After selection, the cells are proliferating CD25+CD44+TIS-21low precursor T cells and CD25low CD44−TIS21low cells. By transgenic overexpression of TIS21 in precursor T cells and HPC, it can inhibit the expansion of thymocytes. By transgenic overexpression of TIS21 in precursor T-cells and HPC, it can inhibit the expansion of thymocytes (Konrad and Zuniga-Pflucker, 2005). Thus, somatic and genetic manipulations would become easier with cloning and identifying such "quiencenting genes" and their ability to express at stage-specific matter.

Other genes expressed which might play a role in the function of cells at this stage are the cell cycle control genes such as the D3 cycline of D type, the preTCR (pre-T Cell Receptor gene) and the BCL2A1 (antiapoptotic A1 gene). PreTCR+ cells are selected to survive and differentiate further, whereas preTCR− cells are selected to die. The induction of the BCL2A1 gene will induce pre-T cell survival by inhibiting activation of caspase-3. The knock-down of BCL2A1 expression can compromise survival, even in the presence of a functional preTCR. However, the overexpression of preTCR-induced BCL2A1 can contribute to T cell leukemia in mice and humans. Both OLIG2 and LMO1 were overexpressed in large thymic tumor masses. Gene expression profiling of thymic tumors that developed in OLIG2-LMO1 mice revealed upregulation of Notch1. Two genes considered to be downstream of Notch1 − Deltex1 (Dtx1) and preTCR-alpha − are also upregulated. The established OLIG2-LMO1 leukemic cell line was suppressed by a gamma-secretase inhibitor, suggesting that Notch1 upregulation is important for the proliferation of OLIG2-LMO1 leukemic cells. Thus Notch-Delta signaling is

critical to trigger the T cell development program. In addition, commitment to the T cell lineage is also shown to depend on BCL1b, while initiation of the T cell differentiation program begins earlier with the induction of TCF-1 (Tcf7 gene product) and GATA-3 [107].

T lymphocytes are generated in the thymus, where developing thymocytes must accept one of two fates: they either differentiate or they die. These fates are determined by signals that originate from the TCR. CD4+CD8+ thymocytes undergo one of three fates in the thymus: positive selection, negative selection (TCR-mediated apoptosis), or death by neglect. Only 5% of developing thymocytes are exported as mature T cells. Negative selection of thymocytes that express TCRs with high affinity for self-peptide-Major HistoCompatible antigen (MHC) deletes potentially self-reactive thymocytes, generating a largely self-tolerant peripheral T cell repertoire. Most negative selection is thought to occur in the thymic medulla, for this contains two types of specialized antigen-presenting cell — DCs (dendritic cells) and thymic epithelial cells (TECs). Medullary TECs transcribe genes that are normally expressed in peripheral tissues. Negative selection can occur before or after, and is thus independent of, positive selection and in thymocytes at all stages of development. Negative selection in response to high-affinity ligands might be due to increased TCR occupancy or a slower off rate (kinetic proof-reading). Although discrepancies exist between blocking experiments and genetically deficient mice, TCR signal and a second co-stimulatory signal might be required for negative selection. The kinetics of MAPK (mitogen-activated protein kinase) signaling might determine positive vs. negative selection signals. ERK (extracellular signal regulated kinase) is induced more rapidly during negative selection, which might determine the triggering of transcriptional factors, NUR77 and NF-B (ref. cited in [4]). The recent view on roles of TCR signaling required for activating T cells and for generating memory T cells has been reviewed [55].

In the thymus, two major T cell lineages — alpha-beta T cells and gamma-delta T cells developed from common lymphoid precursors. Their differentiation requires outside signaling. Development of alpha-beta T cells is driven in its early stages by signaling from the pre-TCR, most likely in a ligand-independent fashion, and later, by signals delivered by alpha-beta TCRs binding to their ligands — MHC molecules. Similarly, gamma-delta T cells require TCR signaling for their differentiation. However, most gamma-delta TCRs remain orphan receptors, and it is not clear whether ligands are required for gamma-delta TCR signaling in the development of gamma-delta T cells.

Work with transgenic and knockout mice affecting the TCR pathways suggests that TCR signaling ensures the developmental progression towards mature alpha-beta T and gamma-delta T lineages and that the strength of TCR signal instructs lineage fate; i.e., a stronger TCR signal results in gamma-delta T lineage commitment, and a weaker TCR signal results in alpha-beta T lineage commitment. This decision of alpha-beta vs. gamma-delta fate choice during thymus development is called the first TCR-controlled checkpoint.

Distinct molecular programs have been revealed to govern this lineage decision choice. ERK, Egr (early growth response), Id3 (inhibitor of differentiation 3) were identified to be potential molecular switches operating downstream of TCR which determine lineage choice. Removal of Id3 was sufficient to redirect gamma-delta TCR transgenic cells to the alpha-beta T cell lineage, even in the presence of a strong TCR signal. However, in TCR non-transgenic Id3 knockout mice, the overall number of gamma-delta T cells was increased due to an outgrowth of a Vgamma1Vdelta6.3 subset, suggesting that not all gamma-delta T cells depend on this molecular switch for lineage commitment. Thus, two or more lineages not sharing a common molecular program might exist in the gamma-delta T cell lineage. In addition to signaling through TCR, signaling from Notch and CXCR4 receptors, cooperate with the TCR in controlling the development of alpha-beta T cells and gamma-delta T cells [56].

Precursor T cells are not self-renewing. Apoptosis occurs easily, and only some cells of this compartment differentiate into double-positive mature T cells. A scale-up expansion of this

population presents a challenge. Fetal thymus organ culture systems and suspension cultures with dissociated TECs and, a cell line, OP9, which requires a very high concentration of FBS, (20%, in the culture medium) transgenic with notch-ligand such as DL1 (Delta-Like-1), for studying this particular population are available (ref. cited in reference [4]). It is possible to expand transiently para-aortic splanchnopleurally-derived precursor T cells in culture but such cells perish quickly. Variations in the physical culture environment, such as the O_2 level of the incubator where the fetal organ culture was set up, were shown to help the survival of (precursor) T cells. Free radicals promote molecules that lead to the apoptotic pathway. Thus, reducing the concentration of local O_2 and increasing the level of N_2 in the incubator, or supplying the cultured cells with a high density of stroma cells, favors the survival of (precursor) T cells.

To engineer the thymic niche *in vitro*, Shukla and Zandstra (personal communication, unpublished) have engineered an artificial thymus niche containing an immobilized ligand-hydrogel system containing Notch signaling ligands: DL4 (Delta-like 4) and Jagged-1 which are essential for T cell development. This system might allow a robust and scalable generation of mouse proT cells from HSCs and iPS (stage 2, above). It is also possible to grow mouse pre-T cells by using solid phase DL4, and cytokines such as IL7, SCF (Rolink, personal communication, unpublished).

Beaudette-Zlatanova et al. [57] described a hTEC (human thymic epithelial cell) line culture system to support T lymphopoiesis from hHSCs. The cells were immortalized by infection with an amphotropic retrovirus from a cell line containing the HPV E6E7 early genes. The parental cell line, expressing low levels of DL1 and DL4, permits HPCs to differentiate to a B cell lineage. This cell line was engineered to overexpress mouse DL1, called TEC-DL1. In co-cultures with HPCs from cord blood or BM, TEC-Dl1 cells promote the generation of CD7+CD1a+ T-lineage-committed cells, most of them being CD4+CD8+ cells. CD3+(lo) cells were detected within the Double Positive (DP) and Single Positive (SP) CD4 and CD8 populations. The CD3+(lo) SP cells expressed lower levels of IL2R and IL7R. However, this cell line is not sufficient to generate CD3+/high mature single positive T cells. OP9DL1 (and OP9) cell lines have been shown to be able to support the differentiation of T cells (and B cells), however due to its mouse origin, its clinical application for cell therapy is challenged by the regulatory authorities. Thus, this hTEC line might become a promising candidate for clinical application.

The role of miR-142, -181, and -223 in mouse hematopoiesis has been studied [58] and it was found that ectopic overexpression of miRNA-181 in mouse HSPCs resulted in an increase in B lymphopoiesis, and miRNA-142 and -223 resulted in small but significant increases in T lymphopoiesis. However, the relevant target mRNAs were not determined.

The analysis of human and mouse HSCs/HPCs showed large differences in miRNA expression. In human BM, miRNA-181 is expressed more weakly than miRNA-146, which was found to be expressed strongly in all mouse hematopoietic tissues. miRNA-142 is not expressed in human HSPs [59]. The *in silico* model indicates that miRNA-181 and miRNA-146 might block differentiation very early in human lymphopoiesis.

The production of miRNAs by Dicer is required for efficient T cell and B cell development. miR-146, miR-223, and miR-150 target the developmental stage from lymphoid committed stage to T cell development in mouse.

T cell development is controlled by complex protein signaling networks, which are subjected to regulation by miRNAs (Fig. 50.1). The expression patterns of miRNAs are found to vary between T cell subsets and stages of development. Mature miRNAs of a given miRNA species can have several variants, which can vary in length at either the 3′ or 5′ end, or can contain mutated sequences. Proliferating T cells express genes with shorter 3′UTRs than those in resting T cells, rendering these mRNAs less susceptible to regulation by miRNAs due to the

loss of miRNA binding sites. These findings suggest that miRNA-mediated regulation of mRNA targets in T cells is a dynamic process that is influenced by a broad range of factors [16].

Only a few selected individual mRNAs which play a role in early T cell development will be mentioned in this section.

Xiao et al. [60] generated mice with elevated miR-17−92 expression in lymphocytes. These mice developed lympho-proliferative disease and autoimmunity, and died prematurely. Lymphocytes from these mice showed increased proliferation and reduced activation-induced cell death. The miR-17−92 cluster was shown to target mRNAs encoding for the pro-apoptotic protein BIM (BCL-2-interacting mediator of cell death) and the tumor suppressor, PTEN (phosphatas and TENsin homologue) during DN (double negative) thymocyte stage of development by increasing T cell survival. This mechanism likely contributed the disease and death of miR-17−92 transgenic mice, and to lymphoma development in patients carrying amplifications of the miR-17−92 coding region.

The strength of TCR signaling influences whether thymocytes are positively or negatively selected during thymic development, and specific miRNAs have been implicated in this process, for example, Li et al. [103] showed that miR-181a enhanced TCR signaling strength by targeting multiple protein phosphatases, such as DUSP5 (DUal-specificity protein phosphatase 5), DUSP6, SHP2 (SH2-domain-containing protein tyrosine phosphatase 2) and PTPN22 (protein tyrosine phosphatase, non-receptor type 22), which lead to elevated steady state levels of phosphorylated intermediates and a reduction of the TCR signaling threshold. They showed that inhibiting miR-181a expression in the immature T cells reduced sensitivity and impaired both positive and negative selection. The miR-181a was also shown to increase the expression in mature T cells augmented the sensitivity to peptide antigens.

Stage 5: Mature T cells and memory T cells

Using cultures of mature T cells in the presence of APCs and recombinant cytokines, one can study the growth and differentiation of T cells from a variety of sources. Long term mouse and human T cell clones are also available. T cells can be maintained as clones in culture far longer than B cells. Human T cells (mainly NK cells) grown in the presence of cytokines in a short term culture have been re-implanted into autologous cancer patients. Most CD8+ and CD4+ T cells have a short lifespan. Memory T cells have been well studied, but in reality they do not exist in abundance and can be demonstrated *in vivo* only by repeated priming with antigen. They are very difficult to define at the cellular and molecular levels. There is no isotype 3′-end downstream of the TCR constant region for class switching to occur. Somatic hypermutation of the TCR-beta gene has never been claimed, and it is debatable whether the TCR-alpha gene is hypermutable. Memory T cells are thus defined using criteria such as accelerated cellular responses, distinct pathways of lymphocyte recirculation *in vivo*, distinct DNA motifs of TCR genes, cytokine- producing pattern and diminishing expression of surface markers (ref. cited in [4]), and functional and antigen requirements. In mice, memory CD4+ T cells are CD45RO, L-selectin (MEL-14)low. This is the equivalent of human CD45RO, keeping in mind that the CD45R family (A, B, C, and O) may not be the best marker to define naïve versus memory T cells. Because foreign antigens do not always quickly elicit unprimed T cells, memory T cells must exist, but the commitment, the mechanism of development, and the maintenance of these cells are unknown. Memory T cells are thought to be generated, either when T cells acquire specificity to kill, or to help during thymic education, or they are generated during the mature stage.

MEMORY CD8+ T CELLS

Because cell-bound antigen on APCs (see later for details) and more than one signal are required for educating T cells to perform effector functions instead of becoming tolerated,

work on the generation of CD8+ memory T cells has been mainly performed *in vivo*, using viruses. Is it possible to prevent cytotoxic T cells from performing their function by releasing granzymes. If this is the case, what happens to committed CD8+ T cells? Do they die, become anergic, or become memory cells? A study addressing the avoidance of granzyme B-induced apoptosis in target cells is interesting in this regard (ref. cited in reference [4]). Dephosphorylation of cdc2 was shown to be a critical step in granzyme B-induced apoptosis in the targets of cytotoxic T cells. A nuclear kinase encoded by the wee1 gene was transiently expressed and shown to induce phosphorylation of the tyrosine residues of cdc2 kinase, and that in turn provoked mitosis and the rescue of target cells. Because cytotoxic T cells are subject to being killed by their colleagues, the apoptosis pathway in these cytotoxic T cells could be similar to that of the target cells. The priming, clonal expansion, and differentiation into memory T cells can be achieved. The expansion of memory T cells can be demonstrated in culture. Besides viral peptide antigens, recent other examples are the use of mycobacterial glycolipids as the antigen, the antigen processed by CD1b, CD1e (ref. in [4]). Primed T cells are harvested from a human adult and stimulated *in vitro* with antigen with hIL2. The T cell clone can demonstrate the killing of cells infected with *M. (Mycobacterium) tuberculosis* (see later).

It is interesting that DNA methylation may contribute to regulation of mouse T effector cell function (ref. cited in [4]). In Dnmt1−/− (the maintenance DNA methyl-transferase), silencing of IL4, IL5, IL13, and IL10 in CD8 T cells was abolished, and expression of these Th2 cytokines increased drastically as compared with that of control CD8 T cells. Th2 cytokine expression also increased in Dnmt1−/− CD4 T cells, but the increase was less than for CD8 T cells. As a result, both Dnmt1−/− CD4 and CD8 T cells expressed high and comparable amounts of Th2 cytokines. Loss of Dnmt1 had more subtle effects on IL2 and IFN expression and did not affect the normal bias for greater IL2 expression by CD4 T cells and greater IFN expression by CD8+ T cells or the exclusive expression of perforin and granzyme B by the CD8 T cells. Dnmt1 and DNA methylation seem to be necessary to prevent cell-autonomous Th2 cytokine expression in CD8 T cells, but were not essential for maintaining proper T cell subset-specific expression of Th1 or CTL effectors. Thus, transcription factors and DNA methylation are complementary and non-redundant mechanisms by which the Th2 effector program is regulated.

MEMORY CD4+ T CELLS

Like memory CD8+ T cells, the generation of memory CD4+ T cells has been studied *in vivo*. This population of cells has been well characterized in mice. Effector memory CD4+ cells in mouse spleen against the soluble protein antigen KLH (keyhole limpet hemocyanin) and other protein antigen were found to be CD45RO, L-selectin−, CD44+ and to produce elevated levels of IL4 (ref. cited in reference [4]). Whether this population of cells can be expanded in culture remains a question. Because T cells from IL2 knockout mice survive much longer than those of conventional mice, they may be useful for studying the development of memory T cells. Moreover, molecules such as Fas and Fas ligands of the apoptotic pathways play critical roles in determining the fate of cells after activation. The Bcl-2/bcl-x family seems to function by helping the survival of cells via action on the intermediate steps of apoptosis. Genes controlling the cell death pathway may play critical roles in the development of, and the subsequent genetic manipulation of, memory T cells. It is important to improve the conditions for growing CD8+ T cells *in vitro*, because for tumor therapy, the generation of tumor-specific CD8+ T cells is a critical step. Although surface markers and the life span of a population of T cells equivalent to those of the mouse system have been documented, ethical and safety considerations have prevented a systematic study of human memory T cells *in vivo*. The possibility of producing human CD4+ T cells on a large scale has been reported. By stimulating peripheral, resting T cells with cytokines such as IL2, anti-CD28, and solid phase anti-CD3, survival and proliferation of CD4+, but not CD8+, T cells could be greatly

promoted. What remains to be shown are the specificity and function of these cells and how they are related to the regulation of the Bcl-2/bcl-x family and CD95 (Fas/apo-1) ligand and whether they are candidate memory CD4+ T cells or abortive T cells.

ROLE OF APC, TOLL-LIKE RECEPTOR, IN THE ACTIVATION OF CD4+, Th1 VS. Th2 PATHWAYS

The recognition and stimulation of antigen epitopes by T cells requires that antigen molecules first be processed to become fragmented epitope and presented properly by APCs. The APCs identified are macrophages, dendritic cells, and B cells when in the process of T–B cell cooperation. T cells will only recognize the antigenic epitope when it is embedded in MHC (major histocompatibility complexes, H2 in mouse and HLA in humans), which enables the immune system to distinguish its own cells from foreign cells.

Foreign antigens are great engineers. The nature of the antigen to be processed by the APCs decides which processed antigenic epitopes are to be presented by which kind of T cells and how to influence the reaction of the immune system. For example, when cytosol enters the host, viral proteins will be digested by proteasomes to become fragmented epitopes and be presented on the surface of APCs in complex with class I MHC. Their interaction with the CD8+ T cells will enable them to become cytotoxic T cells to perform cell-mediated immunity.

Soluble bacterial toxins (with exceptions, see next section), phagocytized into acidic vesicles in APCs, will be processed by the vesicular proteases, and the fragmented epitopes will be presented on the surface of APCs in complex with class II MHCs. In macrophages, foreign proteins within the acidic phagocytic vesicles will be digested and presented on their surface in complex with class II MHC, similar to APCs. These epitopes are recognized by the CD4+ helper T cells and will cause immune responses, such as the activation of phagocytes and antibody production by activated B cells.

Two major subsets, Th1 and Th2, were originally recognized in the population of CD4+ T cells, though more recently Treg, Th17 cells were also described. Th1 has been shown to secrete IFN-gamma and to help cell-mediated responses. Th1 stimulation results in local inflammatory responses, including the activation of macrophages, and the production of complement-fixing and -opsonizing antibodies IFN-gamma and IL12 drive the differentiation of IFN-gamma-producing Th1. Th2 is shown to secrete IL4, IL3, IL5, IL13, and IL25 and to help the generation of antibody-mediated responses. IL4 is also required for Th2 cell differentiation. Th2 activation leads to the production of IgG and IgE and to the activation of basophil and eosinophils to fight against allergens and large mucosal parasites [61].

It has been a puzzle as to how Th1 vs. Th2 cells recognize and distinguish the epitopes of foreign antigen on APCs, since both require the presence of class II MHCs. The mammalian equivalent of TLRs (Toll-like receptors) derived from Drosophila 'Toll' was discovered and helped to address this question. TLRs are found on the surface of APCs. More than 11 different receptors have been shown to bind to various cellular components of microorganisms.

The binding of most of these components to the preferred TLRs produces a differential signaling cascade that stimulates an immune response in favor of Th1 over Th2. Stimulation of TLR7, and TLR8 in humans by synthetic imidazoquinoline compounds leads to activation of the Th2 pathway; while stimulation of TLR4 by MPL (monophosphoryl lipid A) and Lipo-PolySaccharide (LPS) or viral peptides such as VIPER [104], and stimulation of TLR9 by unmethylated CpG of bacterial DNA lead to activation of the Th1 pathway. TLR11 and several other TLRs are also used for activation of Th cells, but by which specific pathways remains unknown [62,63], and other refs. cited in reference [4]. As shown in Fig. 50.2, signaling by TLRs involves five adaptor proteins: MyD88, MAL, TRIF, TRAM and SARM. Apart from NF-kappa-B activation and IFN-gamma signaling, MyD88 was shown to activate the transcription factors IRF1, IRF5 and IRF7. MAL and TRAM act as bridging adaptors, with MAL

recruiting MyD88 to TLR2 and TLR4, and TRAM recruiting TRIF to TLR4 to allow for IRF3 activation. The fifth adaptor, SARM, was shown to negatively regulate TRIF [64].

THE ROLE OF CD1 IN THE T CELL RECOGNITION OF LIPID ANTIGEN

Besides peptide-protein antigen, which stimulates the Th1, Th2 pathway via TLR on APCs, lipid antigen activates T cells by CD1 molecules, independently of TLR. Studies have shown that CD1 molecules are lipid antigen-presenting molecules that offer the lipid antigens to the TCR of T cells, resembling MHC presentation of peptides, a MHC class III molecule. They have no structural homology with TLR, though they are also present on APCs. Some lipids are recognized by the immune system as classical antigens, resembling peptides associated with MHCs, whereas other lipids trigger the TLR innate receptors. A panel of T cell clones with different lipid specificities isolated from *M. tuberculosis* was established. A novel lipid antigen belonging to the group of diacylated sulfoglycolipids, Ac2SGL(2-palmitoyl or 2-stearoyl 3-hydroxyl-phthioceranoyl-2′-sulfate-alpha-alpha′-D-trehalose) was identified. Ac2SGL is mainly presented by CD1b, after internalization in a cellular compartment with low pH.

Ac2SGL-specific T cells release IFN-gamma, efficiently recognize *M. tuberculosis*-infected cells, and kill intracellular bacteria. The presence of Ac2SGL-responsive T cells *in vivo* is strictly dependent on previous contact with *M. tuberculosis* but is independent of the development of clinically overt disease. These properties identify Ac2SGL as a promising candidate vaccine against tuberculosis.

Another molecule which has been isolated, PIM6 (hexa-mannosylated phosphatidyl-myo-inositols) is another molecule isolated, stimulates CD1b-restricted T cells after partial digestion of the oligomannose moiety by lysosomal alpha-mannosidase, and soluble CD1e, one of the CD1 family members, is required for the processing.

Recombinant CD1e was able to bind glycolipids and assist in the digestion of PIM6 into dimannosylated forms: PIM2, which was stimulatory to specific CD1b-restricted T cells.

CD1 molecules, after exiting from the endoplasmic reticulum, travel to the cell surface via the secretory pathway before being re-internalized into the endosomal compartments. CD1a molecules undergo cycles of internalization into early sorting endosomes followed by early recycling endosomes, whereas CD1c molecules travel to early recycling endosomes and, to a lesser extent, to late endosomes and lysosomes. In contrast, CD1b and human CD1d molecules recycle in late endosome, lysosome compartments where they can co-localize with CD1e. It was debatable whether CD1e participates in presentation of lipid antigens by other CD1 molecules. Recent studies showed that CD1e may positively or negatively affect lipid presentation by CD1b, CD1c, and CD1d. This effect was also shown in APCs from CD1e transgenic mice, which was caused by the capacity of CD1e to facilitate rapid formation of CD1-lipid complexes, and to accelerate their turnover. Thus CD1e helps expand the repertoire of glycolipidic T cell antigens to optimize antimicrobial immune responses through glycolipid editing. (ref. cited in [4,65,66]).

INNATE IMMUNITY, T REGULATORY CELLS, AND Th17 CELLS

Another heterogeneous population of T cells has been re-identified, the so-called Treg (Tregulatory) or suppressor cells. They can be innate or induced.

The subset of Treg cells in mouse can be identified using different surface markers and the secreted cytokines. They include:

1) Naturally occurring T cells possessing CD4+CD25+Nrp1+Foxp3+TGFbeta+, and
2) Cells induced in the periphery following antigen exposure, such as CD4+ Treg1 cells possessing CD4+CD25−/+Foxp3−/+IL10+IFNgamma+TGFbeta+IL5+; Th3 / Th17 cells

possessing CD4+CD25+Foxp3?TGFbeta+IL4+/−IL10+/− and CD8+Treg cells possessing CD8+CD25lowfoxp3-IL10+TGFbeta+/− (ref. cited in [4]).

The CD4+Treg cell subsets can be further classified on the basis of their phenotype, cytokines secreted (such as newly described IL35) function, migration preference to lymphoid or to non-lymphoid tissues [61]. The cells express Foxp3 (fork-head/winged helix transcription factor), which appears to play a key role in supporting their action [67,68]. Microarray study of Treg cells isolated from different tissues of mice transgenic with Foxp3-GFP+ revealed an even more diverse heterogeneity of cell populations (later [69]). The expression of Foxp3 in Treg cells is not stable, several studies have reported the plasticity of Treg in diseases and in inflammation, shown rather complicated mechanisms exerted and influenced by (micro)-environment.

It is also important to keep in mind differences in Foxp3 expression observed in mouse vs. human T cells. In mouse, Foxp3 appears to be a reliable marker for either thymic-derived or induced Treg cells. However, nearly all human CD4+ T cells transiently express Foxp3 during activation, and this is not associated with acquisition of regulatory function. Human MSC have also been shown to express Foxp3 (in Stage 2). Thus, Foxp3 alone is not a reliable marker for human Treg cells, further complicating analyses of their function and stability [61].

In addition, not all antibodies against markers used to characterize mouse Treg work for human Treg, CD45RA marker is used in conjunction with Foxp3 to better classify human Treg phenotypes [106]. So far, three classes of human Treg have been identified based on Foxp3 and CD45RA: (1) Resting Tregs (rTreg) are CD45RA+, Foxp3low; (2) CD4+. Activated Treg (aTreg) cells are CD4+, CD45RA−, Foxp3high; and (3) cytokine-secreting non-Tregs are CD4+, CD45RA−, Foxp3lo. The way that the cytokines which are secreted fit into this classification is not clear.

When stimulated, rTreg can increase Foxp3 expression, convert to an aTreg, and proliferate *in vitro*. However, aTreg rapidly die following proliferation. Furthermore, aTreg inhibit rTreg conversion to aTreg, controlling the balance of active Treg. Naïve CD4+T cells and non-Treg CD4+ cells are also capable of converting to aTreg, but less so than rTreg. Interestingly, CD4+ non-Treg cells have higher levels of Th17 factors (RORgamma, RORalpha, IL17, later), showing a bias toward developing a Th17 phenotype.

APCs are essential in the activation of Treg cells; the immature APCs can support the differentiation of Treg cells. Targeting of antigens to immature dendritic cells has been shown to result in antigen-specific T cell tolerance *in vivo*. The mechanism of suppression could be multiple and is not entirely known. One possibility is to tolerate the APCs, and the other is via the activation of Treg. Treg cells can down-regulate Th1, and Th2 responses. Th17 cells are rather resistant to Treg suppression (later).

Suppression of CD8+T effector cells by Treg could occur to prevent the development of effector function, and could interfere with the execution during the effector phase. Direct cell contact through binding of cell surface molecules such as CTLA4 (cytotoxic T cell-associated antigen 4) on Treg cells to CD80 and CD86 molecules on T cytotoxic cells results in the suppression. The local production of suppressive cytokines such as TGFbeta, IL10, and IL35 also results in suppression. It requires the appropriate co-localization of Treg and T effector cells in different tissue, and may involve interference with the T cell receptor signaling that triggers transcription factors important in regulating effector cell function [61,70].

Treg are usually anergic (do not respond or proliferate to antigen stimulation), IL2 dependent and proliferate poorly in culture. Naïve CD4+ T cells can be converted into Treg cells expressing Foxp3 by targeting of peptide-agonist ligands to dendritic cells or by changing culture conditions, such as adding TGFbeta or reducing IL2, or at the presence of MSC [34]. Treg cell populations induced in low dose antigen conditions could subsequently be expanded by delivery of higher- or immunogenic-dose antigen [70,71].

For a better understanding of transcriptional control in Treg cells, Feuerer et al. [69] compared gene expression profiles of a broad panel of Treg from various origins or anatomical locations of mice such as spleen, peripheral lymph nodes (cervical, axillary, inguinal) of *in vivo* converted Foxp3-GFP+ cells, and lamina propria T cells from Foxp3-GFP mice [68]. The data showed that Treg generated by different means form different sub-phenotypes and could be identified by particular combinations of transcripts. None of which fully encompassed the entire Treg signature. Molecules involved in Treg effector function, chemokine receptors, and transcription factors were differentially represented in these sub-phenotypes. Treg from the gut are different to Treg converted by exposure to TGFbeta *in vitro*, however, they resembled a CD103+Klrg1+ sub-phenotype preferentially generated in response to lymphopenia.

The study of the mechanisms of human MSC-mediated allo-suppression has suggested a sequential process of Treg cell induction involving direct MSC contact with CD4+ cells followed by prostaglandin E2 and TGFbeta1 expression (Stage 2).

In vitro, Treg cells could be generated by T cell stimulation in the presence of high doses of TGFbeta [72]. However, the fast *in vitro* conversion process resulted in Treg with unstable Foxp3 expression that correlated with lack of demethylation of the Foxp3 locus. This *in vitro* conversion method was suboptimal where Treg cell activation was limited for an initial 18 hour culture period; exogenously TGF-beta must be continuously added into the culture and required the inhibition of PI3K (Phosphatidyl Inositol 3-Kinase), Akt/PKB (Protein Kinase B), or protein kinase mTOR (mammalian target of rapamycin) [73]. Thus, how to conduct large-scale production of stable Treg cells *in vitro* is still developing.

Polansky et al. [74] found that the *in vivo* priming with suboptimal antigen resulted in complete demethylation of the TSDR (Treg-specific demethylated region) within the Foxp3 locus, and stable Foxp3 expression. Daniel et al. [75] described an efficient protocol applying the combination of everolimus, a rapamycin analog, and IL2-IL2 antibody complexes to achieve highly effective antigen-driven conversion of naïve CD4+Th cells into Treg and their expansion in transgenic mice. The mechanism of the role of everolimus in enhancing Treg conversion was possibly by interfering with T cell co-stimulation, reducing cell division and thereby activation of DNA methyltransferase1; and by reducing T cell activation through the ATP-gated P2×7 receptor controlling Ca2+ influx. The resulting Treg cells exhibit increased stability of Foxp3 expression even when generated in TGFbeta-containing media in culture. This protocol constitutes an important tool to achieve immunological tolerance by Treg vaccination. Whether it can be applied to therapeutic usage remains to be investigated.

Th17 (T helper 17) cells were first described in mice [76,77] then in humans [78] and other groups) to be a CD4+ T cell subset characterized by production of IL17, which is a highly inflammatory cytokine with robust effects on stromal cells in many tissues. Studies in RA (rheumatoid arthritis) have shown that cytokines secreted by Th17 cells can be further divided: IL17A (originally described), IL17F (a total of six members), IL22, and IL21 [79].

Besides differentiate to Th1, Th2 and Treg, naïve CD4+ T helper cells can be induced to differentiate to Th17 lineage according to the local cytokine milieu. The differentiation towards Th17 and Treg phenotypes is in a mutually exclusive manner. Each phenotype is characterized by unique signaling pathways and transcription factors, such as T-bet for Th1, GATA-3 for Th2, Foxp3 for Treg and RORalpha (Receptor-related Orphan Receptor) and RORgammat for Th17 cells [80–82].

Very little is known about the pathways that control the expression of IL17 in humans. Evans et al. [78] showed that the factors that determine the expression of IL17 in human CD4+ T cells are different from mice. IL6 and IL21 were unable to induce IL17 expression in either naïve or activated T cells, and TGFbeta actually inhibited IL17 expression. The expression of IL17 was maximally induced from pre-committed precursors present in human PBL by cell-cell contact with Toll-like receptor-activated monocytes / APCs in the context of TCR ligation.

Although the pro-inflammatory activity of Th17 cells can be beneficial to the host during infection, uncontrolled or inappropriate Th17 activation has been linked to several autoimmune, including RA, multiple sclerosis, psoriasis, lupus, inflammatory bowel disease, asthma, and might also be involved in tumor and transplant rejection. In the absence of the Th1 *in vivo*, Th17 cells are capable of rejecting cardiac allografts. A report suggests that Th17 cells might be involved in the auto-inflammatory disorder AOSD (adult-onset Still's disease) [83]. Addition of inflammatory signals to a Treg inducing environment leads to Th17 development. Established Treg can be converted to Th17 cells under inflammatory conditions. These findings might explain some of the dys-regulation seen in autoimmune diseases [84,85].

T cell differentiation is modulated by several miRNAs: miR-148a is shown to repress mRNAs encoding for BIM, affecting the commitment of Th1 memory (Haftmann and Radbruch, personal communication, unpublished). miR-155 is shown to have a role in B cells (later), and dual roles in T cells:

1) To repress mRNAs of MAF (Macrophage-Activating Factor) which leads to in favor of the differentiation of CD4+Th0 cells towards Th1 cells;
2) To repress mRNAs of SOCS1 (Suppressor Of Cytokine Signaling 1) which is implicated in the survival of Treg cells.

The miR-155 maps within, and is processed from an exon of its precursor, called bic. In activated B, T cells, and APC, bic/miR-155 shows greatly increased expression. Bic/miR-155 knockout mice are immune-deficient and developed lung pathology with age. Bic/miR-155 was shown to modulate levels of transcription factor c-Maf in CD4+T cells, contributing to bias towards Th2 differentiation [86,87]. Global analysis of the network of genes regulated by Foxp3 has identified the miR-155, which is highly expressed in Tregs, as a direct target of Foxp3. The miR-155 knockout mice have reduced numbers of Treg in the thymus and periphery. However, there was no evidence for defective suppressor activity in miR-155−/− Treg cells, either *in vitro* or *in vivo*, suggesting that additional unidentified miRNAs control Treg function [88]. Lu et al. [89] have shown that miR155 deficiency in Treg cells results in increased SOCS1 expression accompanied by impaired STAT5 activation in response to limiting amounts of IL2.

Drosha and Dicer, the two RNaseIII enzymes, mediate the stepwise maturation of miRNAs. Chong et al. [90] found that miRNA biogenesis is indispensable for the function of Treg cells, mice specific deletion of either Drosha or Dicer phenocopies lack a functional Foxp3 gene or Foxp3+ cells, whereas deletion throughout the T cell compartment also results in spontaneous inflammatory disease later in life.

In order to analyze the role of miRNAs in the development and function of Treg cells, Zhou et al. [91] have crossed a Treg cell-specific, Foxp3-GFP-hCre bacterial artificial chromosome transgenic mouse to a conditional Dicer knockout mouse. The peripheral Treg cells showed altered differentiation and dysfunction. Dicer-deficient Treg cells failed to remain stable, as a subset of cells down-regulated the FoxP3, whereas the majority expressed altered levels of multiple genes and proteins including Neuropilin 1, glucocorticoid-induced TNFR, CTLA4. A significant percentage of the Treg cells possessed a memory Thelper cell phenotype including increased levels of CD127, IL4, and IFNgamma. Dicer-deficient Treg cells lost suppression activity *in vivo*. The mice rapidly developed fatal systemic autoimmune disease resembling the foxp3 knockout phenotype.

Th17 cells have been identified as important mediators of inflammatory disease. Du et al. [92] found that miR-326 promotes Th17 cell development *in vitro* and *in vivo* by targeting ETS1, a negative regulator of Th17 differentiation, resulting in promoting the differentiation of CD4+Th0 cells toward Th17 cells. The expression of miR-326 was correlated with disease severity in patients with MS (Multiple Sclerosis) and mice with EAE (Experimental

Autoimmune Encephalomyelitis). *In vivo* silencing of miR-326 resulted in fewer Th17 cells and mild EAE, its overexpression led to more Th17 cells and severe EAE.

Stage 6: Precursor B cells

Mouse and human precursor B cells have been characterized extensively. Surface markers and molecular events of precursor lymphoid to precursor B cells at intermediate stages of development have been defined (Table 50.1, Fig. 50.3) [93]. The markers for peripheral B cells at different stages are summarized in Table 50.1 [94]. More updated markers are summarized by Alinikula and Lassila [95]. For the reason of simplicity, such newly assigned markers are not included in the tables of this chapter.

TABLE 50.1 Expression of cellular and molecular markers during early stages of B cell development

	Progenitor B stage		Precursor B stage		
Human B cell	**I**	**II**	**III**		**Pre-B**
CD34	+	+	+		–
CD38	nd	+	+		+
γL	–	+	+		+
CD10	nd	–+	+		+
CD19	–	–	+		+
lambda-like/Vpre-B	+	+	+		+
Rag 1	+	+	+		+
TdT	+	+	+		–
VH/Cmu	–	+	+		+
V-kappa/C-kappa	–	+	+		+
mbl/B29	+/–	+/–	+		+
cyto-mu	–	–	+/–		+
Mouse B cell	**A**	**B**	**C**	**C'**	**D**
B220	+	+	+	+	+
CD43	+	+	+	+	–
HSA	–	+	+	+	+
BP1	–	–	+	+	+
lambda-5/Vpre-B	+/–	+	+	+	+
Rag 1–2	+/–	+	+	+	+
TdT	+/–	+	+	+/–	–
mbl	+/–	+	+	+	+
D-JH		+	+		
VH-D-JH			+(out)		+(on)
	Pro-B	Pre-B I	Pre-B II		
			Large	Small	
CD43	+	+	+/–	–	
c-Kit	(+)/–	(+)	–	–	
CD25	–	–	+	+	
IL-7Ralpha	+	+	+	(+)	
CD19	–	+	+	+	
γL	+	+	+	+	
lambda5/Vpre-B	+	+	+	+	
Rag 1–2	+	+	–	+	
TdT	+	+	–	–	
cyto-mu	(+)	–	+	+	
D_H–J_H	–	+	+	+	
V_H–D-J_H	–	–	+	+	
V_L–J_L	–	–	–	+	

Data in this table are partially derived from Martensson et al. [102], Matthias and Rolink [94], see Chen [4].

TABLE 50.2 Expression of cellular markers during periphery stages of B cell development

	Stage periphery				
B cell marker	Immature			Mature	
	t1B cell	t2B cell	t3B cell	MZB cell	Follicular B cell
CD21	−	+	+	+	+
CD23	−	+	+	(+)	+
CD93	+	+	+	−	−
IgM	+	+	+	+	+
IgD	(+)	+	+	(+)	+

t: transitional; peripheral immature B cells can be further classified into three transitional stages. MZ: marginal zone.
This table is modified from Matthiasand Rolink [94]. More markers are described recently by Alinikula and Lassila [95].

MOUSE PRE-B CELL LINES

In the mouse system, with the help of several stroma cell lines and recombinant growth factors such as IL7 (in reference [4]), it becomes feasible to expand mouse pre-B cells without gross differentiation. On release from the stroma cells and IL7 and in the presence of the bacterial mitogen LPS, some pre-B cell lines differentiate into plasma cells. Pre-B cell clones have been established from various lymphoid organs of wild-type, transgenic, and knockout mice. Thus, both somatic and genetic manipulation of these cell lines becomes possible. Some pre-B cell lines have been used to repopulate SCID mice and RAG-2 knockout mice. Injected cells migrate to the bone marrow, lymph nodes, peritoneal cavity and spleen. Plasma cells, mature B cells, and pre-B cells were detected in the host. The percentage of cells that mature into various B compartments seems to vary from experiment to experiment.

However, several questions remain. Do these pre-B cell lines retain the capacity to expand? Are these cells self-renewing *in vivo*, as stem cells must be? The critical experiment of repeatedly transferring donor pre-B cells from one host to another, to show that the implanted cells are still precursor B cells, has not yet been done.

Pax5 signatures the commitment of lymphoid precursors to B cell lineage. Pre-B cells established from Pax5−/− mice differentiated into T cells, myeloid cells, dendritic cells and osteoclasts but not mature B cells in SCID mice. The data indicate that the microenvironment, which plays a role in keeping pre-B cells committed to the B cell lineage, may be missing in the Pax5−/− mice. BAFF (B cell activation factor, BLYS) belongs to the Tumor Necrosis Factor (TNF) family, and its receptors have a critical role in the transition from immature to mature B cells (ref. cited in references [4,94,95]).

Due to its potential to develop into mature B- lymphocytes and also into antibody-secreting plasma cells, which can be performed either *in vitro* or *in vivo*, mouse pre-B cells have been subjected to genetic modification to become a vehicle to generate and secrete human antibodies against a variety of infectious antigens in SCID mice. The genetically modified pre-B cells can be subjected to T cell-dependent stimuli, inducing somatic hypermutation in the human antibody constructs. In this way, affinity-matured antibodies can be generated, allowing the development of therapeutic antibodies with the pharmacological efficacies.

CURIOSITIES OF GROWING HUMAN PRECURSOR B CELLS

The question of whether human precursor B cells can expand *ex vivo* is still open. Several reports claim it is possible by using either primary mixed human or mouse stroma cells (ref. cited in reference [4]). When subjected to mixed stroma cells and cytokines such as IL7, most human bone marrow cells expand for a limited period and then either perish or

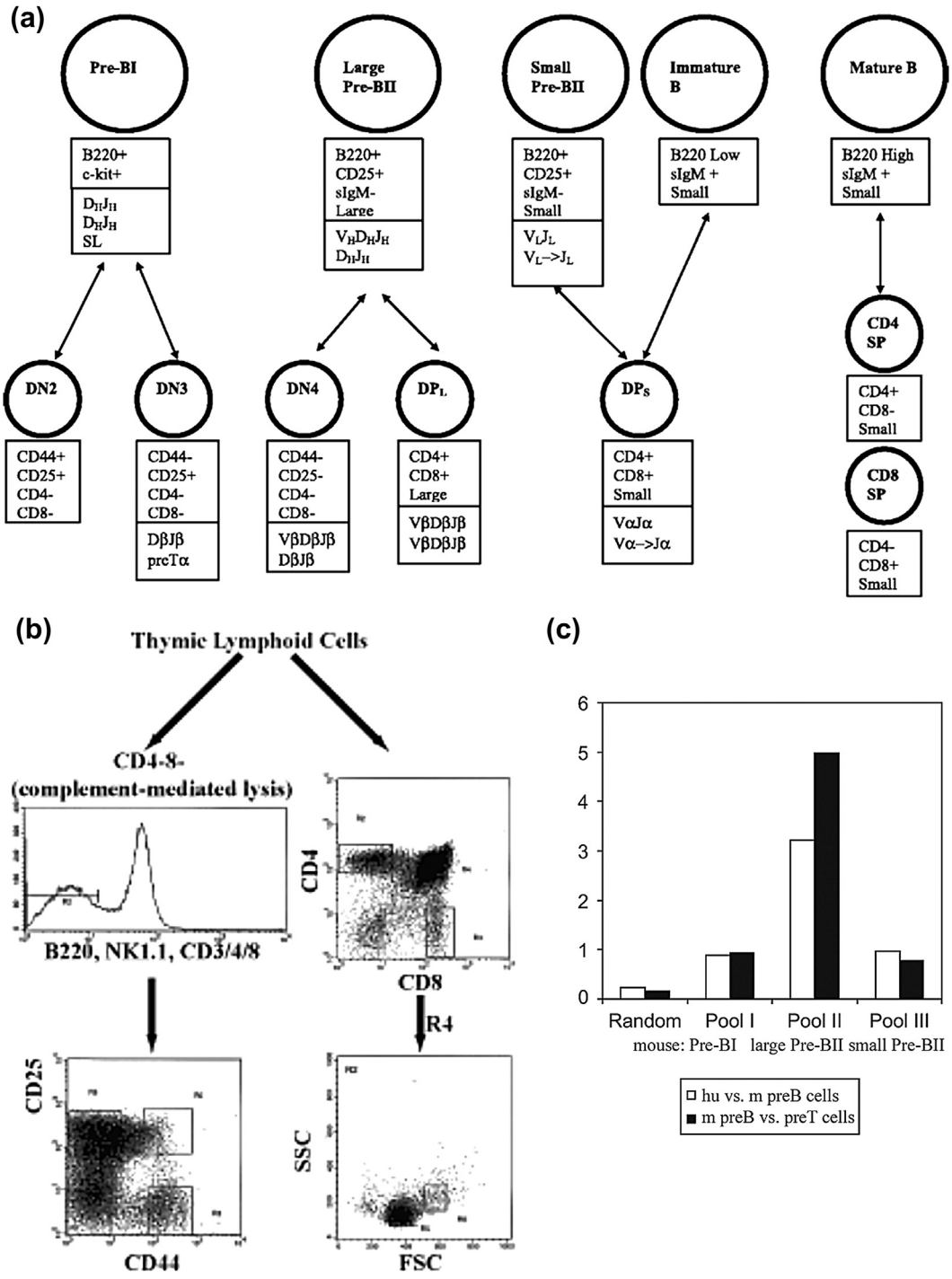

FIGURE 50.3

Surface markers and sorting strategy for isolation of thymic subsets. (a) Synopsis of cellular populations and surface markers used for isolation of lymphocyte developmental stages. Top: B cell development; bottom: T cell development. Cellular stages are depicted as circles. Markers used for separation of the stages appear in the upper boxes; Ig and TCR gene loci rearrangement status appear in the lower boxes. Arrows connect corresponding stages between B- and T cell development. SL, surrogate L chain. (b) Cell-sorting strategy for separation of T cell precursors. To obtain DN thymocytes, CD4+ and CD8+ cells were removed by complement-mediated lysis from thymus single-cell suspension. Remaining lymphocytes were stained with a panel of lineage markers (B220, NK.1.1, CD3, CD4, CD8), and negative cells (R2 gate in top left panel) were gated and analyzed for CD25 and CD44 surface expression (bottom left). For the isolation of DP and SP subsets, single-cell suspension was stained with CD4 and CD8 mAbs (top right); SP cells were sorted according to gates R2 for CD4+ and R3 for CD8+. DP thymocytes (gate R4 in top right panel) were further resolved into large (R5) and small (R6) subsets (bottom right). From Hoffmann et al. [93] with permission. (c) Similarity scores of ordered gene lists between human and mouse precursor B cells (open bars) and between corresponding stages of mouse B and T cell development (filled bars). Pool I, II and III are mouse Pre-BI, large Pre-BII, Small Pre-BII, stages shown in (a). *(Modified from Hoffmann et al. [15]. Fig. 4A.)*

differentiate. The culture conditions established seem to be appropriate only for the short term expansion of pre-B cells. However, no normal human pre-B cell lines have been established. This distinguishes mouse and human precursor B cells.

Global gene screen using microarrays on cells from stages of precursor mouse B, human B and mouse T developments has shown that gene expression patterns differ substantially between human and mouse B cell development. Among 644 genes which were differentially expressed in four early stages of human B cell development, only 48, 86, and 75 genes could be identified, which are upregulated in both human and mouse pre-BI, large pre-BII, and small pre-BII cells, respectively (see Fig. 50.3a for stage definition). A comparison of mouse B and T cell development reveals that gene expression patterns of early mouse B and T precursors are most similar, but not mouse B and human B precursors. However, in more differentiated precursors, human and mouse B precursor have a more similar gene expression profile (panel C) [93]. This finding might partially explain why the culture conditions that are optimal for growing mouse precursor B cells are not optimal for growing human precursor B cells.

The study of human pre-B cells cannot be separated completely from the study of human HSCs and HPCs, given the complexity of the cell types involved and the difficulty in establishing human stroma cell lines. The establishment of human stroma cell lines supporting the growth of human pre-B cells is critical. The failure lies partially in the inability of adherent cells derived from human bone marrow (stroma cells) to proliferate well under normal culture conditions with conventional sera. Methods for immortalizing human cells include transfecting plasmids or retroviruses containing oncogenes such as SV40 T antigen. Dando et al. [40] have done that with human fetal bone marrow, and established many stroma cell lines; a few cell lines were partially characterized for supporting the growth and expansion of cord blood stem cells. However, no further work was performed to test these cell lines using other cell types. Cell proliferation is a prerequisite for the stable integration of transgenes into chromosomes and for immortalization. A breakthrough for human HSCs and pre-B cells would be to establish stroma cell lines for B lymphopoiesis and to optimize the conditions for the growth of stroma cells, as has been done for human MSCs (ref. cited in reference [4]). MSCs have been shown to expand to large quantity when selected lots of FCS are used. The topic of expanding MSCs for clinical trials was discussed in Stage 1 (above).

THE ROLES OF miRNAs IN PRECURSOR B CELLS

The roles of miRNAs in controlling the development of ProB to PreB to B cells are thought to involve the modulation of key factors that control transcription factor networks, V(D)J recombination, selection of BCR, Ig).

Overexpression of miR-181 causes a skewing of hematopoiesis towards the development of B cells, leading to a two- to three-fold increase in the number of B cells and no increase in T cells or myeloid cells, but the target mRNA of miR-161 was not identified [58].

Mice with a conditional deletion of Dicer in early progenitor B cells had a block at the pro-B to pre-B transition [96]. The proapoptotic molecule BIM was highly upregulated. B cell development could be partially rescued by ablation of Bim or transgenic expression of the prosurvival protein Bcl-2 (B cell lymphoma 2). The V(D)J recombination program in developing B cells of Dicer-deficient mice was investigated, and intact Ig gene rearrangements in IgH and IgKappa loci were found, but increased sterile transcription and usage of D(H) elements of the DSP family in Ig Heavy chain (IgH), and increased N sequence addition in Igkappa due to deregulated transcription of the terminal deoxynucleotidyl transferase gene.

Besides affects T cell development (above), the repression of mRNAs coding for the proapoptotic factor BIM (also called BCL2-like 11) by miR-17–92 cluster also affects the differentiation of pro-B to pre-B cells. The changes observed by gene expression profiling of Dicer-

deficient B cell precursors were similar to those observed in B cells lacking the miR-17−92 family.

Constitutive expression of miR-150 causes a block at the pro-B to pre-B cell transition [112], and this inhibition depends on dys-regulation of c-Myb expression. Mice deficient in miR-150 have an accumulation of B-1 B cells in the spleen and the peritoneal cavity, with a relative decrease in the number of short life B-2 B cells. The miR-150-deficient mice have increased levels of antibody secretion both at baseline and following T cell dependent antigenic stimulation [97].

Overexpression of miR-34a causes an increase in cells at the pro-B to pre-B cell transition. miR-34a was shown to target transcription factor Foxp1, which regulates expression of the Rag1 and Rag2 (recombination-activating genes) (in [113]). Because miR-34a is a p53-induced miRNA [98], this repression might link the regulation of DNA damage responses with regulation of RAG proteins.

Besides influence stem cell homeostasis, miR-221 and mir-222 are suggested to repress Pax5−/+ expression, implied in the migration destination of Pre-B/B cells to peripheral lymphoid tissues (Melchers, personal communication, unpublished).

Stage 7: Memory B cells

B cells with surface Ig isotypes such as IgG, IgA, and IgE, which possess higher affinities for antigen, are generally defined as memory B cells. In human beings, CD38+, CD20+ germinal center B cells can be distinguished from memory CD38−, CD20+ B cells and CD38+, CD20− plasma cells. In mice, though still debating, CD38high, CD27+, IgD− memory B cells are thought to be different from long-live and short lived plasma cells, both are CD38+CD138+ [95].

The mechanisms of memory B cell generation are unknown and have been the subject of debate for decades. Memory B cells are mature B cells that have encountered antigen that have been activated but not tolerated and have switched to higher-affinity isotypes. IgM-secreting plasma cells, Syndecan-1/CD138+, are terminal cells destined to die. It is not known how memory B cells develop *in vivo*. *In vitro*, at least two systems may generate memory B cells: the germinal center-like culture system and the suspension culture system, wherein resting B cells are activated by LPS plus anti-mu. These B cells are activated, alive, proliferating, and non-abortive, but they are not plasma cells.

A SYSTEM STIMULATING GERMINAL CENTERS *IN VITRO* TO CULTURE B CELLS

In a culture environment that mimics the germinal center in lymphoid organs, B cells survive better and live longer. A germinal center-like environment is a culture system that provides cytokines and supporting cells from either purified follicular dendritic cells (FDCs) from mouse spleen (ref. cited in [4]) or stroma cells (L cells transfected with CD40 ligand called CD154, or fibroblasts). Both human and mouse B cells survive for two weeks instead of three to four days, and absolute cell numbers increase two- to three-fold.

The *in vitro* germinal center culture system developed was designed to study the differentiation of mouse B cells into plasma cells rather than to maintain long term B cells *in vitro*. Mouse FDCs are non-proliferating, terminally differentiated cells. However, other studies reported that the primary mouse FDCs could be partially replaced by fibroblast cell lines expressing CD154. The cytokines, required to maintain mature B cells in growth phase and differentiation, are controversial. For mouse B cells, combined IL2, IL4, and IL5 induce differentiation into mature cells; for further differentiation into plasma cells, IL6 seems to be essential.

For human B cells, IL2 plus ILI0, combined IL3, IL6, IL7, and combined IL2, IL6, IL10, and more recently discovered cytokines IL21 (later), have been reported to play roles in plasma cell

differentiation. Cellular interactions, including those mediated by CD40 and CD154, are critical in the generation of both memory B cells and plasma cells. If CD154 is removed in the secondary culture, human B cells differentiate into plasma cells. These memory-like cells are neither cell lines nor cell clones; rather, they are a mixed B cell type with a limited life span (up to a few weeks), and they preferentially switch to certain Ig isotypes, such as IgG and IgA. The data suggest that down-regulation of the J chain may not be essential during the development of memory B cells.

IL21, a cytokine affecting T cells, NK cells, and B cells, signaling through IL21 receptor and the common cytokine receptor gamma chain, has been shown to play a role in stimulating the differentiation of mouse (refs cited in reference [4]). In the mouse, IL21 is shown to promote differentiation of B cells into CSR (Ig class switch recombination) and plasma cells, using IL21-transgenic mice and hydro-dynamics-based gene delivery of IL21 plasmid DNA *in vivo* and *in vitro*. IL21 induces expression of Blimp-1 and Bcl-6, which play a role in the development of autoimmune disease. When human B cells were stimulated through the BCR, anti-IgM, a minimal proliferation, IgD down-modulation, and small numbers of plasma cells were shown by IL21 stimulation. In contrast, after anti-CD40 activation of human B cells, extensive proliferation, CSR (Class Switch Recombination), and plasma cell differentiation were demonstrated by IL21 stimulation. On cross linking BCR and CD40, IL21 induced the largest numbers of plasma cells. IL21 drove both CD27+ memory cells and naïve cord blood B cells to differentiate into plasma cells. In the latter, the effect of IL21 was more potent than the combination of IL2 and IL10. IL21 co-stimulation induced the expression of Blimp-1 and AICD (activation-induced cytidine deaminase), required for CSR, secreted IgG from B cells, but did not induce somatic hypermutation. IL2 enhanced the effects of IL21, whereas IL4 inhibited IL21-induced plasma cell differentiation.

A SUSPENSION CULTURE SYSTEM FOR STIMULATING B CELLS WITH LPS PLUS ANTI-MU

Systems for the short-term culture of primary splenic lymphocytes have long existed (in [4]). These systems are valuable for studying the proliferation and differentiation of B cells, T and B cell interaction, the priming of B cells by antigen, and the mechanism of memory B cell generation. If B cells could be kept alive and not tolerated but could be prevented from becoming IgM secretory plasma cells, they might become memory B cells. One example is the finding that when stimulated with a bacterial mitogen, LPS, some B cells die and some proliferate and become plasma cells (in [4]). When stimulated with anti-mu, most B cells die right away, some proliferate and exhibit growth arrest at G1 phase and then die two days later, and none become plasma cells.

When stimulated with LPS plus anti-mu, most B cells proliferate and none become plasma cells. This non-plasma-cell phenomenon has been known as an anti-differentiation effect, and it was postulated to be a way to generate memory B cells in culture. Through the efforts of many including us, the molecular mechanism of this anti-differentiation phenomenon became clear. In the presence of the two stimuli, B cells proliferate maximally, over 90% being in the cell cycle, but IgM secretion is turned off. The block has been shown to be primarily at the level of nuclear RNA processing of the mum-to-mus switch. Inducible nuclear factors binding to the pre-mRNA secretory polyA site have been reported, though the nature of these factors remains unclear.

TRANSCRIPTION FACTORS AND miRNAs ACTIVE IN THE DEVELOPMENT OF PLASMA CELLS

On activation of B cells, many transcription factors (Oct-2, OBF-1, Blimp-1, or PRDI-BF1 in human, Xbp1, IRF4, Bcl6, etc.) become engaged in the production and secretion of Ig genes. Extensive discussion on the transcriptional factors controlling the differentiation of B cells has been summarized in reference [4]. Here I will just briefly mention some key players.

Oct-2 and Blimp-l transcription factors play a role in the decision to switch from mum to mus. Blimp-l is described as a cofactor of transcription factor PU.1 and was shown to bind to multiple Ig-enhancer motifs and the J chain regulatory element. It is crucial for the transcription of mu, kappa, and J chains.

Oct2 is POU-domain-containing transcription factor, binds to the mu intron enhancer octamer motif, on which they can form a ternary complex with the coactivator OBF1, and is essential in transcriptional activation of the mu chain. It was shown that Oct2 and the J chain are highly expressed in LPS-stimulated B cells and are diminished in LPS+ anti-mu-stimulated B cells. It has been shown that Blimp-l is highly expressed in LPS-stimulated B cells and is diminished in LPS+ anti-mu-stimulated B cells. On the other hand, sterile gamma chain is highly expressed in the latter system. Transfection of Blimp-l into LPS+ anti-mu-activated B cells provoked them to become IgM-secreting plasma cells. The data indicated that transcription factors such as Oct2 and Blimp-l are tightly regulated in plasma cell development. If one postulates that LPS+ anti-mu-stimulated B cells represent some stage in memory B cell development, then the down-regulation of Oct2 and Blimp-l reflects the specific transcriptional regulation when B cells make the commitment to the memory cell pathway instead of the plasma cell pathway. The identification of such candidate transcription factors that control memory B cell commitment provided a powerful genetic tool to manipulate the turning on and off of these lineages at will.

The human Blimp-1, PRDI-BF1, is a DNA-binding protein involved in post-induction repression of INFbeta gene transcription in response to viral infection. In terminal differentiation of B cells, it has an essential function in driving differentiation and therein silences multiple genes.

Microarray experiments of the expression of Blimp-1 indicate that it regulates a large set of genes of plasma cell expression signature. Blimp-1 affects numerous aspects of plasma cell maturation, ranging from migration, adhesion, and homeostasis to antibody secretion. It regulates Ig secretion by affecting the nuclear processing of the mRNA transcript and by affecting protein trafficking by regulating genes that impact the activity of the endoplasmic reticulum. The differentiation events that Blimp-1 regulates appear to be modulated, depending on the activation state of the B cell, and hint at the complexity of Blimp-1 and the genetic program that it initiates to produce a pool of plasma cells.

There is a concerted activation-suppression of transcriptional factors during B cell differentiation. In (human) germinal center B cells, a set of genes has been shown to be involved in the differentiation: down-regulation of Bcl-6, which is implied to transform B- cells, activation of Blimp-1/PRDI-BF1, modulation of Myc, and the upregulation of the Mad1 and Mad4 transcription factors. Transcription factor E47 is required for CSR, at least in part, via expression of AICD (activation-induced cytidine deaminase). Id2 has been identified as a negative regulator of E47. Bach2 is a B cell-specific transcription repressor interacting with the small Maf proteins, whose expression is high only before the plasma cell stage. It is critical for CSR and somatic hypermutation of Ig genes. Mitf (microphthalmia-associated transcription factor) is highly expressed in naïve B cells, where it antagonizes the process of terminal differentiation through the repression of IRF-4.

Xbp1 (X-box binding protein 1) is shown to be essential for the differentiation of B cells into plasma cells. By using microarray analyses, a set of genes have been defined whose induction during mouse plasmacytic differentiation is dependent on Blimp-1 and/or Xbp1. Xbp1 increased cell size, lysosome content, mitochondrial mass and function, ribosome numbers, and total protein synthesis. Xbp1 is essential to coordinate diverse changes in cellular structure and function resulting in the characteristic phenotype of professional secretory cells.

Aiolos, a member of Ikaros family, was shown to be required for the generation of long-lived high affinity plasma cells resident in the bone marrow. Recent results on the network of transcriptional factors regulating plasma cell differentiation, have been described by Alinikula and Lassila [95].

In the periphery B cell development, miR-155, besides affecting two steps of T cells development (above), is shown to be upregulated following B cell activation in the germinal center.

B cells lacking miR-155 generated reduced extrafollicular and germinal center responses and failed to produce high affinity IgG1 antibodies [86,87]. Global gene expression profiling of activated B cells indicated that miR-155 regulates an array of genes with diverse function. Among many of the miR-155-mediated regulation of targets are PU.1 and AID (Activation-Induced cytidine Deaminase) and PU.1. PU.1. contributes to class switch and antibody production. When Pu.1 is overexpressed in B cells, fewer IgG1 cells are produced [99].

B cells perform somatic hypermutation and CSR of the Ig locus to generate antibody diversity in affinity and function. These somatic diversification processes are catalyzed by AID, a potent DNA mutator whose expression and function are highly regulated. AID also promotes chromosomal translocations. AID was regulated post-transcriptionally by miR-155. The miR-155 was upregulated in mouse B cells undergoing CSR and it targeted a conserved site in the 3′-untranslated region of the mRNA encoding AID. Disruption of this target site *in vivo* resulted in quantitative and temporal deregulation of AID expression, along with functional consequences for CSR and affinity maturation. Thus, miR-155 does so in part by directly down-modulating AID expression [100].

Mice carrying a mutation in the putative miR-155 binding site in the 3′-untranslated region of AID, designated Aicda(155) mice, were generated [101]. Aicda(155) caused an increase in steady-state Aicda mRNA and protein amounts by increasing the half-life of the mRNA, resulting in a high degree of Myc-Igh translocations. A similar but more pronounced translocation phenotype was also found in miR-155-deficient mice. Thus, miR-155 can act as a tumor suppressor by reducing potentially oncogenic translocations generated by AID.

CONCLUDING REMARKS AND PROSPECTS FOR LYMPHOCYTE ENGINEERING

I have discussed and summarized the current understanding of the expansion of lymphoid cells and their precursors ex vivo at certain stages of lymphopoiesis. I have tried to address the feasibility of expanding lymphoid cells under controlled growth conditions; that is, we need to expand untransformed, non-malignant cells. In general, in order to maintain the status of cell survival and growth without apoptosis and differentiation, cytokines and cell contact with feeder cells are required. Fundamental questions regarding the engineering of lymphoid cells and their precursors for therapeutic purposes remain and can be traced to our current understanding of the immune system. Do we ask too much for the survival in culture of cells that are programmed to die? From extensive studies in gene- manipulated mice, it is possible to generate antigen-specific memory T cells; it remains a puzzle that there is no good systematic study of human memory cells in culture, although the surface markers have been defined.

If there is a massive programmed cell death occurred during the development from HSCs to lymphoid precursors and from pre-T cell to T cell maturation, I wonder whether it is realistic to try to produce enough HPCs and precursor lymphoid cells for therapeutic purposes. In clinical protocols such as ADA trials, expansion of cells ex vivo for the purpose of reinfusion into patients is limited to as few passages as possible in order to avoid mutation and contamination in vitro. Bioreactors for large-scale production of cells in liquid suspension using cytokines are available. However, they are not designed for co-culturing of stem cells with stroma cells, additional micro-particles in culture allowing the attachment of stroma cells in such particles, are needed. With advances in culture technology and bioreactors and with increased supply of recombinant cytokines, it becomes possible to obtain a quantity sufficient for re-implantation from 10 ml of bone marrow cells. However, under these conditions, very few cells engage in lymphopoiesis. Thus, to grow the HPCs consistently and to favor

lymphopoiesis, there is a great need for a better way to grow human stem cells using human stroma cell lines. The current study using transcription factors such as HoxB4, Cdx4, and mMix; small molecules, miRNAs, to manipulate the behavior of hESCs or h-iPS in vitro may be a promising approach.

In view of the massive apoptosis at several stages of lymphopoiesis, it is amazing that mouse precursor B cells can grow normally, become lines and clones, and retain the potential to differentiate in vitro and in vivo. It is still a puzzle why the human precursor B cells cannot grow in culture yet. To grow cells from other stages of lymphopoiesis, it might be advantageous to use cells from the many available mutated or knockout mice.

The future of cell-based immune therapy lies in the ex vivo expansion of cells. Because the techniques to establish ligand-regulatable vectors are available, the derived cell lines will become available and will become valuable resources for many purposes. Other areas remain to be improved, including the search for novel markers of true HSCs and precursor lymphocytes; better sources of HSCs and HPCs, such as cord blood, fetal tissues, hES cells, h-iPS cells, improved retroviral/lentiviral vectors and large-scale culture systems for expansion of HPCs and precursor lymphoid cells.

With advances in genetic tools and mutated-mice technology, it is possible to turn on or off the transcription factors, miRNAs that control the differentiation of cells. With the discovery and understanding of Toll-like molecules on APCs to stimulate Th1 vs. Th2 vs. Treg vs. Th17, the approaches to fight against autoimmune diseases, allergy, inflammation, and transplantation rejection will become accessible. Thus, immunology will continue to be a very exciting field.

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PART 13

Kidney and Genitourinary System

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CHAPTER 51

Stem Cells in Kidney Development and Regeneration

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Acute renal failure (ARF) and chronic kidney disease (CKD) are significant clinical problems whose frequencies are expected to increase with the prevalence of diabetes, glomerulonephritis, obesity and hypertension. Although data regarding the incidence of CKD are still far from being conclusive, it has been estimated that this conditions affects 10% of all adult Americans [1]. Two main clinical problems may arise as consequence of CKD: first, patients present an increased risk to develop cardiovascular complication; second, CKD may ultimately lead to End Stage Renal Disease (ESRD), a condition in which kidneys are no longer functional and dialysis or kidney transplantation are required. The United States has one of the highest ESRD prevalence in the world, as more than 570,000 patients were treated in 2009. It has been forecasted that this cohort will reach approximately 800,000 patients by 2020 (United States Renal Data System; http://www.usrds.org (accessed April 2012)). This increment will be accompanied by a rise in the economic burden on society: in 2009 the average cost to treat ESRD was estimated to be about $50,000 per patient per year and about $100,000 per patient per year when co-morbidities are present [2]. Yet, the overall mortality rate five years after renal replacement therapies still remains above 50% for all patients [1], despite many advances in understanding the cell physiology of renal injury. Clearly, new approaches for the treatment of acute and CKD must be explored. Tissue engineering, cell replacement therapies, and novel growth and differentiation factors are being developed to address the limitations of current therapies. All of these approaches rely on the knowledge base obtained from basic developmental studies in the kidney, regarding the potency of renal stem cells and the genetic basis of epithelial cell differentiation and proliferation. This chapter will outline the basic elements of renal development and renal stem cell biology and discuss current and future applications of emerging technologies for renal disease.

KIDNEY DEVELOPMENT

A detailed description of early renal development and nephron formation is covered in several recent reviews [3–5]. In brief, the mammalian kidney develops from a region of mesoderm, called the intermediate mesoderm, which lies between the axial and lateral plate mesoderm along the medio-lateral axis of the embryo. The earliest morphological indication of unique derivatives arising from the intermediate mesoderm is the formation of the pronephric duct, or primary nephric duct. This single cell thick epithelial tube runs bilaterally beginning from the mid-thoracic region to a posterior cavity called the cloaca. As it grows, the nephric duct induces

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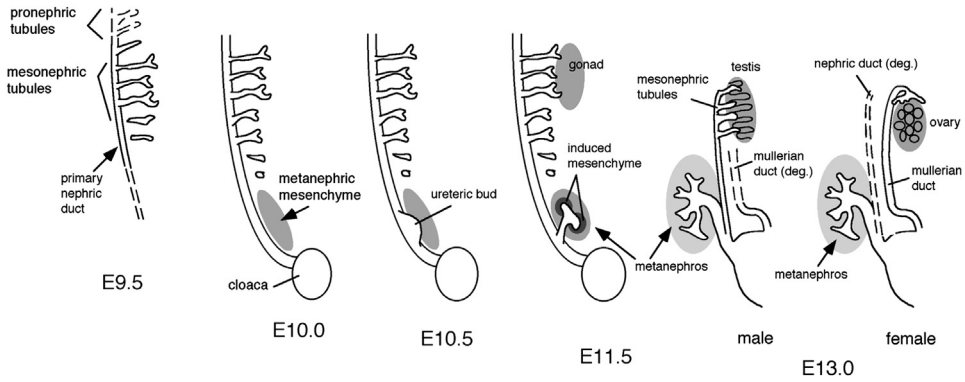

FIGURE 51.1

Urogenital derivatives of the intermediate mesoderm. A schematic of the mammalian urogenital tract during development is shown. The embryonic stages (E9.5) are shown as days post fertilization in the mouse. The primary nephric duct extends anterior to posterior, inducing mesonephric tubules along the ventral aspect. At the posterior end, the metanephric mesenchyme is induced by the ureteric bud, an outgrowth of the nephric duct. Ureteric bud derived signals induce condensation of the mesenchyme, whereas mesenchymal signals induce branching morphogenesis in the bud epithelia. By E13, male and female specific urogenital derivatives become apparent.

a linear array of epithelial tubules, which extend medio-ventrally and are thought to derive from mesenchyme surrounding this duct (Fig. 51.1). The tubules are referred to as pronephric or mesonephric, depending on their position and degree of development, and represent an evolutionarily more primitive excretory system that forms transiently in mammals until it is replaced by the adult or metanephric kidney. The adult kidney, or metanephros, is formed at the caudal end of the nephric duct when an outgrowth, called the ureteric bud or metanephric diverticulum, extends into the surrounding metanephric mesenchyme. Outgrowth or budding of the epithelia requires signals emanating from the mesenchyme. Genetic and biochemical studies indicate that outgrowth of the ureteric bud is mediated by the transmembrane tyrsoine kinase RET, which is expressed in the nephric duct, and the secreted neurotophin GDNF, which is expressed in the metanephric mesenchyme. Once the ureteric bud has invaded the metanephric mesenchyme, inductive signals emanating from the bud initiate the conversion of the metanephric mesenchyme to epithelium (Fig. 51.2). The induced, condensing mesenchymal cells aggregate around the tips of the bud and will form a primitive polarized epithelium, the renal vesicle. Through a series of cleft formations, the renal vesicle forms first a comma then an s-shaped body, whose most distal end remains in contact with the ureteric bud epithelium and fuses to form a continuous epithelial tubule. This s-shaped tubule begins to express genes specific for glomerular podocyte cells at its most proximal end, markers for more distal tubules near the fusion with the ureteric bud epithelia, and proximal tubules markers in between. Endothelial cells begin to infiltrate the most proximal cleft of the s-shaped body as the vasculature of the glomerular tuft takes shape. At this stage, the glomerular epithelium consists of a visceral and parietal component, with the visceral cells becoming podocytes and the parietal cells the epithelia surrounding the urinary space. The capillary tuft consists of capillary endothelial cells and a specialized type of smooth muscle cell, termed the mesangial cell.

While these renal vesicles are generating much of epithelia of the nephron, the ureteric bud epithelia continues to undergo branching morphogenesis in response to signals derived from the mesenchyme. Branching follows a stereotypical pattern and results in new mesenchymal aggregates induced at the tips of the branches, as new nephrons are sequentially induced. This repeated branching and induction results in the formation of nephrons along the radial axis of the kidney, with the oldest nephrons being more medullary and the younger nephrons located towards the periphery. However, not all cells of the mesenchyme become induced and convert to epithelia, some cells remain mesenchymal and migrate to the interstitium. These

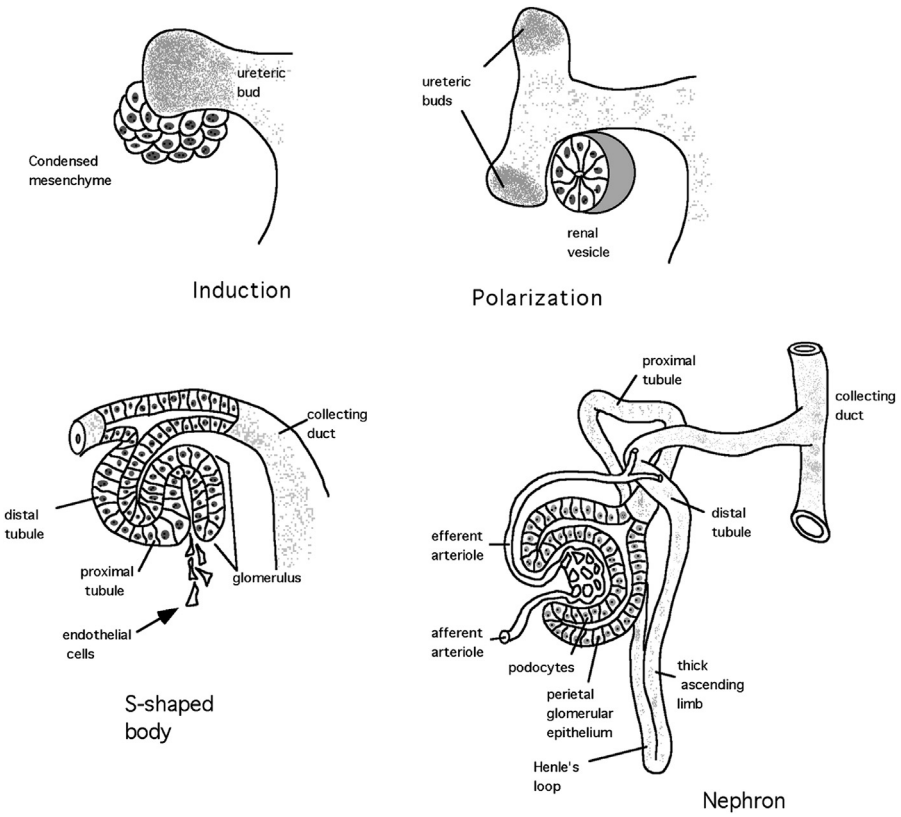

FIGURE 51.2
Sequential conversion of the metanephric mesenchyme to renal epithalia. The metanephric mesenchymal cells are induced to condense around the tips of the invading ureteric bud. These condensation undergo conversion to a primitive epithelial vesicle, the renal vesicle. The renal vesicle is in close proximity to the branching ureteric bud epithelia and fuses to form a continuous epithelial tubule. At the s-shaped body stage, the most proximal cleft becomes vascularized by infiltrating endothelial precursors. The nephron takes shape as the podocyte precursors and endothelial cells intermingle to make the glomerular tuft and the tubular components proliferate and elongate.

interstitial mesenchymal cells, or stromal cells, are essential for providing signals that maintain branching morphogenesis of the ureteric bud and survival of the mesenchyme.

The question regarding the origin of the nephron epithelial cells has been under intense debate for many years. At the time of metanephric mesenchyme induction, which occurs at the embryonic (E) day 11.5 in mouse and at the fifth week of gestation in human, there are at least two primary cell types in the developing metanephros, the mesenchyme and the ureteric bud epithelia. Though these cells are phenotypically distinguishable, they do express some common markers and share a common origin. The ureteric bud epithelium originates the collecting duct network of the nascent kidney; however, it was less clear which subpopulation of progenitor cells within the mesenchyme generates all epithelial cells of the nephron. An elegant study published by Kobayashi and colleagues [6] has answered this question: at the onset of nephrogenesis a domain of the metanephric mesenchyme adjacent to the tips of the ureteric bud, called cap mesenchyme, contains a population of renal progenitor cells that express the transcription factor *Six2*. By using a cell-fate tracking strategy whereby all descendents of the Six2-expressing progenitor cells were permanently labeled, these authors demonstrated that all epithelial cells of the main body of the nephron derive from the $Six2^{+}$ renal progenitor pool of the cap mesenchyme. The cells of the cap mesenchyme are therefore predetermined to make renal epithelia. Thus, their potential as renal stem cells has begun to be explored. To understand the origin of the metanephric mesenchyme, we begin with the patterning of the intermediate mesoderm.

GENES THAT SPECIFY EARLY KIDNEY CELL LINEAGES

The early events controlling the specification of the renal cell lineages may be common among the pro-, and mesonephric regions. Indeed, many of the same genes expressed in the pronephric and mesonephric tubules are instrumental in early metanephric development. Some

of these events that underlie regional specification have been studied in more amenable organisms, including fish and amphibians, in which pronephric development is less transient and of functional significance.

Regionalization of the mesoderm

While formation of the nephric duct is the earliest morphological evidence of renal development, the expression of intermediate mesodermal specific markers precedes nephric duct formation temporally and marks the intermediate mesoderm along much of the A-P body axis. The earliest markers specific for the intermediate mesoderm are two transcription factors of the Pax family (Fig. 51.3), *Pax2* and *Pax8*, which appear to function redundantly in nephric duct formation and extension [7]. *Pax2* is expressed both in the metanephric mesenchyme and the ureteric bud tips, where it is required for mesenchymal-to-epithelial transition (MET) of renal progenitors. At the onset of nephrogenesis *Pax2* is expressed in the comma and s-shaped body and, as the kidney develops, the expression becomes restricted to the epithelial cells of the proximal and distal tubules [5]. In the adult kidney, *Pax2* is only expressed in the collecting duct cells [8]. *Pax2* plays a crucial role for the specification of the nephric lineage: in the mouse, *Pax2* mutants begin nephric duct formation and extension but mesonephric tubules and the metanephros never develop. Besides, mutant embryos lack genital tracts [9].

The *Odd skipped related 1* (*Osr1*) gene is expressed at E7.5 in the common intermediate and lateral plate mesoderm precursors. *Osr1* mutant mouse embryos did not express several key genes important for kidney development, such as *Pax2*, *Eya1*, *Six2*, and *Gdnf* [10]. A more recent study provided evidence that *Osr1*$^{+}$ cells are the precursors of a wide spectrum of cells of

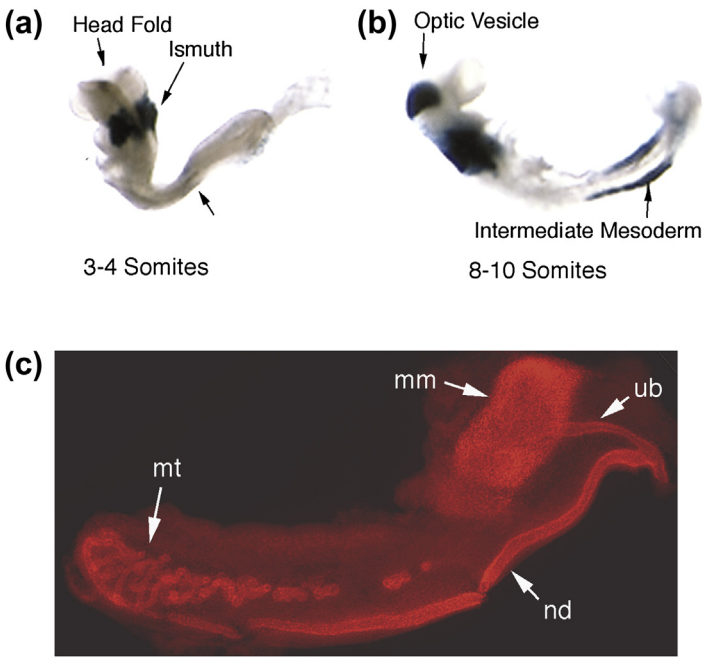

FIGURE 51.3

The activation of Pax2 expression in the intermediate mesoderm. a & b) The embryos shown carry a Pax2 promoter driving the LacZ gene and expression is visualized by staining for beta-galactosidase activity. One of the earliest markers for the nephrogenic region, Pax2 expression is activated around the 4—5 somite in the cells between the axial and lateral plate mesoderm (a). By the eight somite stage (b), Pax2 marks the growing intermediate mesoderm even before the nephric duct is formed. Pax2 is also expressed in parts of the nervous system, especially the mid-brain/hindbrain junction, known as the rhombocephalic ismuth, and in the optic placode and cup. c) By E11, staining for Pax2 protein reveals expression in all epithelial derivatives of the intermediate mesoderm including the nephric duct (nd), the mesonephric tubules (mt), the ureteric bud (ub) branching from the posterior duct, and the metanephric mesenchyme (mm).

the developing kidney. Using a temporal fate mapping strategy, Mugford and collaborators showed that cap mesenchyme, epithelial structures of the nephron, ureteric epithelium of the collecting duct, interstitial mesenchyme progenitors, pericytes, mesangium, vasculature and the kidney capsule all arise from a common *Osr1*$^{+}$ progenitor cell population that are present in the embryo between E7.5 and E11.5 [11].

The transcription factor *Six2* is also essential for kidney development. In the mouse *Six2* is expressed in the cap mesenchyme and its expression is required to maintain a population of self-renewing, multipotent nephron progenitor cells throughout kidney development [6]. Mouse kidneys lacking both copies of *Six2* alleles show ectopic mesenchyme induction, a rapid depletion of the nephron progenitors pool and impaired nephrogenesis [12]. As mentioned earlier in this chapter, *Six2*-expressing cells represent a renal progenitor cell population that generates all epithelial cells of the mature nephron [6].

The homeobox gene *Lhx1* is expressed in the intermediate mesoderm, but is initially expressed in the lateral plate mesoderm before becoming more restricted [13]. As kidney develops, the expression is present in some metanephric mesenchyme-derived structures such as pretubular aggregates, comma and s-shaped body. *Lhx1* regulates urogenital development, as rare *Lhx1* deficient mice, delivered stillborn, lack both kidneys and reproductive tract structures [14]. Indeed, null mutants in *Lhx1* also display reduced expression of Pax2, lack the nephric duct and show reduced ability to differentiate into intermediate mesoderm specific derivatives [13]. In *Pax2/Pax8* double mutants Lhx1 is not expressed and the nephric duct fails to form [7]. Since Lhx1 expression precedes Pax2 and Pax8 and is spread over a wider area in the pre- and post-gastrulation embryo and is Pax independent, it seems likely that maintenance and restriction of Lhx1 expression within the intermediate mesoderm requires activation of the *Pax2/8* genes at the 5–8 somite stage. At this stage *Osr1* expression is detectable in mesenchymal cells that surround the nephric duct, however it is not present in the Pax2 positive cells of the nephric duct.

Are Pax2/8 sufficient then to specify the renal progenitors? This question was addressed in the chick embryo by Bouchard et al. [7] using replication competent retroviruses expressing a Pax2b cDNA. Ectopic nephric ducts were generated within the general area of the intermediate mesoderm upon retrovirally driven Pax2b expression. Strikingly, the ectopic nephric ducts paralleled the endogenous ducts and were not found in more paraxial or lateral plate mesoderm. A more recent study has emphasized the role of *Lhx1* in specifying renal progenitor cells. Cirio and collaborators [15] demonstrated that the expression of a constitutive active form of *Lhx1* in *Xenopus* embryos induced an expansion of the intermediate mesoderm and pronephric anlagen, and this event was accompanied by a reduction of the paraxial mesoderm region. Conversely, the injection of a *Lhx1* antisense oligonucleotide, which suppressed *Lhx1* expression, into *Xenopus* embryos resulted in the absence of pronephric mesenchyme. These studies suggest that nephric duct formation may be induced by Pax2 in a domain of the intermediate mesoderm which is specified by the expression of Lhx1.

If Pax2/8 and Lhx1 restriction in the intermediate mesoderm are the earliest events that distinguish the nephrogenic zone from surrounding paraxial and lateral plate mesoderm, the question then remains as to how these genes are activated. In the axial mesoderm, signals derived from the ventral notochord pattern the somites along the dorsal-ventral axis. Similar notochord derived signals could also pattern mesoderm along the medio-lateral axis. However, this does not appear to be the case. In the chick embryo, the notochord is dispensable for activation of the *Pax2* gene in the intermediate mesoderm. Rather, signals derived from the somites, or paraxial mesoderm, are required for activation of *Pax2* [16]. James and Schultheiss [17] used bone morphogenetic protein 2 (Bmp2) to induce expression of intermediate mesodermal markers in a concentration specific manner. *In vitro*, high levels of Bmp2 induce a lateral plate mesodermal fate whereas low concentrations generate more intermediate mesodermal tissue. Bmp expression is high in the dorso-lateral overlying ectoderm. In an

independent experiment, nephric duct formation can be inhibited if the overlying ectoderm is removed, yet duct formation is restored with exogenous Bmps [18]. The model proposed by James and Schultheiss suggests that factors within the axial mesoderm suppress the expression of intermediate mesodermal markers and that this suppression is lost at low concentrations of Bmps. In the *Xenopus* embryo, retinoic acid and activins are able to expand the pronephric region in animal cap assays, suggesting a positive role for these potential morphogens [19]. Similarly, independent studies have demonstrated that mouse embryonic stem cells express many markers of the intermediate mesoderm and its derivatives when cultured in the presence of activin A, Bmp-4, retinoic acid and lithium chloride [20–22]. Thus, the medio-lateral patterning events appear to be driven by opposing signals to specify the intermediate mesoderm at the interface between lateral plate and axial mesoderm.

If Pax2/8 mark the entire nephric region (Fig. 51.3), then there must be additional factors that specify the position of elements along the anterior-posterior (A-P) axis in the intermediate mesoderm. Such patterning genes could determine whether a mesonephric or metanephric kidney is formed within the Pax2 positive domain. Among the known regulators of A-P patterning are members of the HOX gene family, which are conserved among all metazoan. In mice, *Hox11* paralogs genes display functional redundancy, as neither *Hoxa11* nor *Hoxd11* mutants show any altered phenotype; conversely, *Hoxa11/Hoxd11* double mutants display kidney hypoplasia, though with variable penetrance. Mice that have deleted *Hoxa11*, *Hoxc11* and *Hoxd11* have no metanephric kidneys [23], however it is not clear whether this is truly a shift in A-P patterning or a lack of induction. The Hox paralogs genes seem to regulate the A-P patterning of the posterior intermediate mesoderm, as in the developing kidney Hox11 proteins interact with Pax2 and Eya1 to directly induce the expression of both *Six2* and *Gdnf* in the metanephric blastema [24].

A-P patterning of the intermediate mesoderm may also depend on the FoxC family of transcription factors. *Foxc1* and *Foxc2* have similar expression domains in the presomitic and intermediate mesoderm, as early as E8.5 [25]. As nephric duct extension progresses, *Foxc1* is expressed in a dorso-ventral gradient with the highest levels near the neural tube and lower levels in the BMP4 positive ventro-lateral regions. In *Foxc1* homozygous null mutants the anterior boundary of the metanephric mesenchyme, as marked by GDNF expression, extends rostrally [25]. This results in a broader ureteric bud forming along the A-P axis and eventual duplication of ureters. Similar defects are observed in compound heterozygotes of *Foxc1* and *Foxc2*, indicating some redundancy and gene dosage effects. Thus, *Foxc1* and *Foxc2* may set the anterior boundary of the metanephric mesenchyme, at the time of ureteric bud outgrowth, by suppressing genes at the transcriptional level.

Genes that function at the time of metanephric induction

Induction and conversion of the metanephric mesenchyme to renal epithelial requires the concerted action of many genes. In *Pax2* mutants, there is no evidence of ureteric bud outgrowth despite the presence of a nephric duct. Ureteric bud outgrowth is controlled primarily by the receptor type tyrosine kinase RET, which is expressed on the nephric duct epithelia, the secreted signaling protein GDNF, which is expressed in the metanephric mesenchyme, and the GPi linked protein GFRα1 which is expressed in both tissues. *Pax2* mutants have no ureteric buds because they do not express Gdnf in the mesenchyme and fail to maintain high levels of Ret expression in the nephric duct [26]. Despite the lack of bud, the metanephric mesenchyme is morphologically distinguishable in *Pax2* mutants. While lacking Gdnf, it does express other markers of the mesenchyme, such as Six2 [9]. *In vitro* recombination experiments using *Pax2* mutant mesenchyme, surgically isolated from E11 mouse embryos, and heterologous inducing tissues indicate that *Pax2* mutants are unable to respond to inductive signals [26]. Thus, *Pax2* is necessary for specifying the region of intermediate mesoderm destined to undergo mesenchyme-to-epithelium conversion. In humans, the necessity of *Pax2* function is further underscored as the loss of a single *Pax2* allele is associated

with renal-coloboma syndrome, which is characterized by hypoplastic kidneys with vesicouretral reflux [27].

A second essential gene for the conversion of the metanephric mesenchyme to epithelia is *Eya1*, a vertebrate homologue of the *Drososphila eyes absent* gene. In mice homozygous for an *Eya1* mutation, kidney development is arrested at E11 because ureteric bud growth is inhibited and the mesenchyme remains uninduced, though *Pax2* and *Wt1* expression appears normal [28]. However, two other markers of the metanephric mesenchyme, Six2 and GDNF expression, are lost in the *Eya1* mutants. The loss of Gdnf expression most probably underlies the failure of ureteric bud growth. However, it is not clear if the mesenchyme is competent to respond to inductive signals if a wild-type inducer were to be used *in vitro*. The *eyes absent* gene family is part of a conserved network that underlies cell specification in several other developing tissues. Eya proteins share a conserved domain but lack DNA-binding activity. The Eya proteins interact directly with the Six family of DNA-binding proteins. Mammalian *Six* genes are homologues of the *Drosophila sina oculis* homeobox gene. This cooperative interaction between Six and Eya proteins is necessary for nuclear translocation and transcriptional activation of Six target genes [29]. In humans, mutations in either *SIX1* [30] or *EYA1* [31] are associated with branchio-oto-renal syndrome, further underscoring these genetic and biochemical interactions.

The Wilms' tumor suppressor gene, *WT1*, is another early marker of the metanephric mesenchyme and is essential for its survival. Wilms tumor is an embryonic kidney neoplasia that consists of undifferentiated mesenchymal cells, poorly organized epithelium, and surrounding stromal cells. Expression of *WT1* is regulated spatially and temporally in a variety of tissues and is further complicated by the presence of at least four isoforms, generated by alternative splicing. In the developing kidney, WT1 can be found in the uninduced metanephric mesenchyme and in differentiating epithelium after induction [32,33]. Early expression of *WT1* may be mediated by Pax2 [34]. Initial expression levels are low in the metanephric mesenchyme, but become upregulated at the s-shaped body stage in the precursor cells of the glomerular epithelium, the podocytes. High WT1 levels persist in the adult podocytes. In the mouse, *Wt1* null mutants have complete renal agenesis [35], because the metanephric mesenchyme undergoes apoptosis and the ureteric bud fails to grow out of the nephric duct. The arrest of ureteric bud growth is most probably due to lack of signaling by the *Wt1* mutant mesenchyme.

The transcription factor Gata-3 is also required for proper expression of Ret in the nephric duct [36]. *Gata-3* mutations exhibit increased duct cell proliferation and misdirected ureteric buds [36,37]. Early expression of *Gata-3* in the intermediate mesoderm requires Pax2/8, though direct regulation has not been demonstrated. In humans, Gata-3 is associated with hypothyroidism, deafness, renal abnormalities, the so called HDR syndrome, which is a result of haploinsufficiency [38].

THE ESTABLISHMENT OF ADDITIONAL CELL LINEAGES

At the time of ureteric bud invasion, there appear to be at least two cell lineages established, the metanephric mesenchyme and the ureteric bud epithelia (Fig. 51.4). As branching morphogenesis and induction of the mesenchyme progresses, additional cell lineages are evident. The early E11.5 mouse metanephros contains precursors for most of the cell types present in the adult kidney, including endothelial, stromal, epithelial and mesangial cells. Molecular fate mapping studies have unveiled that all these cell types share a common *Osr1* positive progenitor cell population present in the embryo at E7.5. However, at the induction of the metanephric mesenchyme *Osr1* positive progenitors appears only to generate epithelial cells of the nephron [11]. *Osr1* is expressed in the mesoderm before its subdivision into intermediate and paraxial mesoderm. Interestingly, fate mapping experiments performed in chick embryos

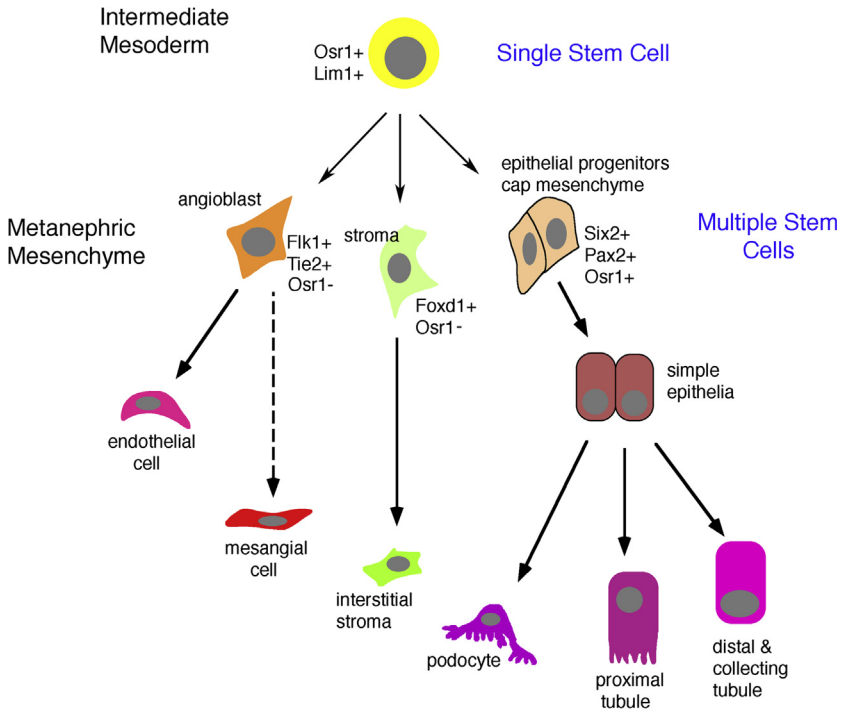

FIGURE 51.4

The major cell lineages of the kidney. Whether the kidney arises from a single renal stem cell or from multiple independent lineages remains to be determined. However, the basic differentiation scheme is becoming clearer. The cell lineage relationships are outlined schematically, with dotted lines reflecting ambiguity in terms of direct lineages. The metanephric mesenchyme contains angioblasts, stromal, and epithelial precursors. Stromal, endothelial and epithelial cells share a common precursor, the metanephric mesenchyme, but segregate at the time of induction. The epithelial cell precursors, found in the cap mesenchyme surrounding the ureteric bud tips, generate all epithelial cells present in the mature nephron.

have indicated that a large population of metanephric stromal cells arises from the paraxial mesoderm, whereas few cells seem to derive from the intermediate mesoderm [39]. Together, these studies suggest that although epithelial and stromal lineages share a common *Osr1* positive lineage, their fate become separate before the beginning of active nephrogenesis. The maintenance of both stromal and epithelial lineages is crucial for renal development, as the ratio of stroma to epithelia is a critical factor for the renewal of mesenchyme and the continued induction of new nephrons.

Epithelia versus stroma

What are the early events in the induced mesenchyme that separates the stromal lineage from the epithelial lineage? There seems to be at least two Wnt proteins involved in triggering renal tubulogenesis, Wnt9b and Wnt4: while the former ligand is expressed in the ureteric bud, the latter is expressed in pretubular mesenchymal aggregate and is placed downstream of Wnt9b signaling. Wnt genes encode a family of secreted peptides that are known to function in the development of many tissues. Mice mutant for *Wnt9b* fail to induce the mesenchyme, while the ureteric bud still undergoes early branching morphogenesis [40]. Mice homozygous for a *Wnt4* mutation exhibit renal agenesis due to growth arrest which occurs shortly after branching of the ureteric bud. Although some mesenchymal aggregation has occurred, there is no evidence of cell differentiation into a polarized epithelial vesicle. Expression of *Pax2* is maintained but reduced [41]. Genetic analyses suggest that Wnt9b is the first inductive signal that triggers renal tubulogenesis. Wnt9b activates Wnt4 which acts as an auto-inducer of nephrogenesis and is required to promote cell polarization in early mesenchymal aggregates [40]. Following induction the cells of the cap mesenchyme downregulate *Six2* and start expressing *Lhx1*, *Pax8*

and *Fgf8*. It has been demonstrated that Wnt9b and Wnt4 induce nephron development by activating the canonical Wnt signaling pathway, which depends on the cytosolic protein β-catenin and the TCF/Lef1 family of transcription factors. However, the canonical pathway is only transiently activated during nephrogenesis and must be downregulated in order to allow early pretubular aggregates to form polarized epithelial structures [42].

The transcription factor Foxd1 is expressed in uninduced mesenchyme surrounding the *Six2* positive cap mesenchyme, then becomes restricted to those cells not undergoing epithelial conversion following induction [43]. After induction there is little overlap between *Foxd1* and the *Pax2* expression domain, prominent in the condensing pretubular aggregates. Clear lineage analysis is still lacking, though the expression patterns are consistent with the interpretation that mesenchyme cells may already be partitioned into a *Foxd1* positive stromal precursor and a *Pax2* positive epithelial precursor prior to, or shortly after, induction. Mouse mutants in *Foxd1* exhibit severe developmental defects in the kidney that point to an essential role for *Foxd1* in maintaining growth and structure [43]. Early ureteric bud growth and branching is unaffected, as is the formation of the first mesenchymal aggregates. However, at later stages (E13–14) these mesenchymal aggregates fail to differentiate into comma and s-shaped bodies at a rate similar to wild-type. Branching of the ureteric bud is greatly reduced at this stage, resulting in fewer new mesenchymal aggregates forming. Bmp4 has been shown to inhibit nephron formation *in vitro*. In *Foxd1* mutant embryos, the ectopic presence of Bmp4 expressing cells in the kidney capsule accounts for mispatterning of the ureteric bud and the consequent delayed and disorganized nephrogenesis. In these mutants the kidney capsule fails to develop correctly and the kidneys remain fused in the pelvis [44]. Thus, the *Foxd1* expressing stromal lineage plays an important role in maintaining growth of both ureteric bud epithelium and mesenchymal aggregates. Perhaps factors secreted from the stroma provide survival or proliferation cues for the epithelial precursors, in the absence of which the non-self-renewing population of mesenchyme is exhausted. It is intriguing to point out that a recent study used time-lapse microscopy and fate mapping to demonstrate that the first pretubular aggregates are only vestigial, since they do not develop into mature nephrons, instead they rapidly degenerate following formation and become part of the stromal compartment [45].

Some survival factors that act on the mesenchyme have already been identified. The secreted TGF-β family member BMP7 and the fibroblast growth factor FGF2 in combination dramatically promote survival of uninduced metanephric mesenchyme *in vitro* [46]. FGF2 is necessary to maintain the ability of the mesenchyme to respond to inductive signals *in vitro*. BMP7 alone inhibits apoptosis but is not sufficient to enable mesenchyme to undergo tubulogenesis at some later time. After induction, exogenously added FGF2 and BMP7 reduce the proportion of mesenchyme that undergoes tubulogenesis while increasing the population of Foxd1 positive stromal cells [46]. At least after induction occurs, there is a delicate balance between a self-renewing population of stromal and epithelial progenitor cells, the proportion of which must be well regulated by both autocrine and paracrine factors. Whether this lineage decision has already been made in the uninduced mesenchyme remains to be determined.

The role of stroma in regulating renal development is further underscored by studies with retinoic acid and its receptors Rarα and Rarβ2. It is well documented that vitamin A deficiency results in severe renal defects [47]. In organ culture retinoic acid, which is the active form of vitamin A, stimulates the expression of Ret to dramatically increase the number of ureteric bud branch points, increasing the number of nephrons [48]. Genetic studies with *Rarα* and *Rarβ2* homozygous mutant mice indicate no significant renal defects when either gene is deleted. However, double homozygotes mutant for both *Rarα* and *Rarβ2* exhibit severe growth retardation in the kidney [49]. These defects are primarily due to decreased expression of the Ret protein in the ureteric bud epithelia and limited branching morphogenesis. Since Rarα and Rarβ2 are expressed both in stromal cells and the ureteric bud, *Ret* regulation could occur via either cell populations. This question has recently been answered by Rosselot and

colleagues using a dominant-negative retinoic acid receptor expressed in ureteric bud cells [50]. These authors have demonstrated that *Ret* expression is induced by retinoic acid produced in the stroma which in turn activates retinoic acid signaling in ureteric bud cells. Together, these findings support the hypothesis that paracrine signals between the stroma and the ureteric bud are required for maintaining *Ret* expression during nephrogenesis and that retinoids are required for stromal proliferation. Reduced expression of the stromal cell marker Foxd1, particularly in the interstitium of Rar double mutants, supports this hypothesis.

Cells of the glomerular tuft

The unique structure of the glomerulus is intricately linked to its ability to retain large macromolecules within the circulating bloodstream while allowing for rapid diffusion of ions and small molecules into the urinary space. The glomerulus consists of four major cell types, the endothelial cells of the microvasculature, the mesangial cells, the podocyte cells of the visceral epithelium, and the parietal epithelium. The development of the glomerular architecture and the origin of the individual cell types are just beginning to be understood.

The podocyte is a highly specialized epithelial cell whose function is integral to maintaining the filtration barrier in the glomerulus. The glomerular basement membrane separates the endothelial cells of the capillary tufts from the urinary space. The outside of the glomerular basement membrane, which faces the urinary space, is covered with podocyte cells and their interdigitated foot processes. At the basement membrane, these interdigitations meet to form a highly specialized cell-cell junction, called the slit diaphragm. The slit diaphragm has a specific pore size to enable small molecules to cross the filtration barrier into the urinary space, while retaining larger proteins in the blood stream. The podocytes are derived from condensing metanephric mesenchyme and can be visualized with specific markers at the s-shaped body stage. While there are a number of genes expressed in the podocytes, there are only a few factors known to regulate podocyte differentiation. These include the WT1 gene, which is required early for metanephric mesenchyme survival but whose levels increase in podocyte precursors at the s-shaped body stage. In the mouse, complete *Wt1* null animals lack kidneys but reduced gene dosage and expression of Wt1 results in specific podocyte defects [51]. Thus, the high levels of Wt1 expression in podocytes appear to be required and make these precursor cells more sensitive to gene dosage. The basic helix-loop-helix protein Pod1 is expressed in epithelial precursor cells and in more mature interstitial mesenchyme. At later developmental stages, Pod1 is restricted to the podocytes. In mice homozygous for a *Pod1* null allele, podocyte development appears arrested [52]. Normal podocytes flatten and wrap their foot processes around the glomerular basement membrane. *Pod1* mutant podocytes remain more columnar and fail to fully develop foot processes. Since Pod1 is expressed in epithelial precursors and in the interstitium, it is unclear whether these podocyte effects are due to a general developmental arrest because of the stromal environment or a cell autonomous defect within the *Pod1* mutant podocyte precursor cells.

Within the glomerular tuft, the origin of the endothelium and the mesangium has yet to be fully understood. At the s-shaped body stage, the glomerular cleft forms at the most proximal part of the s-shaped body, furthest from the ureteric bud epithelium. Vascularization of the developing kidney is first evident within this developing tuft. The origin of these invading endothelial cells has been studied in some details. Under normal growth conditions, kidneys excised at the time of induction and cultured *in vitro* do not exhibit signs of vascularization. However, hypoxygenation or treatment with vascular endothelial growth factor (VEGF) promotes survival or differentiation of endothelial precursors in these same cultures, suggesting that endothelial precursors are already present and require growth differentiation stimuli [53,54]. It is likely that VEGF secretion by podocyte precursor cells helps to attract neighboring endothelial cells into the proximal cleft [55]. The mesangial cells are located between the capillary loops of the glomerular tuft and have been referred to as specialized pericytes. The

pericytes are found within the capillary basement membranes and have contractile abilities, much like a smooth muscle cell. Genetic and chimeric analyses in the mouse have revealed a clear role for the platelet derived growth factor receptor (PDGFr) and its ligand (PDGF) in the development of pericytes. In mice deficient for either PDGF or PDGFr [56,57], a complete absence of mesangial cells results in glomerular defects, including the lack of microvasculature in the tuft. PDGF is expressed in the developing endothelial cells of the glomerular tuft, whereas the receptor is found in the presumptive mesangial cell precursors. Thanks to the development of cell-fate tracking techniques, the origin of both endothelial and mesangial cells of the glomerular tuft have recently become more clear. As discussed earlier in this chapter, Mugford and co-workers [11] used a temporal fate mapping strategy to demonstrate that descendents of *Osr1* positive cells present in the intermediate mesoderm originate most of all cell types of the adult kidney, including the epithelium of the nephron, interstitial mesenchyme, kidney capsule, smooth muscle, vasculature and pericytes. As kidney development goes on, the fate of *Osr1* positive cell descendants becomes more and more restricted in such a way that at the time of metanephric induction *Osr1* expression overlaps the *Six2* positive cap mesenchyme. Intriguingly, investigators from the same research group [6] have provided evidence that parietal epithelial cells and podocytes derive from the *Six2* positive the cap mesenchyme, whereas both pericytes and vascular endothelial cells originate from the cell population that had turned off *Osr1* expression at the onset of nephrogenesis. These studies strongly suggest that the epithelial lineage separates from mesangial and vascular endothelial progenitors prior to the beginning of nephron development.

STEM CELLS IN KIDNEY REGENERATION

The ability of renal epithelial cells to regenerate after injury has prompted much speculation regarding the source of adult renal stem cells and the potential for utilizing such stem cells to further enhance recovery from both acute and chronic renal failure. Acute tubular necrosis (ATN) is the most common cause of ARF in a clinical setting. Often the result of hypotension, surgical cross clamping of the aorta or renal arteries, exposure to toxicants or muscle trauma, ATN has been studied in humans and in a variety of well characterized animal models. The kidney has a remarkable capacity to recover even from an acute insult; however, in some circumstances the recovery seems to be inadequate [58]. The disease may therefore progress to CKD and, ultimately, lead to ESRD. Despite many advances in understanding the cell physiology of ischemic and toxic renal injury, the high frequency of mortality in the clinical setting has not dipped significantly below 50% [59]. In animal models, ATN is the result of sub-lethal damage that is characterized by Adenosine 5′-Triphosphate (ATP) depletion, mitochondrial dysfunction, loss of epithelial cell polarity, and loss of adhesion to extracellular matrix [60]. Such changes can trigger the intrinsic apoptotic response, most likely through cytochrome C release from mitochondria. Cells within the S3 segment of the proximal tubules and the thick ascending limb seem to be particularly sensitive to damaging agents [61–63]. Despite significant cell death after ischemia/reperfusion, folic acid, glycerol, or mercury chloride treatment, renal function in animal models of ATN can recover and proximal tubule morphology is restored if the extent of damage is sub-lethal.

Renal stem and progenitor cells

The process of proximal tubule regeneration in rodent models of ATN has been characterized in much detail. After clearing of cellular debris, cells begin to repopulate the damaged area within a few days. The proliferation of cells within the S2 and S3 segments is accompanied by the expression of many immediate early genes, such as *c-myc* and *AP1* [64], and the re-expression of developmental regulatory genes such as *Pax2* [65]. While it is clear that sub-lethal injury promotes cell proliferation and the expression of dedifferentiation markers along the damaged proximal tubules, the origin of these newly differentiating cells has been the subject of intense debate. There are at least two different models of tubular regeneration that

may explain the origin of these repopulating cells. The conventional model presumed that a subpopulation of viable, remaining cells could repopulate the damaged tubules [60,66]. Alternatively, the existence of a small population of renal stem cells within the environment of the adult kidney was postulated as a potential source of renewable epithelia after injury [67]. The latter model has been supported by several lines of evidence showing that the adult kidney hosts different populations of cells expressing stem or progenitor cell markers that appear to be involved in the process of regeneration. For instance, Gupta and collaborators isolated a population of renal multipotent progenitor cells from the adult kidney that, under particular culture conditions, expressed *Pax2* and the pluripotent marker *Oct4*. *In vivo* localization studies hinted these cells may have a tubular origin. When injected under the renal capsule of a rat model of ischemia-reperfusion injury, some cells seemed to be able to incorporate into damaged tubules; however, many cells also became logged in glomeruli and formed cellular casts [68]. Other investigators have suggested that renal progenitor cells may instead reside in the renal papilla. A population of slowly dividing cells capable of differentiating into multiple epithelial and parenchymal cell types in response to hypoxia have been identified in the papilla of adult rat [69,70]. These findings, however, have recently been contested by a study showing that slowly dividing papillary cells neither proliferate, nor migrate to the proximal tubules after ischemia-reperfusion injury [71]. In a different study, a progenitor cell population of papillary origin has been shown to be able to differentiate into myogenic, adipogenic, osteogenic and neural lineages in culture. Following inoculation into the renal parenchyma of an ischemia-reperfusion injury mouse model, these cells could engraft into damaged tubules and, to a lesser extent, into glomeruli [72]. A more recent study has prompted the hypothesis that the renal interstitium may well be a *niche* where stem/progenitor cells reside. Multipotent kidney progenitor cells were isolated using a transgenic strategy in which the last part of the sequence of *Myh9*, a gene expressed in all interstitial cells and encoding myosin heavy chain IIA, was exchanged with the sequence for Green Fluorescent Protein (GFP). The isolated cells expressed *Pax2*, *Wt1*, *Wnt4* and *Oct4*. Interestingly, when injected into the renal parenchyma of a mouse model of ischemia-reperfusion injury, multipotent kidney progenitor cells seemed to be able to incorporate into renal tubules and ameliorate renal function [73]. Renal progenitor cells have also been isolated from the glomerulus: as demonstrated by Sagrinati and colleagues [74], a subset of parietal epithelial cells expressing the stem cell markers CD24 and CD133 is present within the Bowman's capsule of the adult human kidney. A subsequent study unveiled that CD24 and CD133 cells represent a heterogeneous population hierarchically distributed within the Bowman's capsule: cells expressing CD24 and CD133, but not podocyte markers, were located at the urinary pole and were able to differentiate into both podocytes and tubular cells. A second population of CD24 CD133 progenitor cells positive for podocyte markers was found between the urinary pole and vascular pole of the Bowman's capsule and could only generate podocytes. Finally, a third population present at the vascular pole of the Bowman's capsule showed characteristics of terminally differentiated cells, as they failed to generate both podocytes and tubular cells. In addition, only the cells present at the urinary pole could ameliorate renal function in a mouse model of glomerulosclerosis [75].

The aforementioned studies strongly support the model whereby kidney regeneration following acute tubular injury is accomplished by different populations of renal progenitor cells that reside in different *niches* of the adult kidney. Nevertheless, as anticipated before, an alternative model hypothesizes that a subpopulation of viable cells that survived to injury could be accountable for repopulating renal tubules after damage. In an elegant study, Humphreys and colleagues used a genetic fate mapping strategy to address this hypothesis [76]. These authors developed a mouse strain in which the expression of the GFP-Cre recombinase fusion protein was driven by the *Six2* gene promoter, which is expressed in the cap mesenchyme at the onset of nephrogenesis [14]. Crossing of this mouse strain with a different mouse line carrying a reporter gene, such as *Lac-Z* or *Ds-Red*, allowed for heritably

labeling of all cap mesenchyme-derived cells. Therefore, all epithelial cells of the adult nephron, from the Bowman's capsule to the distal convolute tubule, permanently expressed the reporter gene. Conversely, all non-epithelial cells did not express the reporter gene, as they originated outside the cap mesenchyme. The rationale of this study was that a dilution of the marker following a complete cycle of injury and repair would indicate that unlabeled interstitial cells, or even circulating cells, contributed to the epithelial lineage and tubular regeneration. By contrast, if tubules retained the expression of the reporter gene, then repair of the damaged tubules would instead be accomplished by epithelial cells within the tubules. The results of this study showed that following ischemia-reperfusion injury about 95% of tubular epithelial cells retained the expression of the reporter gene, thus demonstrating that tubular regeneration is accomplished by intrinsic epithelial cells that undergo rapid proliferation following injury. These findings were further confirmed by the converse experiment, in which all interstitial cells were labeled by using a *FoxD1-Cre* driver: in this case the authors did not detect any increase in labeled tubular epithelial cells following renal injury. Nonetheless, this study does not answer the question of whether the tubular repair is carried out by a specialized population of intratubular progenitors, or whether it is the result of self-proliferation of surviving epithelial cells. In a follow-up study Humphreys and his team [71] made use of thymidine analogs to clearly demonstrate that following ischemia-reperfusion injury renal tubules are repopulated by a mechanism of self-division that involves tubular epithelial cells, most of which were non-lethally injured, and not by the presence of intratubular progenitor cells.

Bone marrow derived stem cells

Several investigators have pursued the potential of bone marrow derived cells as a source of renal stem cells. Two early reports demonstrated bone marrow derived proximal tubule cells in mice subjected to ischemia and reperfusion [77,78]. In these experiments, the majority of regenerated proximal tubule cells appeared to be derived from donor bone marrow stem cells either transplanted prior to or injected intravenously directly after injury. However, these findings lead to a great deal of skepticism and have since been disputed. The potential of whole bone marrow to contribute to proximal tubule regeneration many months after transplantation was examined in detail by Szczypka et al. [79], who found little evidence for bone marrow derived renal epithelial cells after induction of ATN by folic acid, despite numerous bone marrow derived leukocytes within the renal interstitium. Similarly, Duffield et al. [80] found no evidence for bone marrow derived renal epithelial cells after injury after careful examination of histological sections by deconvolusion microscopy. In a follow-up article, Lin et al. [81] were not able to demonstrate a significant fraction of bone marrow derived renal epithelial cells after injury, despite their promising initial findings [78].

In addition to whole bone marrow transplants, purified human mesenchymal stem cells have been used for testing renal replacement in animal models. Such cells have been recombined with developing rodent kidneys *in vitro* and *in vivo* and show the potential to differentiate into tubular epithelium [82,83]. Injection of mesenchymal stem cells in rodent models of ischemia reduces the severity of renal failure, although this does not appear to be due to transdifferentiation into epithelia [80,84]. Bone marrow derived cells were used to rescue a genetic defect in a Collagen IV chain (Col4A3), a mouse model of Alport's syndrome, such that the glomerular basement membrane was partially restored [85]. Again, this correction does not necessarily mean that bone marrow derived cells contributed to epithelial structures, rather replacement of capillary endothelial cells by genetically corrected bone marrow cells could help remodel the aberrant glomerular basement membrane. More recently, it was demonstrated that the infusion of human umbilical cord derived mesenchymal stem cells into a mouse model of ATN could ameliorate renal function and greatly reduce mortality [86], thereby suggesting that these cells may be of clinical relevance for the treatment of acute kidney injury. However, it is

important to point out that the renoprotective effect of bone marrow derived cells may be due to the secretion of protective factors in a paracrine manner, rather than being a consequence of cell engraftment into damaged renal structures and subsequent differentiation into tubular cells. For instance, Togel and colleagues noticed that mesenchymal stem cells injection into a rat model of ischemia-reperfusion injury stimulated the expression of anti-inflammatory cytokines and inhibited the expression of pro-inflammatory cytokines, and improved renal function [87]. Though, cell engraftment into the injured kidney was a very rare event . Mesenchymal stem cells released several renoprotective factors, such as VEGF, HGF and IGF-1 that reduced apoptosis in endothelial cells and stimulated cell proliferation [88]. Regardless of the cellular mechanisms involved, the data does point to a possible therapeutic potential for mesenchymal stem cells and other bone marrow derivatives in alleviating certain renal insults.

Human amniotic fluid stem cells

The use of human amniotic fluid stem cells (hAFSC) in renal replacement therapies has recently received great interest. hAFSC can be easily isolated through amniocentesis and propagated *in vitro* for more than 250 population doublings without showing obvious chromosomal rearrangements. These cells express several stem cell markers and were able to differentiate into a broad spectrum of lineages, such as osteogenic, endothelial, myogenic, adipogenic, hepatic and neural lineages [89]. When injected in immunodeficient mice after glycerol-induced tubular necrosis, hAFSC could engraft mostly into damaged tubules, started expressing markers specific of the renal lineage and improved renal function [90]. In a independent experiment Hauser et al. [91] noticed that the infusion of hAFSC into a similar animal model ameliorate kidney function, however cell engraftment occurred mainly in the interstitium and peritubular capillaries.

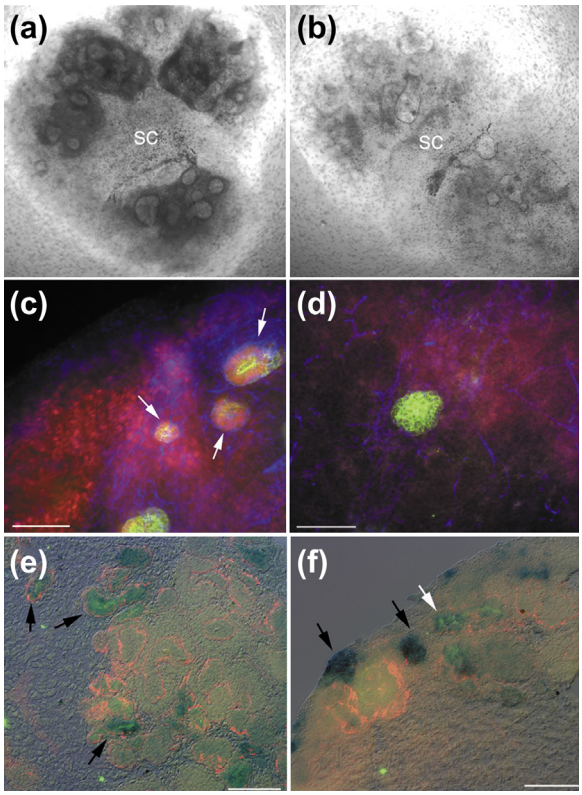

FIGURE 51.5
Embryonic stem cells can differentiate into renal epithelia. ES cells were allowed to form embryoid bodies and cultured with retinoic acid, Activin, and BMP7 (RA7, panels a, c, e) as described [20]. Control ES cells were allowed to form embryoid bodies but were cultured in media without additional differentiation promoting factors (b, d, f). a & b) Embroid bodies co-cultured with inducing tissue, i.e., dorsal spinal cord, show large three-dimensional epithelial structures when treated with RA7 but not with media alone. c & d) Epithelial structures generated from RA7 treated ES cells *in vitro* stain positive for Pax2 (red), E-cadherin (green) and laminin (blue), whereas controls show few Pax2 positive epithelia. e & f) RA7 treated lacZ positive cells (blue) injected into a developing kidney rudiment integrate into tubules (arrows) as indicated by staining for laminin (orange) and LTA lectin (proximal tubules, green). Many control ES cells remain as aggregates of mesenchyme (black arrows), although occasional ES cells are seen within tubules (white arrow).

Embryonic stem cells and induced pluripotent stem cells

An alternative source of renal stem cells may be the true embryonic stem (ES) cells isolated from the inner cell mass of the blastocyst. Upon injection into developing kidney rudiments, ES cells can contribute to tubular epithelium and interstitium suggesting that the local environment is conducive to differentiation of ES cells into renal derivatives [92] (Fig. 51.5). Furthermore, the contribution of ES cells to renal epithelia can be enhanced if the cells are first differentiated *in vitro* by mesodermal factors known to be important for early renal development [20,22]. A combination of retinoic acid, Activin, and BMP7 was shown to stimulate expression of early renal markers in embryoid bodies cultured *in vitro* (Fig. 51.5). Once these renal progenitor cells were injected into a developing kidney they were able to contribute exclusively to the renal proximal and distal tubules. Strikingly, ES cell derivatives were never found in the glomerulus, suggesting that an essential factor for podocyte differentiation was missing [20]. However, the use of ES cells in clinical practice may be limited by ethical concerns and the risk of uncontrolled growth and differentiation which may lead to the formation of teratomas.

The possibility of reprogramming fully differentiated adult cells into a ES-like state, thus obtaining induced pluripotent stem (iPS) cells, may have the potential to revolutionize regenerative medicine. iPS cells were originally generated by retroviral transfection of four genes, namely *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, into mouse and human fibroblasts [93,94]. iPS cells have similar properties to ES cells in terms of morphology, gene expression, differentiation ability, teratomas formation and are capable of forming chimeras when injected into blastocyst. Since embryo destruction is not required for iPS cells generation, it is more than plausible that their use in basic research and clinical applications will not raise ethical concerns. If iPS cells were to be used for regenerative medicine, the main challenge for renal replacement therapies would be to develop culture conditions that could induce iPS cells to differentiate only into desired renal cell types. Studies conducted on animal models of acute and CKDs will be strongly encouraged to evaluate the feasibility of using iPS cells for renal replacement therapies.

The above outlined experiments highlight some of the difficulties regarding development and cell lineage specification in the kidney. A prospective renal stem cell should be self-renewing and able to generate all of the cell types in the kidney, including epithelial, glomerular, mesangial, and stromal cells. Whether this can be achieved by a single cell or requires multiple progenitor cell types is still not clear. In simplest terms, all of the cells in the kidney could be generated by a single stem cell population as outlined in Fig. 51.4. Yet, even at the earliest stage of kidney development there are already two identifiable cell types, the ureteric bud epithelia and metanephric mesenchyme. In turn, at the onset of nephrogenesis the metanephric mesenchyme is already divided into at least two sub-compartments, the first being the cap mesenchyme which hosts epithelial precursors; the second, surrounding the cap mesenchyme, contains the precursors for endothelial and stromal cells. Regarding the repair of renal tissue, it is plausible that the most important lineage is the epithelia. Thus, if we consider the possibility of an epithelial stem cell the following points would be among the criteria for selection:

1) The cells would most likely be a derivative of the intermediate mesoderm.
2) The cells would express a combination of markers specific for the metanephric mesenchyme.
3) The cells should be able to contribute to all epithelial components of the nephron, both *in vitro* and *in vivo*.

The metanephric mesenchyme contains the renal progenitor cells but is essentially quiescent and does not proliferate in the absence of induction. However, growth conditions that mimic induction might be able to allow for the mesenchymal cells to proliferate while suppressing their differentiation into epithelium. If metanephric mesenchymal cells were able

to proliferate *in vitro*, the markers they might express should include: Pax2, Lhx1, Wt1, Gdnf, Six2 and Foxd1. Expression of these markers would indicate a mesenchymal cell that had not decided between the stromal and epithelial lineage. If cells express Pax2, Wt1, and Wnt4, but not Foxd1, they could be an epithelial stem cell. Such epithelial stem cells when injected into, or recombined with, an *in vitro* cultured metanephric kidney should be able to make all of the epithelial cells along the proximal-distal axis of the nephron. Such epithelial stem cells could prove significant in regenerating damaged tubules in acute and chronic renal injury.

At present, the complexity of the kidney still impedes progress in the area of tissue and cell based therapies. Not only must the right cells be made, they must be able to organize into a specialized three-dimensional tubular structure capable of fulfilling all of the physiological demands put upon the nephrons. Developmental biology can provide a framework for understanding how these cells arise and what factors promote their differentiation and growth. While we may not be able to make a kidney from scratch, it seems within the realm of possibility to provide the injured adult kidney with cells or factors to facilitate its own regeneration. Given the high incidence and severity of acute and chronic renal insufficiency, such therapies would be most welcome indeed.

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CHAPTER 52

Renal Replacement Devices

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INTRODUCTION

The rapid understanding of the cellular and molecular bases of organ function and disease processes will be translated in the next decade into new therapeutic approaches to a wide range of clinical disorders, including acute and chronic renal failure. Central to these new therapies are the developing technologies of cell therapy and tissue engineering, which are based on the ability to expand stem or progenitor cells in tissue culture to perform differentiated tasks and to introduce these cells into the patient either via extracorporeal circuits or as implantable constructs. Cell therapy devices are currently being developed to replace the filtrative, metabolic and endocrinologic functions of the kidney lost in both acute and chronic renal failure. This chapter summarizes the current state of development of a wearable or implantable bioartificial kidney (BAK) for full renal replacement therapy that may significantly diminish morbidity and mortality in patients with acute or chronic renal disease.

The kidney was the first solid organ whose function was approximated by a machine and a synthetic device. In fact, renal substitution therapy with hemodialysis or chronic ambulatory peritoneal dialysis (CAPD) has been the only successful long term *ex vivo* organ substitution therapy to date [1]. The kidney was also the first organ to be successfully transplanted from a donor individual to recipient patient as an isograft. However, the lack of widespread availability of suitable transplantable organs has kept kidney transplantation from becoming a practical solution in most cases of chronic renal failure.

Although long-term chronic renal replacement therapy with either hemodialysis or peritoneal dialysis has dramatically changed the prognosis of renal failure, it is not complete replacement therapy, because it provides only the filtration function (usually on an intermittent basis) and does not replace the homeostatic, regulatory, metabolic, and endocrine functions of the kidney. Because of the non-physiologic manner in which dialysis performs or does not perform the most critical renal functions, patients with end stage renal disease (ESRD) on dialysis continue to have major medical, social, and economic problems [2]. Accordingly, dialysis should be considered as partial substitution rather than renal replacement therapy.

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Tissue engineering of an implantable artificial kidney composed of both biologic and synthetic components could result in substantial benefits for patients by increasing life expectancy, mobility, and quality of life, with less risk of infection and with reduced costs. This approach could also be considered a cure rather than a treatment for patients. A successful tissue engineering approach to the kidney depends on a thorough knowledge of the physiologic basis of kidney function.

BASICS OF KIDNEY FUNCTION

The excretory function of the kidney is initiated by filtration of blood at the glomerulus, which is an enlargement of the proximal end of the tubule, incorporating a vascular tuft. The structure of the glomerulus is designed to provide efficient ultrafiltration of blood, to remove toxic wastes from the circulation yet retain important circulating components, such as albumin. The regulatory function of the kidney, especially with regard to fluid and electrolyte homeostasis, is provided by the tubular segments attached to the glomerulus. The functional unit of the kidney is therefore composed of the filtering unit (the glomerulus) and the regulatory unit (the tubule). Together they form the basic component of the kidney, the nephron. In addition to these excretory and regulatory functions, the kidney is an important metabolic and endocrine organ. Erythropoietin, active forms of vitamin D, renin, angiotensin, prostaglandins, leukotrienes, kallikrein-kinins, various cytokines, and complement components are some of the endocrinologic compounds produced by the kidney. Because of the efficiency inherent in the kidney as an excretory organ, life can be sustained with only 5–10% of normal renal excretory function. Accordingly, the approach to a tissue-engineered construct becomes easier to entertain, especially because only a fraction of normal renal excretory function is required to maintain life [3].

The process of urine formation begins within the capillary bed of the glomerulus [4]. The glomerular capillary wall has evolved into a structure with the property to separate as much as one-third of the plasma entering the glomerulus into a solution of a nearly ideal ultrafiltrate. This high rate of ultrafiltration across the glomerular capillary is a result of hydraulic pressure generated by the heart and vascular tone of the preglomerular and post-glomerular vessels as well as by the high hydraulic permeability of the glomerular capillary wall. The hydraulic pressure and permeability of the glomerular capillary bed are at least two times and two orders of magnitude higher, respectively, than those of most other capillary networks within the body [5]. Despite this high rate of water and solute flux across the glomerular capillary wall, this same structure retards the filtration of important circulating macromolecules, especially albumin, so all but the lower-molecular-weight plasma proteins are restricted in the passage across this filtration barrier [6–8].

This ultrafiltration process of glomeruli in normal human kidneys forms approximately 100 mL of filtrate every minute. Because daily urinary volume is roughly two liters, more than 98% of the glomerular ultrafiltrate must be absorbed by the renal tubules. The bulk of reabsorption, 50–65% occurs along the proximal tubule. Similar to glomerular filtration, fluid movement across the renal proximal tubule cell is governed by physical forces. Unlike the fluid transfer across the glomerular capillary wall, however, tubular fluid flux is driven principally by osmotic and oncotic pressures rather than hydraulic pressure. Renal proximal tubule fluid absorption is based on active Na+ transport, requiring the energy-dependent Na+/K+-ATPase located along the basolateral membrane of the renal tubule cell to promote a small degree of luminal hypotonicity [9]. This small degree of osmotic difference (2–3 mOsm/kg H_2O) across the renal tubule is sufficient to drive isotonic fluid reabsorption, due to the very high diffusive water permeability of the renal tubule cell membrane. Once across the renal proximal tubule cell, the transported fluid is taken up by the peritubular capillary bed, due to the favorable oncotic pressure gradient. This high oncotic pressure within the peritubular capillary is the result of the high rate of protein-free filtrate formed in the proximate

glomerular capillary bed [10]. As can be appreciated, an elegant system has evolved in the nephron to filter and reabsorb large amounts of fluid in bulk to attain high rates of metabolic product excretion while maintaining regulatory salt and water balance.

TISSUE-ENGINEERING APPROACH TO RENAL FUNCTION REPLACEMENT

In designing a bioartificial kidney for renal function replacement, essential features of kidney tissue must be utilized to direct the design of the tissue-engineering project. The critical elements of renal function must be replaced, including the excretory, regulatory transport, and endocrinologic functions. The functioning excretory unit of the kidney, as detailed previously, is composed of the filtering unit, the glomerulus, and the regulatory or transport unit, the tubule. Therefore, a bioartificial kidney requires two main units, the glomerulus and the tubule, to replace excretory and metabolic functions of the kidney.

Current technology has developed to replace the glomerular and excretory functions of the kidney via dialytic techniques, either hemodialysis (HD) or peritoneal dialysis (PD). HD utilizes biocompatible membranes and blood circuits; PD utilizes the peritoneal membrane and fluid circuits. Dialysis provides clearance of small molecules by diffusive flow across a semipermeable membrane and control of volume status by bulk flow of water and solutes through that membrane. These short-term effects are sufficient to abrogate the lethal acidosis, volume overload, and uremic syndromes which accompany renal failure but do not protect the patient from the increased mortality associated with dialysis-treated renal failure in either the acute or chronic form. These methodologies all address water and electrolyte balance functional replacement of the kidney. However, they fail to provide for the lost metabolic function. Thus the metabolic, endocrine, and immune roles of the functioning kidney are candidate mechanisms for the difference in survival noted above. The dialytic clearance of glutathione, a key tripeptide in free radical scavenging and protection against oxidant stress, the negative nitrogen balance and energy loss in the clearance of peptides and amino acids in dialysate, loss of oxidative deamination and gluconeogenesis in the tubule cell, and loss of cytokine and hormone metabolic activity by the kidney each impose substantial stress upon the dialyzed patient and as such are appropriate targets for improved renal replacement therapy. Because of this current dialytic renal replacement technology (RRT) only, and substitutes for the excretory function of the kidney, to develop more complete RRT, the last decade has been focused on tissue engineering the development of a renal tubule cell device to add the missing second component of RRT for more complete renal function.

As to the development of a tissue-engineered kidney, a step function approach towards a fully implantable bioartificial kidney (BAK) has been adopted. An initial focus was to develop an extracorporeal bioartificial kidney comprised of a conventional synthetic hemofilter with a renal tubule cell assist device (RAD) in an acute extracorporeal blood circuit. With success of this formulation, proof of concept for a wearable bioartificial kidney (WEBAK) combining PD with a bioartificial renal epithelial cell system (BRECS) has been recently achieved [11]. The final developmental step based upon prior success of the RAD in acute disorders and the WEBAK in preclinical models, is the current approach to design, fabricate and test in preclinical models a functional fully implantable bioartificial kidney (IBAK) [12]. The details of this progressive development sequence from acute short term RRT with cell therapy of WEBAK to IBAK are presented in the following sections.

Development of a renal tubule-assist device

Replacement of the multivariate tubular functions of the kidney cannot be achieved with inanimate membrane devices, as has been accomplished with the renal ultrafiltration process, but requires the use of the naturally evolved biologic membranes of the renal tubular epithelium. In this regard, the tissue engineering of a bioartificial renal tubule as a cell therapy

device to replace these missing functions can be conceived as a combination of living cells supported on synthetic scaffolds [13,14]. A bioartificial tubule can be constructed utilizing renal tubule progenitor cells [15,16], cultured on semipermeable hollow fiber membranes on which extracellular matrix has been layered to enhance the attachment and growth of the epithelial cells [17]. These hollow fiber synthetic membranes not only provide the architectural scaffold for these cells but also provide immunoprotection, as has been observed in the long-term implantation of the bioartificial pancreas in a xenogeneic host [18]. The successful tissue engineering of a bioartificial tubule as a confluent monolayer has been achieved in a single hollow fiber bioreactor system [13]. The scale-up from a single fiber system to a multifiber bioreactor seeded with porcine renal proximal tubule cells has also been successfully demonstrated [19]. Porcine cells were used initially because the pig is currently considered the best source of organs for both human xenotransplantation and immunoisolation cell therapy due to its anatomic and physiologic similarities to human tissue and the relative ease of breeding pigs in large numbers [20]. The potential risk of viral transmission across species, however, requires human cells for clinical use.

The scale-up from a single hollow fiber device to a multifiber bioartificial RAD has proceeded with porcine renal proximal tubule cells grown as confluent monolayers along the inner surface of polysulfone immunoisolating hollow fibers [19]. These hollow fibers are packaged in bioreactor cartridges with membrane surface areas as large as 0.7 m^2, resulting in a device containing up to 108 cells. *In vitro* studies of the RAD have demonstrated retention of differentiated active vectorial transport of sodium, bicarbonate, glucose, and organic anions [19]. These transport properties were suppressed with specific transport inhibitors. Ammoniagenesis and glutathione metabolism, which are important differentiated metabolic processes of the kidney, were also demonstrated in these devices. Synthesis of 1,25-dihydroxyvitamin D3 as a key endocrinologic metabolite was also documented. These metabolic processes were also shown to be regulated by important physiologic parameters and achieved rates comparable to those of a whole kidney.

The next critical step for clinical application of a renal tubule cell therapy device was to ascertain whether the RAD maintained differentiated renal functional performance, similar to that observed *in vitro*, and viability in an extracorporeal hemoperfusion circuit in an acutely uremic dog. The RAD was accordingly placed in series with a standard hollow fiber hemofiltration cartridge; the two cartridges had ultrafiltrate and post-filtered blood connections to duplicate the structural anatomy of the nephron and to mimic the functional relationship between the renal glomerulus and tubule (see Fig. 52.1). The successful

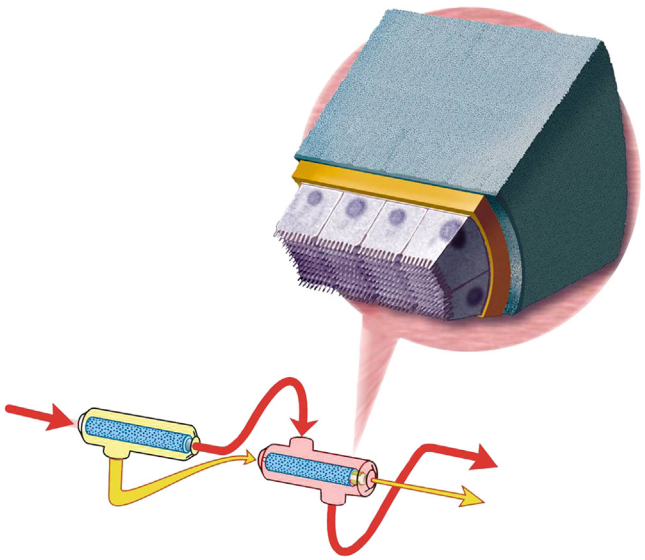

FIGURE 52.1
Schematic of an extracorporeal circuit of a bioartificial kidney used to treat patients with acute renal failure. The first cartridge is a hemofiltration cartridge in series with a renal tubule cell device (RAD). The ultrafiltrate is delivered to the luminal compartment of the RAD which contains the cells and the post-filtered blood is pumped into the extracapillary space of the RAD. The processed luminal ultrafiltrate from the RAD is discarded to waste and the processed blood is returned to the patient.

completion of this step has been reported [21]. Data show that fluid and electrolyte balances in the animals, as reflected by plasma parameters, were adequately controlled with the bioartificial kidney. In fact, plasma potassium and blood urea nitrogen levels were more easily controlled during RAD treatment as compared with sham control conditions. The step-up of oncotic pressure in the post-filtrate blood, which was delivered to the antiluminal space of the RAD, allowed the fractional reabsorption of sodium and water to achieve 40–50% of the ultrafiltrate volume, an amount similar to that seen in the nephron *in vivo*. Active transport of potassium, bicarbonate, and glucose by the RAD was demonstrated. Successful metabolic activity was also observed. These observations resulted in the steps for clinical testing utilizing an extracorporeal blood circuit to evaluate the RAD in a dramatic clinical disease state with substantive immediate medical need.

Bioartificial kidney in acute renal failure

Current therapy for ischemic or toxic acute kidney injury (AKI) or acute tubular necrosis (ATN) is predominantly supportive in nature. Uremia is treated with either intermittent hemodialysis or continuous hemofiltration, treatments that have had substantial impact on this disease process over the last 25 years. Patients with ATN, however, still have an exceedingly high mortality rate of greater than 50%, especially in sepsis [22], despite maintenance of normal electrolyte balance and improvement in the uremic state. The high mortality is caused by the propensity of these patients to develop systemic inflammatory response syndrome followed by multi-organ dysfunction syndrome [23]. The sequential failure of organ systems apparently unrelated to the site of the initial insult has been correlated with altered plasma cytokine levels observed in sepsis [24–26].

Because acute renal failure (ARF), secondary to ischemic or nephrotoxic insults, promotes AKI, predominantly due to renal proximal tubule cell injury, replacement of the functions of these cells during the episode of AKI, and in conjunction with hemofiltration, provides almost full renal replacement therapy. The addition of metabolic activity, such as ammoniagenesis and glutathione reclamation; endocrine activity, such as activation of vitamin D3 (low levels of which seem to correlate with high mortality rates in hospitalized patients) [27]; immunoregulatory support and cytokine homeostasis, may provide additional physiologic replacement activities to change the current natural history of this disease process [3].

Experiments investigated whether treatment with the bioartificial kidney with RAD would alter the course of ARF and AKI with sepsis in animal models. Mongrel dogs that underwent surgical nephrectomy-induced ARF were treated with continuous venovenous hemofiltration (CVVH) and either a RAD containing cells or an identically prepared sham non-cell cartridge. After four hours of therapy, 2 mg/kg intravenous endotoxin was infused over 1 hour to simulate gram-negative septic shock. Mean peak levels of an anti-inflammatory cytokine, IL-10, and mean arterial pressures were found to be significantly higher in cell-treated animals [28].

To further assess the effect of the bioartificial kidney containing the RAD in ARF with bacterial sepsis, dogs were nephrectomized and 48 hours later administered intraperitoneally with 3×10^{11} *Escherichia coli* cells per kilogram body weight [29]. Immediately after bacteria administration, animals were placed in a CVVH circuit with either a RAD with cells or a sham non-cell cartridge. RAD treatment maintained better cardiovascular performance, as determined by mean arterial blood pressure and cardiac output, for longer periods than sham therapy. All sham animals expired within 2–10 hours after bacteria administration, whereas all RAD-treated animals survived greater than 10 hours. Plasma cytokine levels in the bacteremic animals were assessed. Levels of IL-10, an anti-inflammatory cytokine, were significantly elevated in the RAD group as compared with the control group. A significant correlation between the rise in plasma IL-10 levels and the decline in mean arterial pressure was observed. The RAD maintained renal metabolic activity throughout the septic period [27].

In another study, pigs with normal kidney function were administered intraperitoneally with 3×10^{11} *E. coli* cells per kilogram body weight to induce septic shock. Animals were placed in a CVVH circuit containing either a RAD with cells or a sham non-cell cartridge with perfusion beginning immediately following bacterial administration. All animals developed ARF with anuria within 2–4 h after bacteria administration. RAD treatment maintained better cardiovascular performance, as determined by cardiac output and renal blood flow, for longer periods than sham therapy. Consistently, the RAD group survived longer than the controls (10 ± 2 hours versus 5 ± 1 hour, respectively). RAD treatment was associated with significantly lower plasma circulating levels of IL-6, a pro-inflammatory cytokine, and interferon-γ. These data demonstrate that septic shock results in early ARF and that RAD treatment in a bioartificial kidney circuit improves cardiovascular performance associated with changes in cytokine profiles and confers a significant survival advantage [30].

Phase I and II clinical experience with a human renal tubule-assist device

With these encouraging preclinical data, the Food and Drug Administration (FDA) approved an Investigational New Drug application to study the RAD containing human cells in patients with ATN receiving CVVH. Human kidney cells were isolated from kidneys donated for cadaveric transplantation but found unsuitable for such purpose because of anatomic or fibrotic defects. The initial results in the first 10 treated patients in this Phase I/II trial demonstrated that this experimental treatment can be delivered safely under study protocol guidelines in this critically ill patient population for up to 24 hours when used in conjunction with CVVH [31]. These data also indicate that the RAD maintains and exhibits viability, durability, and functionality in this *ex vivo* clinical setting. Cardiovascular stability of the patients was maintained, and increased native renal function, as determined by elevated urine outputs, temporally correlated with RAD treatment, a finding that requires additional study. The isolated and expanded human cells also demonstrated differentiated metabolic and endocrinologic activity in this *ex vivo* treatment. Glutathione degradation and endocrinologic conversion of 25-OH-D3 to 1,25-(OH)2-D3 by the RAD tubule cells were demonstrated.

All 10 patients were critically ill with ARF and multi-organ dysfunction syndrome, with predicted hospital mortality rates between 80% and 95%. One patient expired within 12 hours of RAD treatment because of his family's request to withdraw ventilatory life support. Another patient expired after a surgical catastrophe (toxic megacolon) required discontinuation of RAD treatment after only 12 hours. Of the remaining eight patients, six survived past 28 days with renal function recovery. The other two patients died from non-recoverable complications unrelated to RAD therapy and ARF, including fungal pericarditis and vancomycin-resistant enterococcus septicemia in one patient and ischemic colitis with bowel perforations in the other patient. Plasma cytokine levels suggest that RAD therapy produces dynamic and individualized responses in patients, depending on their unique pathophysiologic conditions. For the subset of patients who had excessive pro-inflammatory levels, RAD treatment resulted in significant declines in granulocyte-colony stimulating factor (G-CSF), IL-6, IL-10, and especially IL-6/IL-10 ratios, suggesting a greater decline in IL-6 relative to IL-10 levels and a lessened pro-inflammatory state. These results were encouraging and led to an FDA approved, randomized, controlled, open-label Phase II investigation, which was completed at 10 clinical sites to assess the safety and early efficacy of this cell therapy approach. The results were equally as compelling as the Phase I/II trial results.

Fifty-eight patients with ARF requiring continuous venovenous hemofiltration (CVVH) in the ICU were randomized (2:1) to receive CVVH + RAD ($n = 40$) or CVVH alone ($n = 18$). Despite the critical nature and life-threatening illnesses of the patients enrolled in this study, the addition of the RAD to CVVH resulted in a substantial clinical impact on survival compared with the conventional CVVH-treatment group. RAD treatment for up to 72 hours

promoted a statistically significant survival advantage over 180 days of follow-up in ICU patients with AKI and demonstrated an acceptable safety profile. Cox proportional hazards models suggested that the risk of death was approximately 50% of that observed in the CVVH-alone group [32]. A follow-up Phase IIb study to evaluate a commercial manufacturing process was not completed due to difficulties with the manufacturing process and clinical study design which led to a suspension of the clinical development of this approach [33].

Technological hurdles to commercialization of cell-based RRT

With the early clinical success of the RAD in RRT in AKI requiring dialytic therapy, two key missing components for the successful commercialization of cell-based therapeutic devices were identified. The first was the need for a reliable and consistent source of cells to manufacture thousands of these cell devices. The second was the requirement to develop a cost-effective manufacturing, storage and distribution process for these devices.

Cell sourcing

A key critical hurdle for any bioartificial organ program, including kidney replacement, is identifying a robust cell source for the replacement device. In this regard, a specific cell-based therapy requires several important methodologic choices and the solution of a number of technological problems. For cell-based therapies, large quantities of cells need to be expanded pathogen free, while maintaining uniformity in activity. Current approaches to ensure robust cell expansion and uniformity requirements are dependent on either stem/progenitor cells or transformed cells. The use of human embryonic stem (ES) cells versus adult stem cells is under rigorous societal debate, with the current political environment strongly favoring adult stem cell processes [34,35]. The plasticity of adult stem cells to transdifferentiate from one lineage pathway to another is also under careful scientific scrutiny. The early support for stem cell plasticity appears to be questioned by recent reports demonstrating stem cell fusion with tissue-specific differentiated cells, resulting in polyploidy rather than true stem cell transdifferentiation with normal diploid chromosomal numbers [36–38]. The ability of bone marrow stem cells to differentiate into a variety of cell types within the kidney, including glomerular and tubular elements, has been demonstrated [39]. These reports, however, demonstrate highly variable engraftment rates and inconsistent phenotypic differentiation. The issue of cell fusion in these experiments has not been addressed. Current cell-based approaches, therefore, are directed toward utilizing adult tissue-specific stem cell expansion, but the potential use of ES cells, as well as induced pluripotent stem cells (iPS) is also being aggressively pursued.

The utilization of transformed cells, including applications to deliver a gene product with gene therapy and iPS cells has recently come under intense scrutiny due to safety concerns. The autologous transplantation of genetically modified hematopoietic stem cells in children with adenosine deaminase deficiency, which leads to severe immunodeficiency, resulted in the development of acute leukemia in some of the patients due to genetic integration of the vector in the hematopoietic stem cells [40]. The ability to retrieve or deactivate these transformed cells following cell implantation is required to mitigate this high risk. Even the use of non-transformed cells may have safety concerns. Implantation of nerve cells in patients with Parkinson's disease leads to a high rate of severe and uncontrollable dyskinetic activity [41]; implantation of myoblasts into the heart has resulted in high rates of cardiac arrhythmias [42].

A choice between autologous and non-autologous human cells is also critical in the formulation of a cell-based application. Non-autologous cells must overcome natural host immunologic rejection processes. Since most indications preclude the use of immunosuppressant drugs to accommodate the discordant cell implant, immunoprotection of non-autologous cells has been approached with micro-encapsulation techniques using ultrathin

synthetic membranes to prevent entry of antibodies and immunocompetent cells of the host. Implantation of cellular micro-capsules has had limited success because of poor long term functional performance secondary to progressive loss of cell viability [43]. Success with short-term cell therapy utilizing hollow fiber bioreactors in an extracorporeal blood perfusion circuit for organ replacement therapy in acute disorders, including ATN, has been more forthcoming [21,29]. The use of autologous cells, although overcoming the immunologic barrier, has its own set of problems. Autologous approaches require obtaining the patient's own cells, expanding them *in vitro* in large quantities over several weeks, and then re-introducing the cells in a site-specific manner. Thus, each treatment is an individualized and non-scalable process, with substantial logistical and regulatory hurdles, including maintenance of the uniform quality of cells, avoidance of introduced pathogens during cell processing, and potential retrievability after implantation.

A final technologic hurdle for cell-based therapies is the maintenance of cell viability during long-term implantation. Maintenance of cell function depends on adequate nutrient and oxygen delivery to the cellular implant [44]. Creative approaches to induce and maintain formation of a neovascular capillary bed in and around the cell implant with various drug-delivery and cell scaffold formulations having been demonstrated experimentally but have not yet been successfully translated to the clinic [45]. An intravascular cell encapsulation implant approach looks promising, but requires further experimentation to determine its successful implementation [46].

Although cell-based therapy has substantial technological, regulatory, and ethical barriers, the potential to develop innovative treatments for a large number of clinical disorders, including acute and chronic renal diseases, is expanding rapidly. Progress in this field is highly dependent on an interdisciplinary approach at the interface of a number of scientific disciplines. This approach is at times empiric rather than reductive in nature, without full understanding of the manner in which cells may alter the complex pathophysiology of a systemic disorder. This limitation should not preclude continued efforts in this field. In fact, this empiric approach may result in unanticipated insights into basic biology, similar to those obtained in immunology as the field of human solid organ transplantation evolved. Cell therapy has the potential to creatively leverage nature's ability to provide new and much needed treatments to patients with acute and chronic diseases.

For renal tubule epithelial cells, cell sourcing issues from human sources has been overcome [47]. This recent development has demonstrated a dramatic increase in the yield of renal epithelial cells, exceeding 10^{16} cells/gm of kidney cortex from a healthy human source. This enhanced propagation methodology can easily produce over 100,000 devices per kidney and allow for autologous use of renal cells from a biopsy specimen from an individual.

Bioartificial renal epithelial cell system (BRECS): A cell therapy approach

The clinical efficacy of the cell-based, multifactorial approach of the RAD, which leveraged the metabolic and synthetic functions of stem/progenitor cells of the kidney directed toward a renal tubule cell fate is well established [30,48,49]. Cell sourcing and storage/distribution issues encountered during RAD manufacturing have now been addressed through the development of a new cell-based device called the bioartificial renal epithelial cell system. The BRECS is an example of a cell therapy device that maintains a high density of renal cells for therapeutic application within an extracorporeal circuit [50] (see Fig. 52.2). In comparison with other cell therapies involving the direct application or injection of stem cells into the body, the extracorporeal approach of the BRECS allows for cell therapy application while maintaining immunoisolation using a series of filters. Critical to the application of the BRECS for acute indications, the BRECS has been designed to enable cryopreservation of cells within the device in a therapeutically ready state, and the cells within the BRECS are from

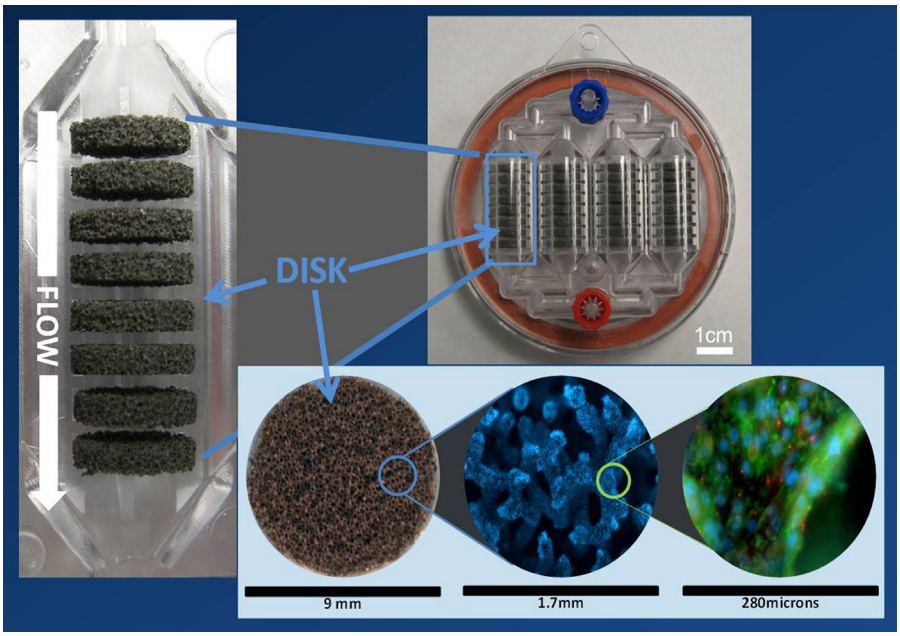

FIGURE 52.2
Previously cryopreserved human renal epithelial cells are reconstituted, followed by seeding onto disks which are then placed into BRECS culture. Post-culture, disks are counter-stained with DAPI (blue, 1.7 mm insert), zonula occludens (ZO)-1 (green, 280 micron insert) and AT-1 (red, 280 micron insert). The cryopreserved, reconstituted cells show the correct renal phenotype with positive ZO-1 and AT-1 staining, indicative of a polarized renal epithelium.

an allogenic source, which enables mass fabrication, cryopreservation for storage and distribution to meet on-demand clinical need.

In brief, the BRECS is a perfusion bioreactor that utilizes primary renal epithelial cells (REC) derived from the kidney, expanded from progenitor cells during *in vitro* culture. Cells are seeded on porous disks, which are placed within a media flow path within the BRECS. Renal progenitor cells are directed toward a renal tubule cell fate, and maintained in perfusion culture prior to therapeutic application. *In vitro* cell viability and metabolic activity were confirmed in the BRECS by measuring lactate production and oxygen consumption. Both metrics of metabolism were consistent throughout the duration of perfusion culture, with an estimated total cell number of over 10^8 cells [49]. Oxygen consumption rates in the nmol O_2/min per 10^6 cells range is similar to previously reported values for metabolically active cells [14].

REC in BRECS maintained renal differentiated phenotypic characteristics over time in perfusion culture, as evidence from human REC-seeded disks from BRECS units that were processed for immunohistochemical (IHC) analysis of selected renal cell differentiated markers, acetylated tubulin (AT-1) and zona occludens (ZO-1). AT-1, a marker for apical central cilia of proximal tubule cells, exhibited regular staining in central regions of cells grown on disks. ZO-1, a marker for epithelial tight junctions, displayed strong expression along the surface of the cells. ZO-1—positive tight junctions and punctate AT-1—positive central cilia are indicative of polarized epithelium and were evident in all disks tested [49].

Renal cell specific function of cells within BRECS has been tested using a non-destructive glutathione (GSH) degradation assay to assess one measure of catabolic function of the cells in the BRECS over time. Exogenous glutathione was supplemented in the BRECS perfusion media, and metabolic degradation of GSH was measured over a 60-minute period. GSH degradation rates in BRECS remained stable, ranging between 600 and 1200 nmoles/hr/BRECS over a 90 day period, indicative of sustained differentiated renal cell function in the device [47].

Cryopreservation and thaw of REC in BRECS was accomplished using a commercially available and FDA approved cryopreservation media. Post-cryopreservation cell retention was optimized when HTS-Purge solution, a hypothermic solution used to prepare cells for the extreme conditions of cryopreservation, was utilized as a pre-cryopreservation rinse buffer, in

combination with a controlled rate freezer, resulting in average cell retention of greater than 80% and viability of greater than 90% [47]. This technology allows for the storage and distribution of cell therapy for on-demand use for acute indications such as sepsis and acute renal failure.

Wearable bioartificial kidney (WEBAK)

A bioartificial kidney for long-term use in ESRD, similar to short-term use in ARF, would integrate tubular cell therapy and the filtration function of a hemofilter. As noted above, ESRD patients on conventional renal replacement therapy are at high risk for cardiovascular and infectious diseases. A recent clinical trial failed to show survival benefit from increased doses of hemodialysis above what is now standard care [51], suggesting that there are important metabolic derangements not adequately treated with conventional dialytic treatment. Data from the survival of renal transplant recipients, which far exceed those from the survival of age-, sex-, and risk-matched controls awaiting transplant, also suggest that there is some metabolic function provided by the kidney that transcends this organ's filtration function. Patients with ESRD display elevated levels of C-reactive protein (CRP), an emerging clinical marker, and pro-inflammatory cytokines, including IL-1, IL-6, and tumor necrosis factor alpha (TNFα) [52–54]. All these parameters are associated with enhanced mortality in ESRD patients. Specifically, IL-6 has been identified as a single predictive factor closely correlated with mortality in hemodialysis patients [52]. Although all ESRD patients could conceivably benefit from a bioartificial kidney, patients in the inflammatory stage who display elevated levels of certain markers of chronic inflammation (most notably IL-6 and CRP) would likely benefit most and will be the target population for clinical study in the near future.

For the ESRD patient population, however, there are obvious limitations in using an extracorporeal RAD connected to a hemofiltration circuit. Ideally, a bioartificial kidney suitable for long term use in ESRD patients would be capable of performing continuously, like the native kidney, to reduce risks from fluctuations in volume status, electrolytes, and solute concentrations and to maintain acid-base and uremic toxin regulation. Such treatment requires the design and manufacture of a compact implantable or wearable dialysis apparatus and the development of miniaturized renal tubule cell devices with long service lifetimes. The ideal design of the next-generation RAD would be like that of an implantable device similar to the pacemaker.

As an intermediary approach to a fully implantable BAK, a WEBAK formulation has been recently evaluated in both preclinical large animal models and in early clinical studies. The WEBAK is comprised of the use of sorbent based technologies to replace the excretory function of the kidney and the compact BRECS device described above to replace the metabolic function of the kidney [55]. This approach is displayed in Fig. 52.3.

Sorbent-based hemodialysis was developed in the late 1960s and introduced into the clinic in the 1970s. Sorbents provide the ability to reduce the large volumes of dialysis solutions utilizing highly purified water from 100–200 liters per dialysis sessions to as little as 6 liters of potable water. With sorbent dialysis, spent dialysis from the dialyzer cartridge is not discarded but is regenerated by processing the dialysate through the sorbent cartridge.

The sorbent cartridge has several layers of sorbent compounds which regenerate used dialysate into fresh, bicarbonate dialysate. The cartridge is based upon carbon binding, enzyme conversion and ion exchange. This compact and disposable regeneration process promotes this sorbent system to remove key uremic toxins and regenerate dialysate. Sorbent cartridges operate without a connection to a water supply or drain, promoting system mobility, portability and measurable RRT.

Attempts have been made to develop wearable dialysis systems to improve the portability of renal replacement therapies. Gura, Beizai, Ezon, and Polaschegg [56] have published research

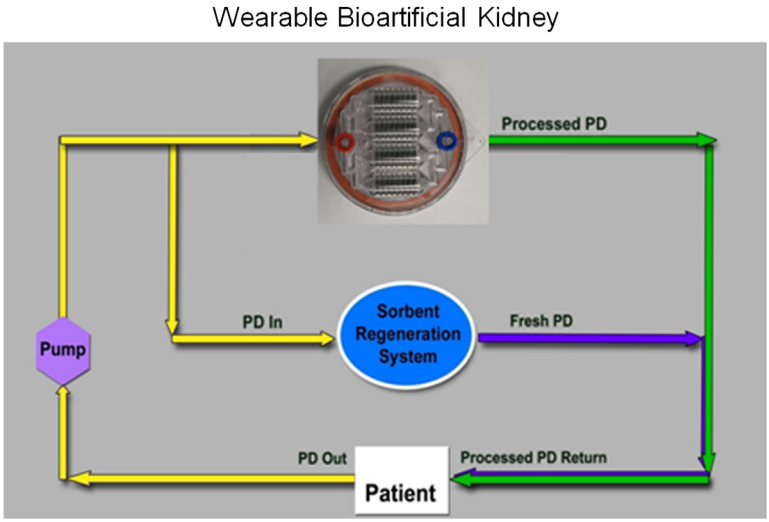

FIGURE 52.3
A circuit design for a WEBAK utilizing peritoneal fluid to maintain cell viability and functionality in the BRECS. Sorbent-based technology is used to regenerate peritoneal dialysis fluid for uremic toxin removal and fluid/electrolyte balance.

into a light-weight, wearable, continuous ambulatory ultrafiltration device consisting of a hollow fiber hemofilter, a battery-operated pulsatile pump, and two micropumps to control heparin administration and ultrafiltration. This device regenerates dialysate with activated carbon, immobilized urease, zirconium hydroxide, and zirconium phosphate, similar to the once commercially available regenerative dialysis (REDY) dialysis system. This approach requires continuous blood access to allow adequate ultrafiltration. Ronco and Fecondini [57] have described a wearable continuous PD system consisting of a double lumen dialysate line with a peritoneal catheter, a miniaturized rotary pump, a circuit for dialysate regeneration, and a handheld computer as a remote control. This latter approach utilizes continuous regeneration of PD fluid relying on continuous-flow PD systems [58–60].

As development towards a sorbent-based, wearable, continuous recycling flow PD is being made, the integration of renal epithelial cell therapy in the PD circuit to treat patients with ESRD is also being evaluated in large animal models of uremia.

Because a cell therapy device requires a continuous source of nutrients and oxygen, the use of blood circuits for this nutrient stream has been avoided due to clotting and infection risks. Accordingly, the use of recycling peritoneal fluid to maintain viability of a renal cell device has been conceived and successfully tested in a uremic sheep model. Further development of this approach of a WEBAK integrating the wearable sorbent dialysis system and renal cell device with recycling peritoneal fluid was dependent upon technologic improvements of human cell sourcing and compact cell device fabrication and storage. These technologic obstacles have been solved as detailed above. In this regard, a major limitation of the widespread adoption of renal cell therapy is the lack of a cryopreservable system to enable distribution, storage, and therapeutic use at point-of-care facilities. Building from the success of the initial renal tubule-assist (RAD), development of the BRECS allows for a device that functions as a combined bioreactor, cryostorage device, and cell therapy delivery system. *Ex vivo* large animal studies suggest using the BRECS in conjunction with standard hemofiltration or PD is a promising approach to treat both ARF and ESRD.

Implanted artificial kidney: successes and barriers

An implantable 'biohybrid' or bioartificial device has the potential to avoid both supply limitations to renal transplant and the burden of therapy of intensive maintenance dialysis [12]. The innovative combination of an implanted hemofilter and a bioreactor of renal cells would provide continuous small solute clearance along with metabolic functions of the proximal tubule, without the morbidity and burden of catheters and dialysis (see Fig. 52.4).

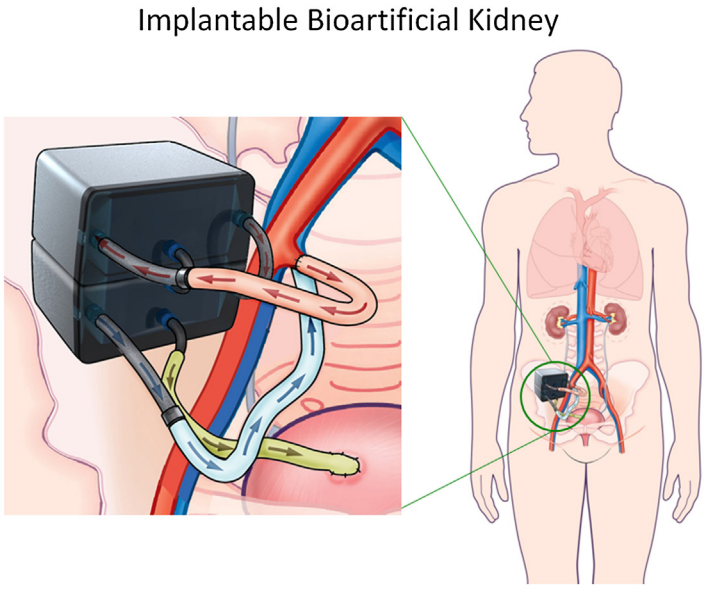

FIGURE 52.4
Concept illustration of implantable bioartificial kidney. The device consists of a silicon hemofilter for toxin filtration and a bioreactor of renal epithelial cells for metabolic and endocrine functions. *(Courtesy of Shuvo Roy.)*

Two factors limit miniaturization and implant of the hemodialysis circuit: the size and pump requirements of modern dialyzers, and the water volume required for dialytic therapy. Hollow fiber polymer membranes have been immensely successful in treating renal failure with extracorporeal therapies and are a lifesaving medical innovation. Hollow fiber dialyzers require super-physiologic driving pressures for blood circulation through the cartridge. The long cylindrical hollow fibers present a high resistance to blood flow, which rises further in the distal portion of the fiber as ultrafiltration increases hemocrit and viscosity, requiring energy-hungry roller pumps to circulate blood through the device. The pores in hollow fiber dialyzers are irregular, approximately cylindrical, and non-uniform in size, typically described as having a lognormal pore size distribution [61]. This polydispersity compromises the trade-off between permeability and selectivity: to prevent albumin leakage through the largest pores in the membrane, the majority of the membrane's pores need to be maintained much smaller than the desired cutoff target of the membrane. Similarly, a four hour dialysis treatment with a dialysate flow of 600mL/min consumes 144 liters of dialysate, which may have required an additional 200 liters of tap water to produce. Dialysate regeneration schemes are an attractive approach to limit water consumption, but require removal and replacement of sorbent cartridges, which precludes an implantable therapeutic strategy [62–64].

Control of pores enables high efficiency filtration

The natural filtration-reabsorption process of renal solute clearance as a model for a new approach to RRT includes the examination of the structure of the glomerular filter for clues to its performance. The kidney's filters appear to be uniform, elongated, slit-shaped structures, rather than the irregular and more cylindrically-shaped pores of polymer membranes. Indeed, nature appears to have evolved slit-shaped filters in a wide range of organisms, from the baleen palates of filter-feeding whales to the beaks of filter-feeding birds, such as shoveler ducks and flamingos. This particular geometry of uniform slits appears to optimize the permeability-selectivity trade-off [65]. The glomerular visceral epithelial cell or podocyte, which appears to be responsible for maintenance of the glomerular filtration barrier, does not assume the same differentiated phenotype in cell culture as it does *in vivo*, limiting its use so far in bioengineering of renal replacement. Thus, we turned to an entirely new technology toolkit, silicon nanotechnology, to develop a membrane incorporating these advantages. Micro-electromechanical systems (MEMS) is a toolkit that applies industrially mature manufacturing techniques from the semiconductor industry to produce precise miniature electromechanical

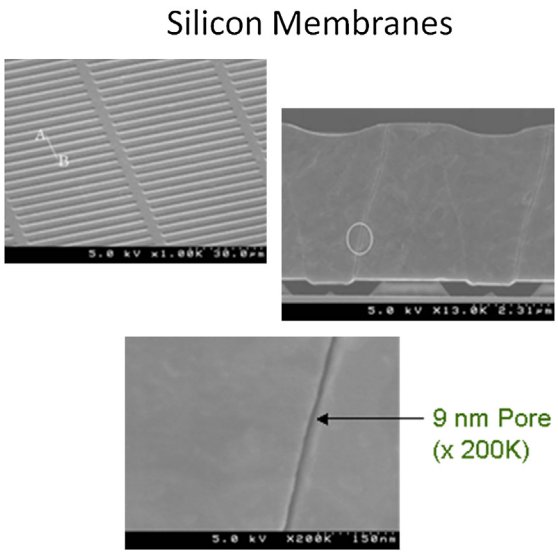

FIGURE 52.5
SEM images of silicon membranes with minimal pore size variation. These membranes can be coated with biocompatible thin films to provide highly selective filtrations of blood at very low driving pressures. *(Courtesy of Shuvo Roy.)*

devices such as pumps, valves, and sensors at low unit cost. Common commercial applications of MEMS include accelerometers for airbag sensors and inertial navigation, pressure sensors for medical and industrial use, and micro-mirror arrays for image projectors. MEMS technology can be used for the production of silicon nanopore membranes with slit-shaped pores that are tailored for implementation in a bioartificial kidney.

The fundamental membrane engineering challenge for the implantable artificial kidney is to simultaneously maximize water permeability while minimizing leakage of albumin and other important macromolecules. Prototyped ultrathin biomimetic silicone nanopore membranes using bulk and surface micromachining techniques derived from MEMS fabrication technology (see Fig. 52.5) and examined macromolecular transport from both analytical and experimental perspectives have been developed and reported [65–67].

Membranes with pore sizes from 5–90 nm were produced and perfused with solutions of a polydisperse tracer (Ficoll) and albumin to characterize their transport characteristics. Silicon nanopore membranes showed a distinct and predictable size-dependant and charge-dependent reduction in sieving coefficient [68]. Another set of experiments measured albumin transmission through membranes with different pore sizes to map the relationship between pore size and albumin retention. The observed sieving coefficients matched a combined electrical/steric hindrance model. Further, an analysis of the trade-off between selectivity (ability to reject albumin) and permeability (ability to transmit water) suggests that a membrane of slit-shaped pores outperforms a comparable membrane of cylindrical pores [65]. Data from the silicone nanopore membranes, when compared to data from polymer membranes, confirms that this theoretical advantage is experimentally observed as well (see Fig. 52.6).

Blood-material interactions can be controlled using hydrated polymer surface modifications

Silicon readily forms a thin oxide coating upon exposure to atmospheric oxygen. This silica film is negatively charged at physiologic pH and both absorbs plasma proteins and activates the coagulation cascade. One strategy to mitigate adverse blood-materials interactions is to modify the silicon surface with a highly hydrated polymer. Various organic polymers have been grafted to silicon surfaces and have been successful in demonstrating the feasibility of using silicon nanopore membranes coated with antifouling films for hemofiltration [69].

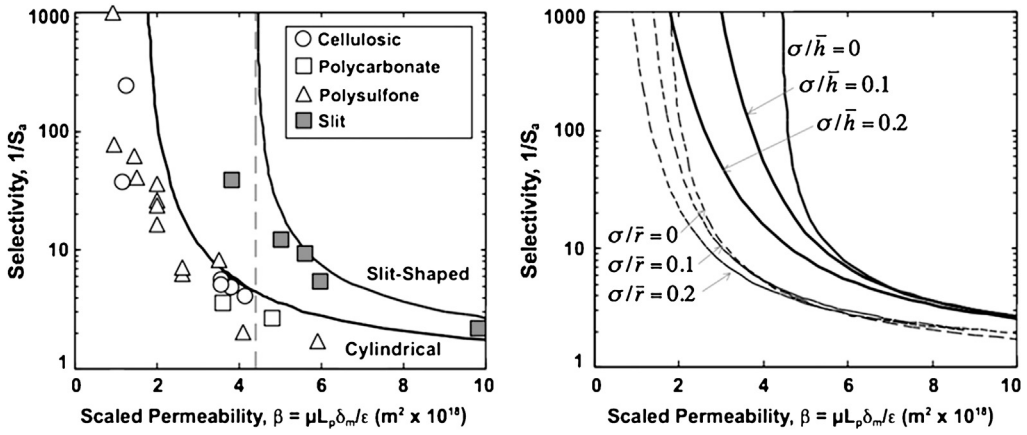

FIGURE 52.6

1. Selectivity-permeability trade-off for ultrafiltration membranes using albumin as the model protein. Open symbols represent data for polymer ultrafiltration membranes. Filled symbols represent data for novel silicon membranes with nanometer-size slit-shaped pores as discussed in the text. The data point shown at $\beta = 9.8\times10^{-18}$ m^2 is actually at $\beta = 20\times10^{-18}$ m^2 but has been moved to accommodate the scaling of the figure. Solid curves represent model calculations for membranes consisting of a parallel array of uniform cylindrical or slit-shaped pores. The dashed vertical line is the permeability of a slit-pore membrane with infinite selectivity. 2. Model calculations showing the influence of a membrane pore size distribution on the trade-off between selectivity and permeability for membranes with cylindrical and slit-shaped pores.

Next steps in implementation of the implantable artificial kidney

Significant preliminary data have been published documenting each of the three platform technologies necessary for an implantable bioartificial device that can provide enough small solute clearance to allow the patient with failing kidneys to avoid dialysis. Significant engineering challenges remain, but these challenges are not new to bioengineering. First and foremost, just as hemodialysis in the treatment of renal failure is more than the hollow fiber dialyzer alone, the design of the implanted device will have to be guided by the planned therapeutic strategy. Specific means for patients to self-monitor and reprogram device function will be critical for truly independent self-care using an implanted device. Lifecycle management of the device, including recognition of impending device failure and a minimally invasive approach to renewing or replacing failed components, modules, or cartridges seems essential. Selection of implant site will be guided by the paramount need to preserve vascular sites for future allografts. These challenges can be met and answered, just as they have been for other implanted devices, from corneas to ventricular assist devices. The remaining work to be completed is more than justified by the overwhelming burden of illness faced by patients with renal failure, the financial burden placed on payers for dialysis, and the revolutionary innovations already achieved in silicon nanopore membrane technology, polymer chemistry, and cell biology.

CONCLUSION

Despite all the advances in renal replacement therapies, a portable, continuous, dialysate-free artificial kidney remains the holy grail of renal tissue engineering. The enabling platform technologies discussed in this review advance this goal from a dream to the laboratory bench and even to the bedside. Future research in renal tissue engineering will need to focus on reproducing mechanisms of whole-body homeostasis. A high priority must be given to sensing and regulating extracellular fluid volume, even if only at the crude level of having the patient weigh him- or herself daily and adjust ultrafiltration and reabsorption by the bioartificial kidney. Chemical-field effect transistors (ChemFETs) offer the possibility of measuring electrolyte levels in a protein-free ultrafiltrate and reading out the potassium level to the patient, who could then alter diet or treat him- or herself with potassium absorbing resins.

The critical building blocks of an autonomous bioartificial kidney are advancing rapidly with revolutionary clinical trials currently underway at multiple medical centers. The technology with which to adapt these advances to a more autonomous, dialysate-free system is under development. In addition, progress has been made in the field of cryopreservation and thus the ability to manufacture, store and distribute bioartificial organs is advancing. The next decade, like the previous, will likely see quantum advances in renal tissue engineering.

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CHAPTER 53

Genitourinary System

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INTRODUCTION

The genitourinary system may be exposed to a variety of possible injuries from the time the fetus develops. Individuals may suffer from congenital disorders, cancer, trauma, infection, inflammation, iatrogenic injuries, or other conditions that may lead to genitourinary organ damage or loss and necessitate eventual reconstruction. Whenever there is a lack of native urologic tissue, reconstruction may be performed with native non-urologic tissues (skin, gastrointestinal segments, or mucosa from multiple body sites), homologous tissues (cadaver fascia, cadaver or donor kidney), heterologous tissues (bovine collagen), or artificial materials (silicone, polyurethane, Teflon). The tissues used for reconstruction may lead to complications because of their inherently different functional parameters. In most cases, the replacement of lost or deficient tissues with functionally equivalent tissues would improve the outcome for these patients. This goal may be attainable with the use of tissue-engineering techniques.

RECONSTITUTION STRATEGIES

The goal of tissue engineering is to develop biologic substitutes that can restore and maintain normal function. Tissue engineering may involve matrices alone, wherein the body's natural ability to regenerate is used to orient or direct new tissue growth, or it may use matrices with cells. When cells are used for tissue engineering, donor tissue (heterologous, allogenic, or autologous) is dissociated into individual cells, which are implanted directly into the host or expanded in culture, attached to a support matrix, and reimplanted after expansion. Ideally, this approach allows lost tissue function to be restored or replaced *in toto* and with limited complications [1–13].

THE ROLE OF BIOMATERIALS

Biomaterials in genitourinary tissue engineering function as an artificial extracellular matrix (ECM) and elicit biologic and mechanical functions of native ECM found in body tissues. Biomaterials facilitate the localization and delivery of cells and/or bioactive factors (such as cell adhesion peptides and growth factors) to desired sites in the body; define a three-dimensional space for the formation of new tissues with appropriate structure; and guide the development of new tissues with appropriate function [14]. While direct injection of cell suspensions without biomaterial matrices has been used [15], it is difficult to control the localization of transplanted cells.

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00053-7

The ideal biomaterial should be biocompatible, promote cellular interaction and tissue development, and possess proper mechanical and physical properties. Generally, three classes of biomaterials have been used for engineering of genitourinary tissues: naturally derived materials, such as collagen and alginate; acellular tissue matrices, such as bladder submucosa and small-intestinal submucosa; and synthetic polymers, such as polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA). While naturally derived materials and acellular tissue matrices have the potential advantage of biologic recognition, synthetic polymers can be produced reproducibly on a large scale with controlled properties of strength, degradation rate, and microstructure.

VASCULARIZATION

A restriction of tissue engineering is that cells cannot be implanted in volumes exceeding 3 mm^3 because of the limitations of nutrition and gas exchange [16]. To achieve the goals of engineering large complex tissues, and possibly internal organs, vascularization of the regenerating cells is essential. Three approaches have been used for vascularization of bioengineered tissue:

1) Incorporation of angiogenic factors in the bioengineered tissue;
2) Seeding EC with other cell types in the bioengineered tissue; and
3) Prevascularization of the matrix prior to cell seeding.

Angiogenic growth factors may be incorporated into the bioengineered tissue prior to implantation, in order to attract host capillaries and to enhance neovascularization of the implanted tissue. Many obstacles must be overcome before large entire tissue-engineered solid organs are produced. Recent developments in angiogenesis research may provide important knowledge and essential materials to accomplish this goal.

PROGRESS IN TISSUE ENGINEERING OF UROLOGIC STRUCTURES

Tissue-engineering techniques are currently being investigated for the replacement of lost or deficient genitourinary structures, including urethra, bladder, male and female genital tissues, ureter, and renal structures.

Urethra

Various strategies have been proposed to regenerate urethral tissue. Woven meshes of PGA, without cells, have been used to reconstruct urethras in dogs [17]. PGA has been used as a cell transplantation vehicle to engineer tubular urothelium *in vivo*. Small-intestinal submucosa (SIS) without cells was used as an onlay patch graft for urethroplasty in rabbits [18], and a homologous free graft of acellular urethral matrix was also used in a rabbit model [19].

Bladder-derived acellular collagen matrix has proven to be a suitable graft for repairing urethral defects in rabbits. The created neourethras demonstrated a normal urothelial luminal lining and organized muscle bundles [20]. Results were confirmed clinically in a series of patients with a history of failed hypospadias reconstruction whose urethral defects were repaired with human bladder acellular collagen matrices [21] (Fig. 53.1). An advantage of this material over non-genital tissue grafts for urethroplasty is that it is 'off the shelf', eliminating the need for additional surgical procedures for graft harvesting and decreasing operative time and potential morbidity from the harvest procedure.

The above techniques, using non-seeded acellular matrices, were successfully applied experimentally and clinically for onlay urethral repairs. However, when tubularized repairs were attempted experimentally, adequate urethral tissue regeneration was not achieved, and complications, such as graft contracture and stricture formation ensued [22]. Seeded tubularized collagen matrices have performed better than their non-seeded counterparts in animal

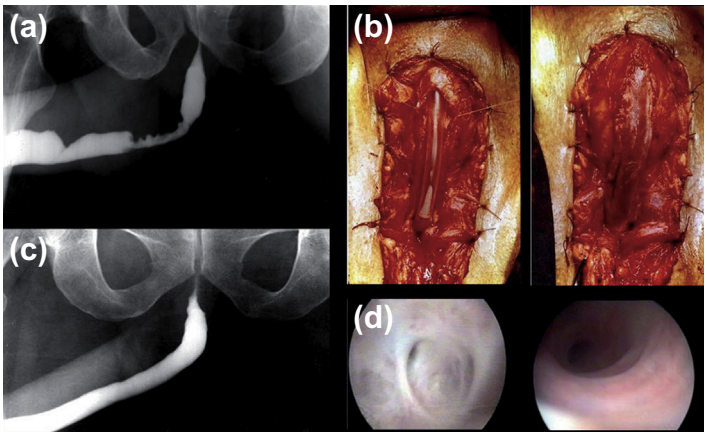

FIGURE 53.1
Tissue-engineered urethra using a collagen matrix. (a) Representative case of a patient with a bulbar stricture. (b) Urethral repair. Strictured tissue is excised, preserving the urethral plate on the left side, and matrix is anastomosed to the urethral plate in an onlay fashion on the right. (c) Urethrogram six months after repair. (d) Cystoscopic view of urethra before surgery on the left side and four months after repair on the right side.

studies. In a rabbit model, entire urethral segments were resected, and urethroplasties were performed with tubularized collagen matrices, either seeded or non-seeded. The tubularized collagen matrices seeded with autologous cells formed new tissue which was histologically similar to native urethra. Those without cells lead to poor tissue development, fibrosis, and stricture formation.

Most recently, we were able to show that synthetic biomaterials can also be used in urethral reconstruction when they are tabularized and seeded with autologous cells [23]. This group used polygycolic acid:poly(lactide-co-glycolic acid) scaffolds seeded with autologous cells derived from bladder biopsies taken from each patient. The seeded scaffolds were then used to repair urethral defects in five boys. Upon follow-up, it was found that most of the boys had excellent urinary flow rates post-operatively, and voiding cystourethrograms indicated that these patients maintained wide urethral calibers. Urethral biopsies revealed that the grafts had developed a normal appearing architecture consisting of urothelial and muscular tissue.

Bladder

Gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, these tissues are designed to absorb specific solutes, and when they come in contact with the urinary tract, multiple complications may ensue, including infection, metabolic disturbances, urolithiasis, perforation, increased mucus production, and malignancy [24]. Because of these problems, investigators have attempted alternative reconstructive procedures for bladder replacement or repair such as the use of tissue expansion, seromuscular grafts, matrices for tissue regeneration, and tissue engineering with cell transplantation.

TISSUE EXPANSION

A system of progressive dilation for ureters and bladders has been proposed as a method of bladder augmentation but has not yet been attempted clinically [25,26]. Augmentation cystoplasty performed with the dilated ureteral segment in animals has resulted in increased bladder capacity ranging from 190% to 380% [25]. A system to progressively expand native bladder tissue has also been used for augmenting bladder volumes in animals. Within 30 days after progressive dilation, neoreservoir volume was expanded at least 10-fold. Urodynamic studies showed normal compliance in all animals, and microscopic examination of the

expanded neoreservoir tissue confirmed a normal histology. A series of immunocytochemical studies demonstrated that the dilated bladder tissue maintained normal phenotypic characteristics [26].

SEROMUSCULAR GRAFTS

Seromuscular grafts and de-epithelialized bowel segments, either alone or over a native urothelium, have also been attempted [27,28]. Keeping the urothelium intact avoids complications associated with the use of bowel in continuity with the urinary tract [27,28]. An example of this strategy is to combine the techniques of autoaugmentation and enterocystoplasty. An autoaugmentation is performed, and the diverticulum is covered with a demucosalized gastric or intestinal segment.

MATRICES

Non-seeded allogeneic acellular bladder matrices have served as scaffolds for the ingrowth of host bladder wall components. The matrices are prepared by mechanically and chemically removing all cellular components from bladder tissue [29,30]. The matrices serve as vehicles for partial bladder regeneration, and relevant antigenicity is not evident. For example, SIS (a biodegradable, acellular, xenogeneic collagen-based tissue-matrix graft) was first used in the early 1980s as an acellular matrix for tissue replacement in the vascular field. It has been shown to promote regeneration of a variety of host tissues, including blood vessels and ligaments [31]. Animal studies have shown that the non-seeded SIS matrix used for bladder augmentation can regenerate *in vivo* [32].

In multiple studies using various materials as non-seeded grafts for cystoplasty, the urothelial layer regenerated normally, but the muscle layer, although present, was not fully developed [29,30]. Often the grafts contracted to 60–70% of their original sizes [33] with little increase in bladder capacity or compliance [34].

Studies involving acellular matrices that may provide the necessary environment to promote cell migration, growth, and differentiation are being conducted. With continued research, these matrices may have a clinical role in bladder replacement in the future. Recently, bladder regeneration has been shown to be more reliable using SIS derived from the distal ileum [32].

CELL TRANSPLANTATION

Cell-seeded allogeneic acellular bladder matrices have been used for bladder augmentation in dogs. Trigone-sparing cystectomy was performed in dogs randomly assigned to one of three groups. One group underwent closure of the trigone without a reconstructive procedure; another underwent reconstruction with a non-seeded bladder-shaped biodegradable scaffold; and the last underwent reconstruction using a bladder-shaped biodegradable scaffold that delivered seeded autologous urothelial cells and smooth muscle cells [12].

The cystectomy-only and non-seeded controls maintained average bladder capacities of 22% and 46% of preoperative values, respectively, compared with 95% in the cell-seeded tissue-engineered bladder replacements (Fig. 53.2). The subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42% total compliance). The compliance of the cell-seeded tissue-engineered bladders showed almost no difference from preoperative values that were measured when the native bladder was present (106%). Histologically, the non-seeded scaffold bladders presented a pattern of normal urothelial cells with a thickened fibrotic submucosa and a thin layer of muscle fibers (Fig. 53.2b). The retrieved tissue-engineered bladders showed a normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle (Fig. 53.2c) [12]. Preliminary clinical trials for the application of this technology have been performed and are under evaluation.

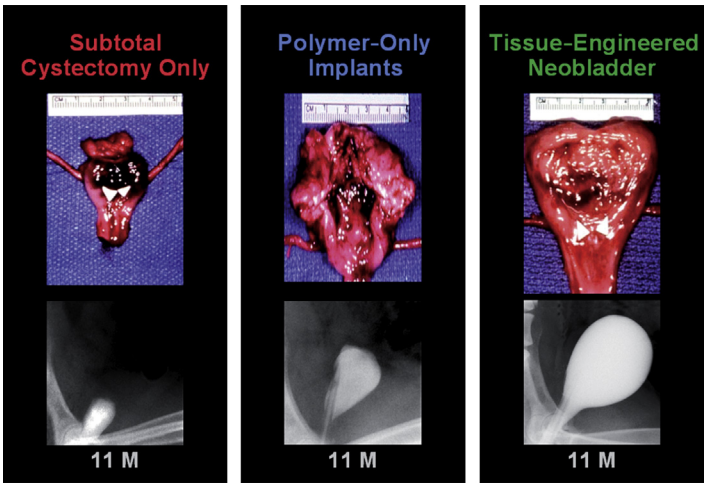

FIGURE 53.2
Comparison of tissue-engineered neobladders. Gross specimens and cystograms at 11 months of the cystectomy-only, non-seeded controls, and cell-seeded tissue-engineered bladder replacements. The cystectomy-only bladder had a capacity of 22% of the preoperative value and a decrease in bladder compliance to 10% of the preoperative value. The non-seeded controls showed significant scarring, with a capacity of 46% of the preoperative value and a decrease in bladder compliance to 42% of the preoperative value. An average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded tissue-engineered bladder replacements, and the compliance showed almost no difference from preoperative values, which were measured when the native bladder was present (106%).

Genital tissues

Tissue engineering techniques have been used to reconstruct male and female genital tissues.

CORPORAL SMOOTH MUSCLE

Because one of the major components of the phallus is corporal smooth muscle, the creation of autologous functional and structural corporal tissue *de novo* would be beneficial. To examine functional parameters of engineered corpora, acellular corporal collagen matrices were obtained from donor rabbit penis, and autologous corpus cavernosal smooth muscle and endothelial cells were harvested, expanded, and seeded on the matrices. The entire rabbit corpora was removed and replaced with engineered scaffolds. The experimental corporal bodies demonstrated intact structural integrity by cavernosography and showed similar pressure by cavernosometry when compared with normal controls. The control rabbits (without cells) failed to show normal erectile function throughout the study. Mating activity in the animals with the engineered corpora appeared normal by 1 month after implantation. The presence of sperm was confirmed during mating and was present in all the rabbits with the engineered corpora. The female rabbits mated with the animals implanted with engineered corpora and also conceived and delivered healthy pups. Animals implanted with the matrix alone were unable to demonstrate normal mating activity and failed to ejaculate into the vagina [35].

ENGINEERED PENILE PROSTHESES

Although silicone is an accepted biomaterial for penile prostheses, biocompatibility remains a concern [36]. The use of a natural prosthesis composed of autologous cells may be advantageous.

A recent study using an autologous system investigated the feasibility of applying the engineered cartilage rods *in situ* [37]. Autologous chondrocytes harvested from rabbit ear were grown and expanded in culture. The cells were seeded onto biodegradable poly-L-lactic acid-coated PGA polymer rods and implanted into the corporal spaces of rabbits. Examination

at retrieval 1 month later showed the presence of well-formed, milky-white cartilage structures within the corpora. All polymers were fully degraded by two months. There was no evidence of erosion or infection in any of the implantation sites.

Subsequent studies assessed the long-term functionality of the cartilage penile rods *in vivo* [37]. To date, the animals have done well and can copulate and impregnate their female partners without problems.

FEMALE GENITAL TISSUES

Congenital malformations of the uterus may have profound implications clinically. Patients with cloacal exstrophy or intersex disorders may not have sufficient uterine tissue for future reproduction.

We investigated the possibility of engineering functional uterine tissue using autologous cells [38]. Autologous rabbit uterine smooth muscle and epithelial cells were harvested, then grown and expanded in culture. These cells were seeded onto pre-configured uterine-shaped biodegradable polymer scaffolds, which were then used for subtotal uterine tissue replacement in the corresponding autologous animals. Upon retrieval six months after implantation, histological, immunocytochemical, and Western blot analyses confirmed the presence of normal uterine tissue components. Biomechanical analyses and organ bath studies showed that the functional characteristics of these tissues were similar to those of normal uterine tissue. Breeding studies using these engineered uteri are currently being performed.

Several pathologic conditions, including congenital malformations and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging due to the paucity of available native tissue.

Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown, and expanded in culture. The cells were seeded onto biodegradable polymer scaffolds, which were then implanted into nude mice for up to six weeks. Immunocytochemical, histological, and Western blot analyses confirmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue-engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, while the non-seeded structures were noted to be stenotic.

Ureter

Ureteral non-seeded matrices have been used as a scaffold for the ingrowth of ureteral tissue in rats. On implantation, the acellular matrices promoted the regeneration of the ureteral wall components [39]. In a more recent study, non-seeded ureteral collagen acellular matrices were tabularized, but attempts to use them to replace 3 cm segments of canine ureters were unsuccessful [40].

Cell-seeded biodegradable polymer scaffolds have been used with more success to reconstruct ureteral tissues. In one study, urothelial and smooth muscle cells isolated from bladders and expanded *in vitro* were seeded onto polyglycolic acid (PGA) scaffolds with tubular configurations and implanted subcutaneously into athymic mice. After implantation, the urothelial cells proliferated to form a multilayered luminal lining of tubular structures, while the smooth muscle cells organized into multilayered structures surrounding the urothelial cells. Abundant angiogenesis was evident. Polymer scaffold degradation resulted in the eventual formation of natural urothelial tissues. This approach has also been used to replace ureters in dogs [41].

Renal structures

Due to its complex structure and function, the kidney is possibly the most challenging organ in the genitourinary system to reconstruct using tissue-engineering techniques. However concepts for a bioartificial kidney are emerging. Some investigators are pursuing the replacement of isolated kidney function parameters with the use of extracorporeal units, while others are working toward the replacement of total renal function by tissue-engineered bioartificial structures.

EX VIVO RENAL UNITS

Although dialysis is currently the most prevalent form of renal replacement therapy, the relatively high rates of morbidity and mortality have spurred investigators to seek alternative solutions involving *ex vivo* systems.

To assess the viability and physiologic functionality of a cell-seeded device to replace the filtration, transport, metabolic, and endocrinologic functions of the kidney in acutely uremic dogs, researchers introduced a synthetic hemofiltration device combined with a renal tubular cell therapy device (containing porcine renal tubules in an extracorporeal perfusion circuit). Levels of potassium and blood urea nitrogen (BUN) were controlled during treatment with the device. The fractional reabsorption of sodium and water was possible, and active transport of potassium, bicarbonate, and glucose and a gradual ability to excrete ammonia was observed. These results demonstrated the technologic feasibility of an extracorporeal assist device that is reinforced by the use of proximal tubular cells [42].

Using similar techniques, a tissue-engineered bioartificial kidney — consisting of a conventional hemofiltration cartridge in series with a renal tubule assist device containing human renal proximal tubule cells — was used in patients with acute renal failure in the intensive care unit. Initial clinical experience with the bioartificial kidney and the renal tubule assist device suggests that such therapy may provide a dynamic and individualized treatment program as assessed by acute physiologic and biochemical indices [43].

IN VIVO RENAL STRUCTURES

Another method of improving renal function involves augmenting renal tissue with kidney cell expansion *in vitro* and subsequent autologous transplantation. The feasibility of achieving renal cell growth, expansion, and *in vivo* reconstitution with tissue-engineering techniques has been explored.

Most recently, an attempt was made to harness the reconstitution of renal epithelial cells to generate functional nephron units. Renal cells harvested and expanded in culture were seeded onto a tubular device constructed from a polycarbonate membrane connected at one end to a Silastic catheter terminating into a reservoir. The device was implanted into athymic mice. Histological examination of the implanted devices over time revealed extensive vascularization, with formation of glomeruli and highly organized tubule-like structures. Immunocytochemical staining confirmed the renal phenotype. Yellow fluid consistent with the makeup of dilute urine in its creatinine and uric acid concentrations was retrieved from inside the implant [9]. Further studies using nuclear transfer techniques have been performed showing the formation of renal structures in cows (Fig. 53.3) [44]. Challenges facing this technology include the expansion to larger, three-dimensional structures.

ADDITIONAL APPLICATIONS

Tissue engineering and cell therapy hold promise for a number of additional genitourinary applications.

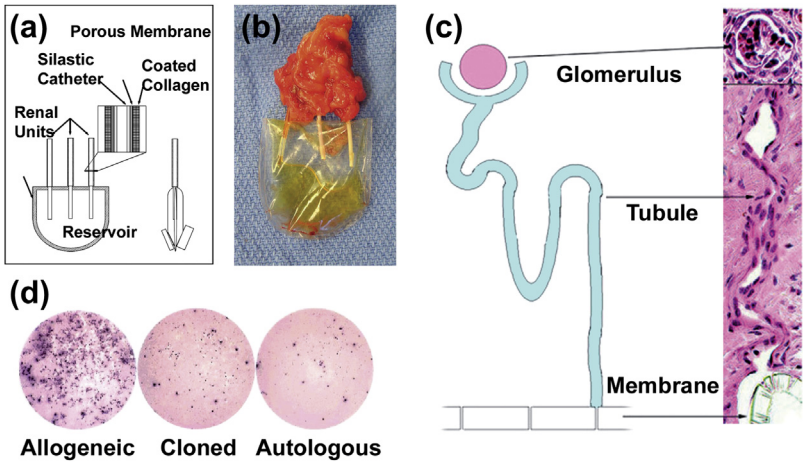

FIGURE 53.3

Creation of kidney tissue from therapeutic cloning and tissue-engineering strategies. (a) Illustration of the tissue-engineered renal unit. (b) Renal unit seeded with cloned cells, three months after implantation, showing the accumulation of urinelike fluid. (c) There was a clear unidirectional continuity between the mature glomeruli, their tubules, and the polycarbonate membrane. (d) Elispot analyses of the frequencies of T-cells that secrete IFN-gamma after primary and secondary stimulation with allogeneic renal cells, cloned renal cells, or nuclear donor fibroblasts.

Fetal tissue engineering

Improved prenatal diagnostic techniques have led to the use of intervention before birth to reverse potentially life-threatening processes. Several strategies may be pursued to facilitate the future prenatal management of urologic disease. Having a ready supply of urologic-associated tissue for surgical reconstruction at birth may be advantageous.

Theoretically, once the diagnosis of the pathologic condition is confirmed prenatally, a small tissue biopsy could be obtained under US guidance. These biopsy materials could then be processed and the various cell types expanded *in vitro*. Using tissue-engineering techniques, reconstituted structures *in vitro* could then be readily available at the time of birth for reconstruction [45].

Injectable therapies

Both urinary incontinence and vesicoureteral reflux (VUR) are common conditions affecting the genitourinary system that can be treated with injectable bulking agents. The ideal substance for endoscopic treatment of VUR and incontinence should be injectable, non-antigenic, non-migratory, volume stable, and safe for human use.

Animal studies have shown that chondrocytes can be easily harvested and combined with alginate *in vitro*; the suspension can be easily injected cystoscopically; and the elastic cartilage tissue formed is able to correct VUR without any evidence of obstruction [46]. The first human application of cell-based tissue-engineering technology for urologic applications occurred with the injection of chondrocytes for the correction of VUR in children and for urinary incontinence in adults [47,48] (Fig. 53.4).

Using cell therapy techniques, the use of autologous smooth muscle cells was explored for both urinary incontinence and VUR applications [49].

The potential use of injectable, cultured myoblasts for the treatment of stress urinary incontinence (SUI) has also been investigated. Use of injectable muscle precursor cells has also been studied for treatment of urinary incontinence due to irreversible urethral sphincter injury or maldevelopment [50]. A clinical trial involving the use of muscle derived stem cells (MDSC) to treat SUI has also been performed with good results. Biopsies of skeletal muscle were

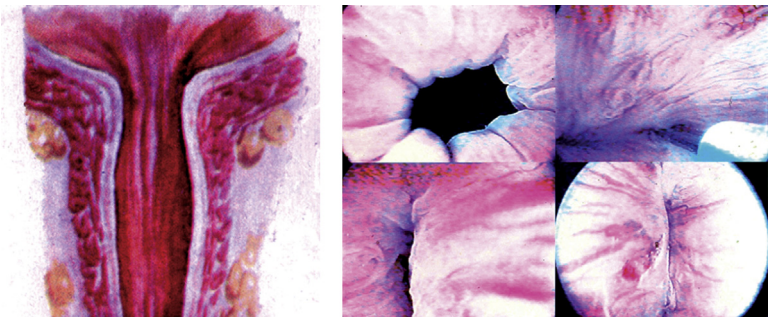

FIGURE 53.4
Tissue-engineered bulking agent. Chondrocytes are harvested and combined with alginate *in vitro*, and the suspension is injected cystoscopically as a bulking agent to treat urinary incontinence.

obtained, and autologous myoblasts and fibroblasts were cultured. Under US guidance, myoblasts were injected into the rhabdosphincter, and fibroblasts mixed with collagen were injected into the submucosa. One year following injection, the thickness and function of the rhabdosphincter had significantly increased, and all patients were continent [51]. These are the first demonstrations of the replacement of both sphincter muscle tissue and its innervation by the injection of muscle precursor cells.

In addition, injectable muscle-based gene therapy and tissue engineering were combined to improve detrusor function in a bladder injury model, and may have potential as a novel treatment option for urinary incontinence [52].

Testicular hormone replacement

Patients with testicular dysfunction require androgen replacement for somatic development. Conventional treatment consists of periodic intramuscular injections of chemically modified testosterone or, more recently, skin patch applications. However, long-term non-pulsatile testosterone therapy is not optimal and can cause multiple problems, including erythropoiesis and bone density changes.

A system was designed wherein Leydig cells were microencapsulated for controlled testosterone replacement. Purified Leydig cells were isolated and encapsulated in an alginate-poly-L-lysine solution. The encapsulated Leydig cells were injected into castrated animals, and serum testosterone was measured serially; the animals were able to maintain testosterone levels in the long term [53]. These studies suggest that microencapsulated Leydig cells may be able to replace or supplement testosterone in situations where anorchia or testicular failure is present.

CONCLUSION

Tissue-engineering efforts are currently being undertaken for every type of tissue and organ within the urinary system. Most of the effort expended to engineer genitourinary tissues has occurred within the last decade. Tissue-engineering techniques require a cell culture facility designed for human application. Personnel who have mastered the techniques of cell harvest, culture, and expansion as well as polymer design are essential for the successful application of this technology. Before these engineering techniques can be applied to humans, further studies need to be performed in many of the tissues described. Recent progress suggests that engineered urologic tissues and cell therapy may have clinical applicability.

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PART 14

Musculoskeletal System

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CHAPTER 54

Mesenchymal Stem Cells in Musculoskeletal Tissue Engineering

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INTRODUCTION

Nearly five decades ago, Friedenstein, et al. [1] described a population of non-hematopoietic cells isolated from human bone marrow with the *in vitro* ability to adhere, proliferate, and differentiate into chondrocytes, osteoblasts, and adipocytes. Such cells were identified in small amounts in multiple tissues throughout the body. Because of their differentiation properties and the ease of their isolation and *in vitro* expansion, these mesenchymal stem cells (MSCs) were naturally considered an attractive candidate progenitor cell type for tissue engineering and regenerative medicine applications. Extensive investigation has yielded knowledge about their biology, particularly tools for controlling their activity and cell fate that are critical to their clinical use. Beyond the three lineages originally identified, MSCs have been shown to differentiate into cells indicative of various mesenchymal lineages, including cellular phenotypes representative of the musculoskeletal tissues, such as cartilage, bone, muscle, ligament and tendon. A plethora of factors control the extensive proliferative and multipotent differentiation capacity of MSCs, including growth factors, cytokines, hormones, various soluble and insoluble signaling molecules, and transcription factors. Musculoskeletal tissues demand specific form and mechanical strength, and the success of a tissue-engineered outcome is thought to be dependent on the dynamic interactions between scaffolds, cells, and various physical and chemical environmental cues. By exploiting current knowledge of MSC differentiation, promising results have been generated towards engineered tissue replacements for cartilage, bone, osteochondral and other musculoskeletal tissues using MSCs. Further development and future clinical success, however, rely on significant knowledge-based improvements. The current understanding, recent advances, and remaining challenges for the deployment of MSCs for musculoskeletal tissue-engineering applications constitute the subject of this chapter.

MSC BIOLOGY RELEVANT TO MUSCULOSKELETAL TISSUE ENGINEERING

An ideal cell source for tissue engineering should exhibit the following characteristics: easy access and high availability of the source cells, capacity for extensive self-renewal or expansion

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00054-9

to generate sufficient quantity, the capacity to differentiate readily into cell lineages of interest upon instructive differentiation cues, and/or the ability to modulate the native environment to promote appropriate tissue repair and integration, including minimal immunogenic or tumorigenic ability. We will discuss below the intrinsic properties of MSC that determine their behavior, as well as external factors that affect the growth, differentiation, and the developmental outcomes of MSCs.

MSC identification

Since their initial description in the 1960s [1], MSCs have since been shown to possess the capacity to differentiate into cells characteristic of several mesenchyme-derived tissues, including cartilage, bone, fat, muscle, tendon, and hematopoietic-supporting marrow stroma (reviewed in [2–7]). In addition to these mesenchymal lineages, MSCs can also differentiate into other tissue types, including hepatocytes and neural tissues.

Experimentally, MSCs are identified first by their ability to adhere to plastic. Next, proliferative and differentiable MSCs are identified, at a minimum, by the expression of a combination of surface markers including STRO-1, SB-10, SH3 (CD73) and SH4 antigens as well as Thy-1 (CD90), TGF-β receptor type III endoglin (CD105), hyaluronic acid receptor CD44, integrin α1 subunit CD29, activated leukocyte-cell adhesion molecules (ALCAM, CD166), and possibly others [2,8]. MSCs are negative for the hematopoietic markers, CD19/CD79a, CD34, CD45, CD11b/CD14 and HLA-DR [2]. CD73, SH4 and STRO-1 antibodies recognize antigens that are present on MSCs and other cells but not hematopoietic cells (for review, see [9–11]). The presence of CD75, CD90, and CD105 and absence of hematopoietic markers, while not sufficient to fully identify an MSC, has been suggested by the International Society of Cell Therapy as the minimum positive criteria necessary to consider a potential cell an MSC [12]. It therefore remains a challenge to isolate MSCs specifically from a mixed cell population. Frequently, a combination of antibodies is used to retrospectively characterize the MSC phenotype. Inconsistency in the literature on the growth characteristics and differentiation potential of MSCs underscores the need for a clear functional definition of MSCs [13]; at present, the sole characteristic uniting disparate MSC sources is their ability to:

1) Proliferate: to self renew and remain in an undifferentiated state until provided the signal to differentiate, and
2) Differentiate along specific mesenchymal lineages when induced (Fig. 54.1).

Tissue sources of MSCs

While bone marrow remains the best studied tissue source for MSCs, MSC-like cells have been identified in a number of other tissues. The endogenous function(s) and exact location of these stem cells in the various tissues remain unclear; they are generally thought to participate in local tissue repair and regeneration. Bone marrow MSCs can give rise to stromal cells that support hematopoiesis *in vitro* and *in vivo*, possibly providing extracellular components as well as various growth factors and cytokines [14].

Although bone marrow is relatively easy to access, the potential donor site morbidity, pain, low cell incidence, and demands of cell therapy have prompted researchers to find alternative autologous sources for MSC isolation, which abound [9–11,15,16] (Table 54.1). MSC yield, proliferation rate, multipotency, differentiation potential, and the previously mentioned accessibility determine the usefulness of MSCs from different tissue origins. Although MSCs from different tissues show similar phenotypic characteristics, it is not clear if these are the same MSCs as they clearly show different propensities in proliferation and differentiation. For example, a study comparing human MSCs derived from bone marrow, umbilical cord blood, and adipose tissue showed that adipose tissue contained the highest number of MSCs while umbilical cord blood contained the lowest; however, umbilical cord blood-derived-MSC could be cultured longest and showed the highest proliferation capacity, whereas bone

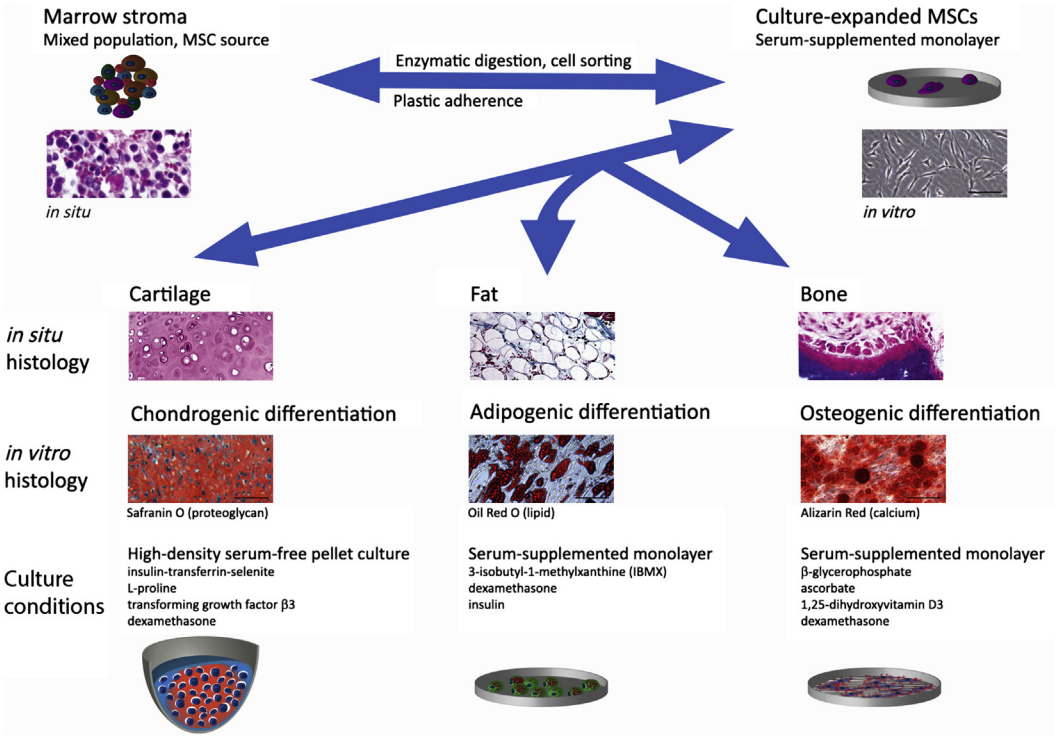

FIGURE 54.1

Minimum multilineage differentiation potential of mesenchymal stem cells. MSCs are able to differentiate into cell types of multiple lineages under appropriate conditions[a]; classically, these lineages include but are not limited to bone, cartilage, and fat. Bone marrow-derived MSCs, specifically, can repopulate the bone marrow stroma under certain conditions. Strong evidence exists that MSCs, once differentiated *in vitro*, can be dedifferentiated and subsequently used without harm to their multipotency. In addition to the standard lineages shown above, additional work suggests this transdifferentiation ability also applies to *in vitro* tenogenesis and musculogenesis[b]. *In situ* and *in vitro* histology is shown for phenotype comparison (scale bar = 100 μm). Adapted[c].
[a]Jackson WM, Lozito TP, Djouad F, Kuhn NZ, Nesti LJ, Tuan RS. Differentiation and regeneration potential of mesenchymal progenitor cells derived from traumatized muscle tissue. J Cell Mol Med 2011;11:2377–88. [b] Song L, Tuan RS. Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow. FASEB J 2004;9:980–2. [c] Tuan RS, Boland G, Tuli R.. Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther 2003;1:32–45.

marrow derived-MSC showed the lowest proliferation capacity [17]. Sakaguchi et al. compared the properties of MSCs isolated from bone marrow, synovium, periosteum, skeletal muscle and adipose tissue, and found significant differences in their differentiation potentials [18]. MSCs from bone marrow, synovium, and periosteum showed greater chondrogenic activities, with synovium-derived MSCs exhibiting the highest capacity. Adipose-derived MSCs exhibit enhanced chondrogenesis if grown with excess FGF-2 then cultured for chondrogenesis with bone morphogenetic protein-6 (BMP6) [19,20]. With regard to osteogenesis, the rate of matrix mineralization was highest in bone marrow, synovium, and periosteum derived MSCs. Therefore, the optimal source of MSCs for therapeutic use for the musculoskeletal system remains to be determined.

In addition to tissue source, donor age and disease stage may directly affect MSC yield, rate of proliferation, and multipotency. Decreasing MSC number and proliferation rate as well as differentiation potentials were observed with increasing donor age [21]. MSCs isolated from osteoporotic women showed significantly reduced proliferative response and osteogenic differentiation [22]. With bone marrow MSCs, advanced osteoarthritis condition, regardless of age, had a significantly deleterious impact on proliferative capacity and chondrogenic and adipogenic activities when compared to healthy donors [23]. Decreases in MSC quantity and quality due to age and disease may limit the use of autologous MSCs in clinical settings,

TABLE 54.1 Tissue sources of human mesenchymal stem cells

Tissue source	Representative references
Bone Marrow	Friedenstein, et al. J Embryol Exp Morphol. 1966;16:381–90. Prockop. Science. 1997;276:71–4. Pittenger et al. Science. 1999;284:143–7.
Trabecular bone	Tuli, et al. Stem Cells. 2003;21:681–93. Osyczka, et al. Ann N Y Acad Sci. 2002;961:73–7.
Muscle	Young, et al. Dev Dyn. 1995;202:137–44. Bosch, et al. J Orthop Res. 2000;18:933–44. Williams, et al. Am Surg. 1999;65:22–6.
Adipose	Zuk, et al. Mol Biol Cel. 2002;13:4279–95. Erickson, et al. Biochem. Biophys. Res. Commun. 2002;290:763–769.
Periosteum	De Bari, et al. Arthritis Rheum. 2001;44:85–95. Nakahara, et al. J Orthop Res. 1991;9:465–76.
Synovial membrane	De Bari, et al. Arthritis Rheum. 2001;44:1928–42. Nishimura, et al. Arthritis Rheum. 1999;42:2631–7.
Articular cartilage	Alsalameh, et al. Arthritis Rheum. 2004;50:1522–32. Dowthwaite, et al. J Cell Sci. 2004;117:889–97. Tallheden, et al. J Bone Joint Surg Am. 2003;85–A Suppl 2:93–100.
Skin	Young, et al. Dev Dyn. 1995;202:137–44. Toma, et al. Nat Cell Biol. 2001;3:778–84.
Pericyte	Brighton, et al. Clin Orthop 1992;275:287–99.
Peripheral blood	Kuznetsov, et al. J Cell Biol. 2001;153:1133–40. Zvaifler, et al. Arthritis Res. 2000;2:477–88.
Deciduous teeth	Miura, et al. Proc Natl Acad Sci U S A. 2003;100:5807–12.
Periodontal ligament	Lekic and McCulloch. Anat Rec. 1996;245:327–41. Seo, et al. J Dent Res. 2005;84:907–12.

Further sources of human MSCs include the intestinal [4], limbal [5], knee-joint [6–9] and prostate [10] stroma, trachea [11], nasal mucosa [12], Wharton's jelly (WJ) [13,14], cord blood [15] and placenta [16].

Lanzoni G, Alviano F, Marchionni C, Bonsi L, Costa R, Foroni L, Roda G, Belluzzi A, Caponi A, Ricci F, Luigi Tazzari P, Pagliaro P, Rizzo R, Lanza F, Roberto Baricordi O, Pasquinelli G, Roda E, Paolo Bagnara G: Isolation of stem cell populations with trophic and immunoregulatory functions from human intestinal tissues: potential for cell therapy in inflammatory bowel disease. *Cytotherapy* 2009, 11:1020–1031.

Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK: Mesenchymal cells from limbal stroma of human eye *Mol Vis* 2008, 14:431–442.

Morito T, Muneta T, Hara K, Ju YJ, Mochizuki T, Makino H, Umezawa A, Sekiya I: Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. *Rheumatology (Oxford)* 2008, 47:1137–1143.

Nimura A, Muneta T, Otabe K, Koga H, Ju YJ, Mochizuki T, Suzuki K, Sekiya I: Analysis of human synovial and bone marrow mesenchymal stem cells in relation to heat-inactivation of autologous and fetal bovine serums. *BMC Musculoskelet Disord* 2010, 11:208.

Segawa Y, Muneta T, Makino H, Nimura A, Mochizuki T, Ju YJ, Ezura Y, Umezawa A, Sekiya I: Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. *J Orthop Res* 2009, 27:435–441.

Ju YJ, Muneta T, Yoshimura H, Koga H, Sekiya I: Synovial mesenchymal stem cells accelerate eariy remodeling of tendon-bone healing. *Cell Tissue Res* 2008, 332:469–478.

Santamaria-Martinez A, Barquinero J, Barbose-Desongles A, Hurtado A, Pinos T, Seoane J, Poupon MF, Morote J, Reventos J, Munell F: Identification of multipotent mesenchymal stromal cells in the reactive stroma of a prostate cancer xenograft by side population analysis. *Exp Cell Res* 2009, 315:3004–3013.

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Jakob M, Hemeda H, Janeschik S, Bootz F, Rotter N, Lang S, Brandau S: Human nasal mucosa contains tissue-resident immunologically responsive mesenchymal stromal cells. *Stem Cells Dev* 2010, 19:635–644.

Hsieh JY, Fu YS, Chang SJ, Tsuang TH, Wang HW: Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. *Stem Cells Dev* 2010, 19:1895–1910.

Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, Mcintosh KR: Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* 2008, 26:2865–2874.

Tark KC, Hong JW, Kim YS, Hahn SB, Lee WJ, Lew DH: Effects of human cord blood mesenchymal stem cells on cutaneous wound healing in Lepr db mice. *Ann Plast Surg* 2010, 65:565–572.

Miao Z, Jin J, Chen L, Zhu J, Huang W, Zhao J, Qian H, Zhang X: Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int* 2006, 30:681–687.

warranting further study of allogeneic MSCs for tissue repair. In addition to disease transmission, these non-autologous tissues might carry the risk of immune rejection and allogeneic reaction by the host and will be discussed in detail later.

MSC isolation and *in vitro* culture

MSCs are rare in bone marrow, occurring with a frequency of 0.001–0.01 % of the total nucleated cells [5]. Various protocols of MSC isolation are available, and some are species specific. Human bone marrow MSCs can be isolated by simple direct plating after isolation from native tissue, sometimes involving enzymatic digestion. Frequently, this is performed with the layer of mononuclear cells obtained after Percoll or Ficoll density gradient centrifugation. With culture time and medium changes, non-adherent hematopoietic cells are washed away, and MSCs appear as small, adherent, spindle-shaped fibroblast-like cells. Surface marker-based positive selection or negative selection using either magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS) can further purify the MSC population. Positive selections have been based on the detection of markers that are expressed, albeit not exclusively, by MSCs; negative selection is based on the exclusion of markers that are expressed by hematopoietic cells, using antibodies against CD34, CD45 and CD11b, as mentioned above. Growth cocktails, designed to maintain the multipotent and proliferative capacities of MSCs vary widely, and typically involve culture in serum-supplemented Dulbecco's Modified Eagles Medium (DMEM) or α-Minimum Essential Medium (α-MEM) (Table 54.2).

After initial expansion, cultures are maintained at densities of 50–100,000 cells/cm^2 [4,24,25]. The extensive expansion capacity of MSCs depends on the harvesting techniques, culture conditions, and the health condition and age of the donor. Culture conditions, such as initial seeding density, play a role in the expansion capacity of MSCs. For example, higher proliferation profiles of MSCs was seen when plated at low initial plating density (1.5–3 cells/cm^2) but not at high density (12 cells/cm^2), resulting in a dramatic increase in the fold expansion of total cells (2,000-fold vs. 60-fold increase, respectively) [26].

TABLE 54.2 Human bone-marrow-derived MSC *in vitro* media formulation and growth characteristics

Culture conditions: Expansion media	Population doublings	References
αMEM, 17% FBS, 2 mM L-glutamine	10	Pochampally, et al. Blood. 2004;103:1647–52.
Coon's modified Ham's F12 medium, 10% FCS	22–23	Muraglia, et al. J Cell Sci. 2000;113:1161–6.
αMEM, 10% FBS	15	Lee, et al. Cell Physiol Biochem. 2004;14:311–24.
αMEM, 10% FCS	24 ± 11 to 41 ± 10	Stenderup, et al. Bone. 2003;33:919–26.
αMEM, 20% FBS, 100 uM L-ascorbate-2-phosphate	20	Gronthos, et al. J Cell Sci. 2003;116:1827–35.
Coon's Modified Ham's F-12, 10% FCS, 1 ng/ml FGF-2	22–23	Banfi, et al. Exp Hematol. 2000;28:707–15.
DMEM, 10% FBS	38 ± 4	Bruder, et al. J Cell Biochem. 1997;64:278–94.
αMEM, 20% FBS	30	Colter, et al. Proc Natl Acad Sci USA. 2000;97:3213–8.
αMEM, 10% FBS	20	Lee, et al. Cell Physiol Biochem. 2004;14:311–24.
DMEM, 2% FBS, MCDB-201, 10 ng/ml EGF, 1x insulin-transferrin selenium, 1x linoleic acid BSA, 10 nM Dex, 10 mM ascorbic acid phosphate	30	Hu, et al. J Lab Clin Med. 2003;141:342–9.

MSC self-renewal and proliferation capacity

In vivo, MSCs remain in a mitotically quiescent state (G0 stage of the cell cycle) as demonstrated by analysis of fresh bone marrow cell harvests continuously exposed to tritiated thymidine labeling [27]. BrdU labeling of cells in a human trabecular bone explant culture also reveals that cell proliferation, including that of STRO-1 positive mesenchymal stem/progenitor cells, was inhibited completely within the explant tissue milieu [28]. Since most of the tissues/organs from which MSCs are derived exhibit a relatively slow turnover rate in the adult organism, it is not unreasonable to propose that self-renewal or proliferation of MSCs is normally suppressed *in vivo* in the course of tissue homeostasis, possibly regulated by intrinsic factors present in the tissue microenvironment and/or through direct or indirect interaction with neighboring cells.

In vitro cell cycle studies revealed that the majority of MSCs (between 78.7% and 96.45%) are in the G0/G1 phase of the cell cycle while a very small fraction of MSCs are engaged in active proliferation. During the exponential phase of cell growth, human MSCs isolated from bone marrow and fetal pancreas exhibited a similar population doubling time, ranging from 10 to 30 hours [29].

Unlike embryonic stem cells, MSCs do not expand indefinitely *in vitro* when cultured in the presence of serum, which could reflect on their intrinsic properties as adult stem cells or a suboptimal culture environment. After extensive propagation, MSCs change their phenotype from a fibroblastic shape to a more flattened morphology with extensive podia and actin stress fiber formation, a phenomenon usually referred to as 'replicative senescence'. Consistent with their limited expansion capacity, human MSCs do not express telomerase activity, and their telomere length is reduced as the cells grow older [30]. Furthermore, murine MSCs derived from telomerase knockout mice failed to differentiate into adipocytes and chondrocytes and lost telomere at late passage [31]. Therefore, telomerase activity and telomere maintenance appear to play a crucial role in maintaining the self-renewal and multidifferentiation potential of MSCs. Indeed, hMSCs stably expressing telomerase reverse transcriptase can undergo 80 [32,33] or 260 [34] population doublings without growth arrest.

In addition, growth factors also play a role in the expansion capacity of MSCs. Basic fibroblast growth factor (bFGF) can prolong the replicative capacity of hMSCs and increase total cell numbers by several fold when included in the basal culture medium [35]. Other growth factors and cytokines, including canonical Wnts, e.g., Wnt 3A [36], and interleukin-6 (IL-6) [37], have also been found to enhance the proliferative activity of adult human MSCs. Growth factor pre-treatment may also help define the differentiation potential of the cultured MSCs [38]. Detailed study of MSC biology and proliferation, as well as variations due to culture conditions, may further extend the number and potential efficacy of MSCs.

Skeletogenic differentiation of MSCs

MSC differentiation can be controlled by several factors including growth factors, hormones, and extracellular matrix molecules working through various signaling pathways. Relevant to musculoskeletal tissue engineering, *in vitro* chondrogenesis of MSCs, or cartilage formation, is accomplished by culturing the cells in a three-dimensional culture condition, either as pellet culture, micromass cultures, or three-dimensional scaffold, in the presence of a member of the transforming growth factor family (TGF-β1, TGF-β2, or TGF-β3, or bone morphogenetic proteins, BMPs) [35]. Supplementation with dexamethasone also promotes the expression of chondrocyte phenotype. Chondrogenesis is demonstrated histologically as well as on the basis of the expression of genes encoding cartilage matrix components, including aggrecan, collagen types II and IX, and COMP (cartilage oligomeric matrix protein) [39]. Control of MSC chondrogenesis will be discussed in more detail in the cartilage tissue engineering section of this chapter.

Osteogenesis, i.e., bone formation, can be induced *in vitro* by treating MSCs with the synthetic glucocorticoid dexamethasone, β-glycerophosphate, ascorbic acid, and 1,25-dihydroxyvitamin D_3. Alkaline phosphatase (ALP) activity and calcification of the extracellular matrix are typical markers used for detecting preosteoblasts and mature osteoblasts, respectively. Dexamethasone stimulates MSC proliferation and increases ALP activity of osteogenic hMSCs. Matrix mineralization only occurs in the presence of β-glycerophosphate, ascorbate, and dexamethasone, and is further enhanced by 1,25-dihydroxyvitamin D_3[40]. Although used less uniformly, a number of growth factors also contribute to osteoinduction. For instance, treatment of human MSCs with insulin-like growth factor 1 (IGF-1), epidermal growth factors (EGF), and vascular endothelial growth factor (VEGF) increases the activity of ALP and mineralization *in vitro*. On the other hand, platelet-derived growth factor (PDGF) has little inductive effect on hMSC osteogenesis [41].

Also relevant to tissue-engineering applications, osteogenesis can be stimulated *in vitro* by various physical stimulants, including mechanical stimulation, pulsed electromagnetic field (PEMF), and hydrostatic pressure [42–44]. Such models, in addition to offering insight into bone-forming processes, can also be used to analyze various injury mechanisms and to develop potential therapies.

Plasticity of MSCs

Plasticity/transdifferentiation refers to the ability of a cell type committed to and progressing along a specific developmental lineage to switch into another cell type of a different lineage through epigenetic reprogramming. As demonstrated by several studies, terminally differentiated cells can switch their phenotype under appropriate stimulations. For example, chondrocytes can become osteocytes [45,46], and adipocytes can convert to osteoblasts [47–49]. Since all of these cells are the mature progenies of MSCs, the conversion between each other might reflect the plastic property of MSCs. By using an *in vitro* differentiation strategy, we have demonstrated that human MSCs pre-committed to a given mesenchyme cell lineage can transdifferentiate into other cell types in response to inductive extracellular cues [50]. Understanding the molecular mechanisms that control the transdifferentiation potential of MSCs will facilitate the identification of regulatory factors, thus providing tools to manipulate adult stem cells for cell-based tissue-engineering applications. Recent evidence suggest that mesenchymal cell populations contain a subpopulation of multilineage stress enduring (MUSE) cells that possess pluripotency and may account for some of the plasticity observed [51].

MSC heterogeneity

Individual colonies derived from single MSC precursors exhibit a heterogeneous nature in terms of cell proliferation and multilineage differentiation potential. For instance, only a minor proportion of colonies (17%) derived from adult human bone marrow continued to grow beyond 20 population doublings, while the majority of the colonies exhibited early senescence [52]. There is also a marked difference in the differentiation capacity of MSC colonies. For example, Pittenger et al. [5] reported that only one-third of the initial adherent bone-marrow-derived MSC clones are multipotent to differentiate along the chondro-, osteo- and adipo-genic pathways (osteo/chondro/adipo). Furthermore, non-immortalized cell clones examined by Muraglia et al. [53] demonstrated that 30% of the *in vitro*-derived MSC clones exhibited a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage potential (osteo). These observations are consistent with other *in vitro* studies using conditionally immortalized clones. Even derived from a single colony, cells seem to be heterogeneous. Kuznetsov et al. demonstrated that only 58.8% of the single colony-derived clones had the ability to form bone within hydroxyapaptite-tricalcium phosphate ceramic scaffolds after implantation in

immunodeficient mice [54]. Similar results were reported by using purer populations of MSCs maintained *in vitro* [52] as well as human trabecular bone derived MSCs [55]. Taken together, these results suggest that MSCs are heterogeneous with respect to their developmental potential, and place a significant challenge on selecting the most potent cells for clinical application in tissue regeneration.

There exist a number of inconsistencies and discrepancies among currently published results on MSCs, perhaps reflecting the heterogeneity of the population, different isolation and culture methods, as well as the different stimuli used for the differentiation procedures [13]. There has not been a single MSC specific marker to unequivocally identify MSCs, and there is heterogeneity in MSC populations. Most likely distinct and unrelated cells types are being studied, and only a subpopulation of the MSCs are true stem cells.

MSC effect on host immunobiology

Ideally, autologous MSCs would have advantages over allogeneic MSCs for regenerative medicine as autologous MSCs pose few immunological complications. Due to the low abundance of MSCs and the possible decrease in quantity as well as quality of the MSCs with age and disease, it is more feasible to consider using allogeneic MSCs for replacing or repairing damaged tissues. Before this is considered acceptable, it is important to understand how MSC elicit host immunological reactions. Allogeneic cells are normally detected and deleted by the host immune systems. However, MSCs have been surprisingly different in this aspect and offer several advantages, including possible immuno-modulatory, including immunosuppressive, effects on the host response.

The first immunological advantage of MSCs is that they are hypo-immunogenic and can evade host immune system, as shown by several *in vitro* experiments [56]. This makes MSCs attractive for allogeneic transplantation whose major limitation is host immune rejection. MSCs express low (fetal) to intermediate (adult) major histocompatibility complex (MHC) class I molecules and do not express MHC class II molecules on their cell surface [57,58]. The expression of MHC class I molecules helps to protect MSCs from deletion by natural killer cells. The lack of surface MHC class II expression gives the MSCs the potential to escape recognition by alloreactive CD4+ T cells. MSCs contain an intracellular pool of MHC class II molecules that can be mobilized onto cell surface by treatment with interferon-γ (IFN-γ). However, induced surface expression of MHC class II still does not render the MSCs immunogenic [58]. After differentiation, MSCs continue to express MHC class I but not class II molecules on their cell surface, and continue to be non-immunogenic [57,58].

Experimental evidence suggests that MSCs can interact directly with immune cells and modulate and suppress alloreactivity. MSCs inhibit T cell proliferation *in vitro* [59–61]. MSCs do not seem to express co-stimulatory molecules, CD40, CD40 ligand, CD80, and CD86 (formerly B7–1 and B7–2, respectively) and probably do not activate alloreactive T cells [61]. In fact, MSCs suppress T cell activation and proliferation. For example, MSCs have been shown to suppress CD4+ and CD8+ T cells in mixed lymphocyte cultures [59,61]. Even though T cell proliferation can be induced by exogenous co-stimulation, when they are co-cultured with MSCs in the presence of a stimulant, T cell proliferation is not observed [61]. MSCs can also induce apoptosis of activated T cells but not resting T cells [62]. Supporting these observations, Bartholomew [60] showed that allogeneic baboon MSCs inhibited the proliferation of lymphocytes *in vitro*, and prolonged skin graft survival *in vivo*. In addition to the effect of MSCs on T cells, MSCs can affect dendritic cell differentiation and maturation and interfere with their function [63,64]. They also alter the phenotype of natural killer cells, and can suppress the proliferation, cytokine secretion and cytotoxicity of these cells against MHC class I targets [65].

In addition to cell-cell interaction mediated inhibition, MSCs are capable of secreting soluble factors to create a local immunosuppressive environment. These factors have been shown to

include hepatocyte growth factor (HGF), TGF-β1, IL-10 and prostaglandin E2 [59,66]. When anti-HGF antibody and anti-TGF-β1 are included, MSC inhibition on T cell proliferation is lifted [59]. IL-10 is a cytokine for regulatory T cells and can suppress inflammatory immune response. Similarly, MSCs are shown to produce IL-10 either constitutively, or in co-culture, and MSCs can mediate the suppressive activities partially through secretion of IL-10 [64,66,67]. Collectively, this *in vitro* evidence has suggested that MSCs can interact with the various subsets of cells of the immune system, alter the response of the immune cells, and shift the response from a pro-inflammatory response to an anti-inflammatory response, possibly through inhibition of the pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) and stimulation of the immunosuppressive cytokines including IL-10 and prostaglandin E2 [66].

The role of secreted factors in the immuno-regulatory action of MSCs remains an actively investigated area [68,69]. Contradictory results exist as to which factors are important for this function. For example, although a role for IL-10, TGF-β, and prostaglandin E2 has been suggested, in other studies, none of these factors was found to be responsible for the immuno-suppressive action of MSCs [61]. Despite the discrepancies on the mechanism of action, the above studies suggest that MSCs can be transplanted between MHC incompatible individuals.

The *in vitro* studies showing that MSCs possess immuno-modulatory and immuno-suppressive activities suggest that MSCs can be potentially used *in vivo* for enhancing the engraftment of other tissues (e.g., hematopoietic stem cells), or for the prophylactic prevention and even possibly as a treatment of graft-versus-host-disease, to prevent rejection, and promote transplant and patient survival. Indeed, a search of clinicatrials.gov (October 29, 2012) returned 66 studies using the terms 'mesenchymal stem cell' and 'immune' [70]. However, before MSC treatment can be used as standard therapy on humans, more *in vivo* animal studies need to be performed, and the biology and mechanism of MSC immunomodulation effect need to be better elucidated.

Safety of using MSCs for transplantation

The ability of MSCs to undergo extensive self-renewal via proliferation raises some concern as to whether MSCs, after prolonged *in vitro* culture, can become tumorigenic. Although most MSC transplantations did not show obvious malignant transformation, there has been evidence suggesting otherwise. Rubio et al. [71] showed that although standard short-term *in vitro* culture (6–8 weeks) seems to be safe, MSCs can undergo spontaneous transformation after long-term culture of 4–5 months.

The immunosuppressive properties, especially the potential systemic immuno-suppressive ability of MSCs, bring caution to the use of MSCs under certain clinical conditions, such as cancer. Using a murine melanoma tumor model, it has been shown that co-transplantation of an MSC cell line (C3H10T1/2) favor tumor growth of subcutaneously injected B16 melanoma cells [72]. However this tumor promoting effect was not observed in another study of co-culture of a rat MSC line, MPC1cE, with rat colon carcinoma cells in a gelatin matrix. In this case, inhibitory effect of MSCs on the outgrowth of the tumor cells was observed [73]. The effect of MSCs on tumor growth requires further investigation to rule out the potential side effect of therapeutic use of MSCs [74].

MSCs IN MUSCULOSKELETAL TISSUE ENGINEERING

The musculoskeletal system of the human body is designed to sustain and maintain its form and function in the face of enormous load bearing demands throughout a normally active life. Form and mechanical strength are vital for the function of this system. The mechanical and biochemical properties of musculoskeletal system that define its structure and function also define the functional requirements of a tissue-engineered substitute. Successful tissue

engineering-based replacement of native tissues will likely require constructs that possess functional properties similar to those of the native tissues to minimize premature failure. There are two basic approaches in tissue engineering. The first one is *ex vivo* tissue engineering, in which the construct is cultivated *in vitro* to achieve appropriate functionality before implantation. The second approach is *in vivo* tissue engineering, in which the construct is allowed to mature *in vivo* for tissue repair and regeneration. For both approaches, three components govern the eventual outcome of tissue-engineered constructs: appropriate scaffold, instructive environment, and responsive cells.

Cartilage tissue engineering

The need for engineered cartilage arises from the fact that while the tissue often functions well through a lifetime of use, over 30% of adults in the US over 30 years of age have radiographic evidence of cartilage degeneration, with 9–10% of the US population aged 30 suffering symptoms of osteoarthritis of the hip or knee [75,76]. The total cost of osteoarthritis is estimated at $36 billion dollars per year in the USA alone [77], with >200,000 knee replacements performed each year. The intrinsic healing capacity of the native tissue is limited, and given the increasing incidence of osteoarthritis and increasing life expectancy of the population, there is a growing demand for novel repair strategies. Effective treatment of cartilage injuries may eliminate or forestall the need for joint replacement, thus enhancing the quality of life.

GENERAL PROPERTIES OF ARTICULAR CARTILAGE

Articular cartilage is the dense white tissue that lines the surfaces of joints and functions to transmit the high stresses associated with joint motion. The tissue consists of both a solid extracellular matrix (ECM) component as well as a fluid phase [78,79]. The solid ECM is composed of a dense network of specialized molecules that engender the unique mechanical properties of the tissue. Collagen content (predominantly collagen type II) of the tissue ranges from 5–30% by wet weight while proteoglycan content ranges from 2–10%. In addition to these major elements, the ECM also includes numerous minor collagens (e.g., types VI, IX, and XI), and linking molecules (COMP, hyaluronan, link protein, fibronectin). The highly charged ECM, and the proteoglycans in particular, trap a large amount of water within the cartilage matrix, which comprises the fluid component of cartilage matrix. Together, these elements make up the fluid-filled fibrous network with larger collagen fibers interwoven throughout an array of large proteoglycan aggregates (aggrecan core protein with its covalently linked keratin and chondroitin sulfate moieties) attached to long hyaluronan chains.

The exact composition of cartilage changes slightly but sequentially as one progresses from the surface zone to the calcified zone that interacts with the subchondral bone. The surface zone, that is well adapted to tensile forces, comprises relatively little matrix with finer fibrillar structure rich in the proteoglycan lubricin arranged parallel to the articular surface as revealed by polarized microscopy and the squamal morphology of the cells in this region. The deep zone, well adapted to compressive forces, comprise larger collagen fibrils with greater cross-linking arranged perpendicular to the articular surface. These core constituents change with age, resulting in the emergence of adult functional properties, and deteriorate with disease processes, such as osteoarthritis [80–82].

The cellular component of cartilage, the chondrocytes, comprises less than 10% of the tissue volume [83]. The sparse distribution of chondrocytes belies their critical importance in maintaining the balance between anabolic synthesis of ECM constituents and continual remodeling and degradation over time [84]. Chondrocytes display a rich transcriptional profile *in situ* [85], reflective of their specialized cartilaginous phenotype, which is lost when these cells de-differentiate with expansion in monolayer culture [86]. This process may be substantially reversed, with chondrocytes re-differentiating towards their mature phenotype when returned to three-dimensional culture [87]. However, the exposure to two-dimensional

expansion in culture leads to certain 'irreversible' phenotypic changes [88,89], such as the expression of collagen type I that leads to fibrosis, reducing the mechanical properties of the matrix comprising repair tissue or engineered cartilage, and harkening matrix and tissue degeneration.

The mechanical properties of articular cartilage are complex and underlie its ability to act as a low friction, weight-bearing surface over a lifetime of use. These properties are a result of both the solid (matrix) and fluid phases of the tissue. Regarding the solid, matrix phase, the dense, negatively charged, proteoglycan-rich ECM results in an equilibrium compression modulus of 0.2–1.4 MPa [90,91]. Meanwhile, the collagen content engenders a tensile modulus that is higher, ranging from 1–30 MPa [92,93]. Other structural elements of cartilage, including collagen type IX [94] and COMP [95], participate in the crosslinking of the collagen type II ECM, increasing matrix connectivity.

The highly charged ECM, and the proteoglycans in particular, trap a large amount of water within the cartilage matrix [96]. These electrostatic forces and the physiological cyclic loading (0.1–2 Hz) of the tissue maintain elevated interstitial fluid pressure. Fluid pressurization increases with contact and supports >90% of applied stress, shielding the matrix from excess deformation [97]. This fluid pressurization also results in a higher dynamic modulus than equilibrium modulus, stiffening the tissue with higher frequency and/or higher rate of loading. Finally, this fluid pressurization, coupled with molecules that participate in boundary lubrication, maintains the frictional coefficient of cartilage at extremely low values (lower than ice on ice), further protecting the tissue from excess wear with physiologic use [98,99].

CELLS FOR CARTILAGE TISSUE ENGINEERING

In the quest to repair or replace damaged articular cartilage, any replacement material, tissue or their composite must be fabricated to retain the complex mechanical properties described above. The development of such a material begins with the consideration and combination of appropriate and responsive cells, biocompatible and mechanically conductive scaffolds, and inductive environment for the optimal differentiation and proliferation of the cell type of interest. As discussed in this and other chapters, MSCs derived from various adult tissues have emerged as promising cell sources because of the ease with which they can be isolated and expanded and their multilineage differentiation capabilities.

When tissue engineering was originally proposed as a strategy for repairing diseased or damaged tissues [100], it was believed that articular cartilage would be one of the first successes in this new field, owing to its relatively simple composition (possessing a single cell type) and lack of neural and vascular supply. First approaches focused on the chondrocyte itself, postulating that as these cells make and maintain matrix *in vivo*, exogenous cells should be able to reconstitute the tissue when implanted. Indeed, early reports on high density chondrocyte monolayer culture demonstrated the development of high tensile moduli in thick cell sheets [101]. Early *in vivo* successes demonstrated enhanced repair when high density chondrocyte solutions were transplanted to focal defects beneath a periosteal flap [102], a procedure now commonly referred to as autologous chondrocyte implantation (ACI), and commercialized as the Carticel® method by Genzyme Biosurgery. While questions remain regarding the efficacy and cost-effectiveness of this approach [103], it nevertheless stands as the first clinically available tissue-engineering strategy to enhance cartilage repair. New embodiments of this cell-based approach have incorporated scaffold matrix for better cell retention (matrix-assisted ACI or MACI)[103a].

Although significant progress has been made in the production of engineered cartilage constructs with chondrocytes, several significant impediments exist that limit their clinical application. First, chondrocytes are present in limited supply, and are often of an aged and/or diseased state in patients presenting with osteoarthritis. While most tissue-engineering studies

in the literature report on newborn or juvenile derived chondrocytes, a recent study has shown that aged chondrocytes are less able to form functional constructs in agarose culture [104]. Finally, chondrocytes must be isolated from the joint tissue itself, a process that may further complicate an already damaged joint [105]. One opportunity for overcoming such concerns is the use of chondroprogenitor cells, such as adult MSCs. As described above, these cells are readily obtained from the adult bone marrow and other tissues, and retain a multilineage potential. These cells are expandable in culture [106], and may be grown in sufficient numbers to populate engineered scaffolds.

The use of MSCs for cartilage regeneration and tissue engineering has shown enormous potential and initial success. Of note, one current surgical strategy for enhancing the repair of cartilage defects is via microfracturing of the subchondral bone. This technique provides entrance for marrow elements, including MSCs, to the wound site, and has been shown to generate an enhanced, albeit fibrous, repair response [107]. MSCs have also been used as cellular therapeutics directly, via injection into the joint space (with and without a carrier matrix) in an undifferentiated state [108–110] and after differentiation down a cartilage lineage *in vitro* [111,112]. These cells may be further transduced to express morphogenetic proteins that drive their own chondrogenesis after implantation [113].

MSC CHONDROGENESIS

MSC chondrogenic potential

Control of chondrogenesis in MSCs is a complex and developing research area, with much of our understanding of the relevant molecules and processes stemming from a continuing elucidation of the events that control healthy cartilage homeostasis as well as cartilage formation in the developing limb. Elements including soluble factors such as growth factors, cytokines, hormones, various intracellular signaling pathways and transcription factors, environmental factors such as mechanical loading and oxygen levels, and seeding density all affect chondrogenic differentiation of MSCs.

The standard *in vitro* system of MSC chondrogenesis involves a three-dimensional culture of MSCs under the stimulation of a suitable chondrogenic stimulus. High density cultures of pellet or micromass are frequently employed, modeling early chondrogenesis in development where condensation of the early progenitor cells initiates the cascade of events leading to cartilage formation [114]. These MSCs can then effectively differentiate under standard chondrogenic conditions (serum-free medium supplemented with insulin-transferrin-selenite (ITS), ascorbate, proline, dexamethasone, and TGFα3). In addition, MSCs may also be seeded on various three-dimensional scaffolds to induce chondrogenesis. While the maintenance of a spherical shape in these scaffolds may be enough to induce a chondrogenic phenotype by MSCs, a chondroinductive growth medium is still required to realize the maximum chondrogenic potential of these cells. The hallmarks of an articular chondrocyte are initially high levels of Sox9 expression followed by ultimately high levels of collagen types II, IX, and XI and concomitant expression of aggrecan, COMP and link protein, among others. Together these extracellular components produce a highly crosslinked and hydrated matrix surrounding the cells and their pericellular and intercellular matrix. This phenotype represents the prehypertrophic state of cartilage in the parlance of developmental biology describing the growth plate, the cartilage anlage of the endochondral skeleton [114].

MSCs that have undergone chondrogenic differentiation assume a chondrocyte-like phenotype characterized by increases in proteoglycan deposition and expression of aggrecan, COMP, and collagen type II [115] as described above, and microarray analysis has shown that numerous other cartilage extracellular matrix elements increase in their expression as well [50,116,117]. A major challenge to articular cartilage engineering is to prevent the apparently natural progression of the cells down the path of chondrocyte hypertrophy, matrix

mineralization and apoptosis that promotes the ossification of the tissue to mature bone. In this latter process, cells express Runx2, collagen types I and X, and matrix degradative enzymes such as matrix metalloproteases (e.g., MMP13). These markers are also characteristic of osteoarthritic cells that can rapidly cause matrix degradation, scaffold and construct failure [118].

Signaling in MSC chondrogenesis

Growth factors that have regulatory effects on MSCs include members of the TGFβ superfamily, the IGFs, the fibroblast growth factors (FGF), and the PDGFs. Among these growth factors, members of the TGFβ family, including TGFβ1, TGFβ2, and TGFβ3, as well as BMPs are the most potent inducers to promote chondrogenesis of MSCs. For human MSCs, TGFβ2 and TGFβ3 were shown to be more active than TGFβ1 in promoting chondrogenesis [115]. BMPs, known for their involvement in cartilage formation, act alone or in concert with other growth factors to induce or enhance MSC chondrogenic differentiation. For example, BMP2, BMP4, or BMP6, combined with TGFβ3, induced the chondrogenic phenotype in cultured human bone-marrow-derived MSC pellets, with BMP2 seemingly the most effective [119]. Other growth factors, such as IGF, FGF, and PDGF, are important signaling molecules that mediate chondrocyte physiology rather than promoting chondrogenesis of MSCs, and therefore commonly work with TGFβs to promote chondrogenesis and enhance chondrocytic activities of differentiated MSCs. The pro-mitotic activity of the FGFs has also been exploited for cell expansion purposes [120]. Interestingly, FGF2-supplemented human MSCs proliferated more rapidly, and exhibited greater chondrogenic potential than untreated controls [121]. Canonical Wnt signaling has been shown to enhance MSC differentiation [122] and Wnt signaling in chondrogenesis has been shown to crosstalk with TGFβ signaling [123–125]. The current challenge in finding the most efficient growth factor(s) for MSC chondrogenesis is that the regulatory effects of signal molecules are dependent on property, dose, and timing of the molecules administered to the cells. This may explain some of the contradictory results regarding the effects of specific growth factors on chondrogenesis. Furthermore, the exact mechanisms of articular cartilage development *in vivo* remain unknown; however, we may surmise that it occurs in a very complex biochemical environment, and that some level of this complexity must be replicated *in vitro* in order to produce functional articular cartilage. Replication of this complexity begins in part with the application of growth factor cocktails, applied combinatorially or sequentially, and as the effects of growth factors are changed in the presence of other growth factors and environmental cues, the repetoire of pro-chondrogenic formulations are likely to become more confused before the field achieves clarification.

Growth factors act on cells and induce various intracellular signaling pathways to coordinate transcription factors and change cellular phenotype. The most important molecule intrinsic to the assumption of the cartilaginous phenotype is the transcription factor, Sox9. The role of Sox9 in cartilage formation was first observed in condensations in the developing mouse limb, and its presence is thought to be required for cartilage formation [126]. The expression of Sox9 is considered a master regulatory step of chondrogenesis defining the commitment of a cell such as the MSC down the chondrogenic lineage. The action of Sox9 is enhanced by the related molecules, sox5 and sox6 [127], with recent evidence suggesting that the exogenous introduction of the combination of the three is sufficient to induce chondrogenesis in a variety of cell types [128]. In human bone-marrow-derived MSCs, exogenous expression of Sox9 led to increased proteoglycan deposition and expression of link protein [129,130]. It has been shown that addition of BMP-2 resulted in dose-dependent increase in Sox9 expression in C3H10T1/2 cells, a mesenchymal progenitor cell line [131].

In addition to growth factors, several 'physical' environmental factors influence MSC chondrogenesis. These include, oxygen tension, mechanical loading and hydrostatic pressure. Articular cartilage is an avascular tissue, therefore nutrients and metabolites are provided via

diffusion from the synovial fluid and the subchondral bone and aided by the movement and resulting fluid flow in the joint. Oxygen tension in the deep zones of articular cartilage has been reported to be between 1−2% with an increasing oxygen gradient towards the articular surface [132,133]. Hypoxia is a positive regulator of the chorocytic phenotype and MSC chondrogenic differentiation. [134−136]. Regular, cyclic loading of cartilage is vital to its homeostasis *in vivo*, and cyclic loading has been shown to be chondroinductive *in vitro* [137−139] and even prevent the induction of hypertrophic markers *in vitro* [140]. Hydrostatic pressure itself, a component of the increased interstitial pressure experienced by cartilage under load, is chondro-stimulatory [141], although not in isolation, and its effects are quite variable between individuals [142]. These examples illustrate the value in understanding normal cartilage homeostasis in order to engineer better articular cartilage.

SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

The three-dimensional substrate upon which the cells are grown can have profound effects upon MSC differentiation. Considerations include the materials composition, its structure and topography, and its biodegradability. In general, scaffolds may be made of natural or synthetic biomaterials and may be in the form of hydrogels, sponges, fibrous meshes and nanofibers. Only some of the many available scaffolds are appropriate for cartilage tissue engineering. Among artificial materials, poly-glycolic acid (PGA), poly-L-lactic acid (PLA), poly-ε-caprolactone (PCL) polyethylene oxide (PEO) and polyethylene glycol (PEG) among others have shown potential in cartilage tissue engineering (Fig. 54.2) [143−149]. These polymers may be employed as either sponges formed through phase separation and salt leaching or as micro- or nanofibers formed by electro-spinning. The small diameters of nanofibers closely match the geometry of collagen fibrils comprising a natural cartilage matrix, making these structures a potentially useful biomimetic scaffold [150]. The high porosity, high surface-to-volume ratio and their unique mechanical properties account for the popularity of these structures in cartilage engineering as well. In recent studies, natural materials such as chitin [151], collagens [152], hyaluronan [153], among others, have been processed into nanofibrous scaffolds. These biomaterials have the added value of providing natural epitopes for cell attachment as well as sites for covalent attachment of signaling molecules. Co-spinning different materials together can be useful in creating complex surface topographies, greater porosity, increasing biological activities, and adjusting mechanical properties of the resulting scaffold [154]. Finally, fibers may be spun directionally to yield the proper matrix alignment as

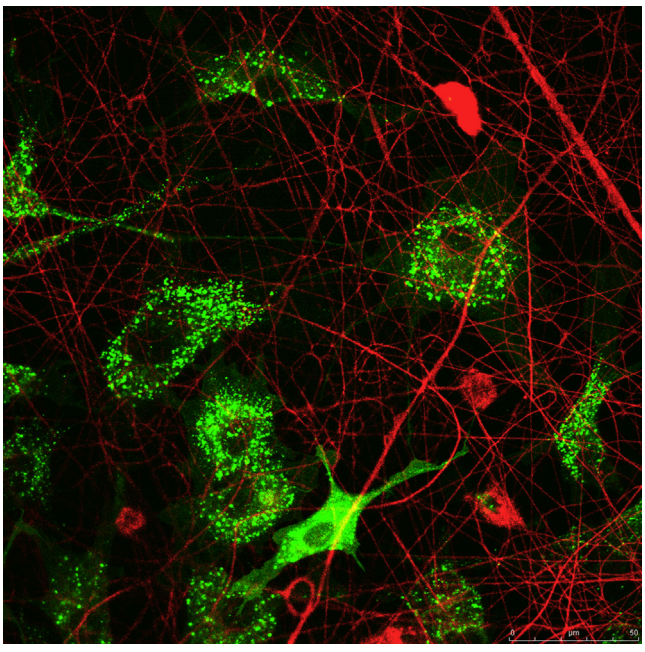

FIGURE 54.2
Confocal laser scanning fluorescence microscopy of adult human mesenchymal stem cells (MSCs; green) seeded on to an electrospun nanofibrous scaffold (red). MSCs appear to be well integrated within the nanofibrous matrix, interacting with individual fibers via cellular processes, as well as filling the 'pores' within the scaffold. Scale bar = 50 μm.

well as potentially transmit or absorb mechanical forces necessary to the protection and function of the tissue.

In addition to fibrous scaffolds, various biocompatible hydrogels have also been applied for the *in vitro* and *in vivo* growth of cartilage constructs. Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of retaining large amounts of water or biological fluids and commonly used to mimic the chondrogenic environment. Frequently used hydrogels include synthetic materials such as PEG, self-assembling peptides, and natural materials including agarose [79,155,156], alginates [157–159], the cartilage matrix components collagen type I [160–162], collagen type II [163], hyaluronan-based gels [164,165], and fibrin gels [166,167]. The exploration of each of these scaffolds has contributed to our understanding of MSC chondrogenesis for tissue engineering.

Agarose gels have been employed extensively in cartilage tissue engineering and are unique in that they offer cells no epitopes for adhesion. The agarose maintains the cell in a spherical shape, which alone has been shown to maintain and even promote chondrogenesis. The otherwise inert agarose has been useful in elucidating the effects of mechanical loading, TGFβ exposure, and differences between chondrocytes and MSCs.

Synthetic PEG hydrogels, although relatively inert, have been shown to support cartilage tissue formation by both chondrocytes and MSCs [168,169]. Unlike many other synthetic polymers, PEG may be modified to include Arginine-Glycine-Aspartyl (RGD) and other bioactive peptides [170,171] to enhance viability [172] and chondrogenic differentiation of encapsulated cells [173], and to control the mechanical [174] and degradation [175] properties of the hydrogel itself. These features have made it a staple of tissue engineering. The inclusion of cartilage matrix molecules or peptide derivatives greatly enhances the PEG scaffold support of chondrogenesis and may be tuned to create multilayered constructs [176–178]. The addition of hyaluronan is particularly potent in inducing MSC chondrogenesis [179]. Not only is hyaluronan a natural component of cartilage, but it is also functionally involved in biological processes, including proliferation, morphogenesis, inflammation, and wound healing. Hyaluronan hydrogels also support chondrogenic differentiation of MSCs, making it a promising scaffold for cartilage regeneration [180].

FACTORS INFLUENCING OUTCOMES OF TISSUE-ENGINEERED CARTILAGE

Since the quality of the extracellular matrix and the mechanical properties of engineered constructs generally fall short of the characteristics of native cartilage, many studies have focused upon the combination of chondrogenic stimuli. The evolution of a tissue-engineered construct is a time-dependent process, controlled by numerous (and sometimes unanticipated) factors. These factors include an evolving cell phenotype, changing biochemical and biophysical environments, and continuous remodeling of the matrix from the engineered scaffold to native extracellular matrix. Two philosophies govern the engineering of each component. On the one hand, tissue engineers may strive to build and control each component throughout development of the construct. On the other hand, components and processes may be permitted to proceed as naturally as possible *in situ*. In both cases, a perfect scenario involves the development of all construct components in unison to ultimately form a functional tissue. The fact that no tissue-engineered construct based upon MSCs has successfully repaired articular surfaces over the long term shows that such a coordinated development is not occurring; it demands that we seek to understand each process better, particularly as they relate to each other.

In recent years, there has been a rapid growth in the number of studies analyzing the synergistic effects of various chondrogenic stimulants

1) Different growth factor combinations,
2) Scaffolds comprised of complex materials with different mechanical, biological and biodegradation properties,

3) The addition of mechanical stimulation and or hypoxia to the differentiation equation, and
4) The inclusion of various biological matrices including devitalized tissue matrices and blood fractions including platelet-rich plasma.

While clearly all components of the tissue-engineered construct are under continuous study and improvement, but the scaffold in particular is undergoing rapid evolution. Investigators have focused on how to recapitulate the multiple roles of native extracellular matrix including its roles in structural integrity, mechanotransduction, and repository and co-receptor for various signaling molecules. Towards this end, scaffolds have been decorated with peptide sequences derived from the bioactive motifs of collagen, fibronectin and laminin (among others) through adsorption or covalent bonding. These peptides have also been modified to include substrate sequences for specific proteolytic enzymes to allow targeted release, a property which is particularly useful when non-degradable scaffolds, such as agarose, are used.

Most bioresorbable scaffolds undergo a degree of degradation that is essentially incomplete during tissue regeneration. Growth factors may be incorporated in the scaffolds using microparticles of select composition, micelles, and composite structures with specific time-release profiles to deliver changing combinations of stimulatory factors to enhance the chondrogenic phenotype of the MSC. A major advance in the use of hydrogels has been the development of photo-polymerizable monomers that make hydrogels amenable to photo-stereolithography. This technique allows the design and fabrication of detailed internal and surface microarchitecture of large scaffolds such as those required for joint resurfacing. With precise spatial control, scaffolds can be loaded with microparticles of varying composition, tuned to release encapsulated growth factors at specified times. Thus, spatio-temporal specific differentiation of the seeded MSCs can be controlled to approximate and re-create the zonal organization of articular cartilage, osteochondral plug, or musculoskeletal entheses.

Despite such excellent design and fabrication strategies, many challenges remain in using such large complex scaffolds, including enhancement of cell survival as well as efficient and spatially targeted cell seeding. The latter may be largely overcome by centrifugation or vacuum aspiration, traditionally used to seed fibrous scaffolds, or direct incorporation of cells within the scaffold during fabrication, a technique commonly used with hydrogels. In the bottom-up approach to tissue engineering using photo-stereolithography, another challenge is the cytotoxic conditions generated by the UV-activation of photoinitiators during polymerization of the monomer solution. A solution has recently been reported by the development of a visible light-activatable photoinitator-based method of projection stereolithography [181].

Bone tissue engineering

Bone has a vigorous potential to regenerate itself after damage; however, the efficacious repair of large bone defects resulting from resection, trauma, or nonunion fractures still requires the implantation of bone grafts. Five hundred thousand surgical cases of bone grafting procedures are performed annually [182], and the demand for bone grafts is expected to be even greater over the next decade as the population ages. Natural bone grafts have been used extensively in clinical settings. Autografts, considered the gold standard for bone implantation, have the advantage of immunocompatibility over allografts and xenografts. However, complicating issues such as donor site morbidity, risk of infection, and the availability of bone tissue of the correct size and shape, limit the use of autografts in orthopedic applications. One possible remedy for the shortage of bone grafts is a functional tissue-engineered bone graft possessing one if not all of the following properties – an osteoconductive matrix, osteoinductive factors, and osteogenic cells [182].

MSCs are an attractive cell type for bone tissue engineering due to their extensive expansion and osteogenic differentiation capacities. Traditionally, MSCs are induced to undergo osteogenesis *in vitro* by the use of chemical supplements as described earlier. Growth factors, such as Wnt family members, have also been shown to play a role in osteogenesis [183]. *In vitro*, exposure of MSCs to Wnt3a-conditioned medium or overexpression of ectopic Wnt3a inhibits osteogenesis. The expression of several osteoblast specific genes, e.g., ALP, bone sialoprotein, and osteocalcin, was dramatically reduced, while the expression of Cbfa1/Runx2, an early osteoinductive transcription factor was not altered, implying that the Wnt3a-mediated canonical signaling pathway is necessary, but not sufficient, to completely block MSC osteogenesis. On the other hand, Wnt5a, a typical non-canonical Wnt member, has been shown to promote osteogenesis. Since Wnt3a promotes MSC proliferation during early osteogenesis, it is very likely that canonical Wnt signaling functions in the initiation of early osteogenic commitment by increasing the osteoprogenitor reservoir, while non-canonical Wnt drives the progression of osteoprogenitor to mature functional osteoblasts. The exact identity and actions of intracellular mediators of Wnt signaling in regulating MSC osteogenesis remain to be elucidated.

In order for MSCs to generate a successful bone graft, the cells require three-dimensional biomaterial scaffolds to secure them at the implantation site, provide physical protection, and maintain and direct tissue shape. In MSC-based bone tissue engineering, various biomaterial scaffolds have been evaluated for their potential as cell carriers. These scaffolds may be made from natural or synthetic materials that have been fashioned into structures with different shapes and sizes. In general, natural polymers, such as collagens, contain bioactive domains favorable for biological activities involved in tissue regeneration, whereas synthetic polymers, such as poly(α-hydroxyesters), feature controllable material properties that can approximate the physical properties of native tissue. Among the materials that have been used in bone tissue engineering, hydroxyapatite (HA) and their derivatives, such as β-tricalcium phosphate (β-TCP), are the most common scaffold materials for osteogenic induction of MSCs. Bioresorbable β-TCP based scaffolds, compared to HA based ones, showed comparable results on ectopic bone formation [184] and, moreover, both synthetic ceramics support osteogenic differentiation more efficaciously than demineralized bone matrix [185]. To improve the affinity of osteoconductive ceramics for cells, HA has been coated with bioactive peptides [186] or proteins [187], to enhance MSC attachment and osteogenic differentiation.

Composite scaffolds, composed of multiple materials, are expected to be physically and biologically superior to single-material-based scaffolds, as the properties of a composite may be programmatically varied by mixing different materials in various ratios. Both the composition and the relative ratio of the constituent materials can affect bone formation. HA has been used as a primary material combined with other materials such as TCP [188], poly-lactic-co-glycolic acid (PLGA) [189] and chitin [190] to produce various composite scaffolds. It was reported that scaffolds with different ratios of HA/TCP loaded with MSCs showed different extents of bone formation *in vivo*. Composites in which the HA/TCP ratio was designed to coordinate scaffold degradation with tissue deposition seemed optimal in promoting the greatest ectopic bone formation [191].

Finally, growth factors, cytokines, and other non-proteinaceous chemical factors are critical for osteogenic differentiation of MSCs, as mentioned earlier. To successfully augment bone formation, it is necessary to continuously introduce osteoinductive molecules, most of which have a short half-life, into the cell culture or the defect site. One strategy for enhancing bone formation is to use biomaterial scaffolds both as a cell carrier as well as a reservoir for the release of growth factors in a controllable manner. BMP2 is the most efficacious growth factor among the BMP family members, and has been incorporated in various forms of biomaterial scaffolds to induce osteogenesis in *ex vivo* cultures [192], or to stimulate bone formation *in vivo* [193], as well as in clinical applications (e.g., INFUSE® Bone Graft/LT-CAGE® Lumbar Tapered Fusion Device, Medtronic).

Osteochondral tissue engineering

Severe joint defects often extend to damage or destruction of subchondral bone, leading to associated pain and mechanical instability of the joint. Clinical results show that, even for a partial thickness cartilage defect, there are beneficial effects in exposing subchondral bone by drilling, as well as the incorporation of a bone layer as an anchor to securely integrate grafts with host tissue. Osteochondral grafts or plugs are used clinically in the treatment of both chondral and osteochondral defects.

Tissue engineering offers a promising alternative to autologous osteochondral grafts through the combination of biocompatible materials possessing widely varying physical properties with the multilineage differentiation potential of MSCs. Chondrogenesis and osteogenesis of MSCs require different physical and biochemical cues from matrices and soluble growth factors/cytokines, respectively. A commonly used approach is to fabricate cartilage and bone independently before integrating them together using sutures or glues. MSCs loaded into a hyaluronan or a TCP ceramic scaffold were separately induced to undergo chondrogenic or osteogenic differentiation, respectively. Then the two components were integrated together with fibrin sealant, becoming a single unit of an osteochondral construct [194,195]. One drawback with this method is the less-than-optimal integration between the chondral and osteo constructs; poor integration leads to discontinuous cell distribution at the interface and/or the possibility of eventual separation of the two components. Several groups have reported different alternatives to solve the problem of discontinuous interface. In one approach, MSCs after *in vitro* chondrogenic or osteogenic differentiation were loaded into two separate PEG hydrogel layers, then the cell-loaded PEG gels were photopolymerized together. Since the two components were combined before gel solidification, the osteochondral construct exhibited a less defined gap-line [196]. Another approach was to apply a press-coating process [197] to fabricate osteochondral constructs. A PLA scaffold was pressed into a high-density pellet of chondrogenically-induced MSCs then seeded at the opposite end with osteogenically differentiated MSCs. Macroscopically, the osteochondral composite consisted of a cartilage-like layer adherent to, and overlying, a dense bone-like component [198]. Since both cartilage and bone were produced in a single unit, no gap developed between the two tissues; instead, an interface resembling the native osteochondral junction was observed. Recent attempts have included the combination of MSCs encapsulated in microbeads of collagen type I, separately induced into osteogenic and chondrogenic lineages, with an interface consisting of undifferentiated MSCs, which resulted in a trilaminar osteochondral construct with an interzonal 'tidemark'-like structure [199].

The ideal scenario for the fabrication of bi-phasic osteochondral constructs would be to differentiate MSCs cultured in a single unit scaffold into chondrocytes on the top and osteoblasts on the bottom. To this end, the biomaterial scaffold should chemically and structurally support both chondrogenesis and osteogensis. Nanofibrous scaffolds morphologically resembling natural ECMs have been shown to successfully support both chondro- and osteogenesis of MSCs *in vitro* [200]. Another need for achieving this goal is a culture system in which an MSC-laden construct can differentiate into cartilage and bone simultaneously. A double-chamber bioreactor with a unique two-compartment design allowing the storage of different media was used to culture osteochondral constructs [201]. With this double-chamber bioreactor, it would be feasible to engineer an autologous osteochondral graft using MSCs by means of a nanofibrous scaffold.

Engineering other skeletal tissues with MSCs

In addition to their clear roles in engineering bone and cartilaginous tissues, MSCs may be readily applied for the repair of numerous other musculoskeletal tissues, including tendon and ligament repair. In early studies, it was observed that rabbit MSCs contract collagen type I carrier gels and that the delivery of these constructs to patellar and Achilles tendon defects

improved the biomechanical properties of the repair tissue as compared to acellular controls [202]. Newly emerging silk scaffolds that have been modified with RGD moieties and/or formed into nanofibrous scaffolds may also prove useful for tendon/ligament tissue engineering with MSCs [203]. Hybrid scaffolds consisting of silk and bFGF-releasing PLGA stimulated MSC proliferation and increased expression of tendon specific markers [204]. Studies applying tensional and torsional mechanical stimulations to MSC-seeded collagen gels *in vitro* have shown enhanced tenogenesis [205]. These studies suggest a potential role for MSCs in regenerative applications for tendon and ligament defects that may be modulated by the mechanical loading environment.

Another tissue in which exogenous MSC application has been used to effect repair is the fibrocartilaginous knee meniscus. It has been reported that intra-articular injection of MSCs derived from synovium [206] (absent a tissue engineering delivery vehicle) can have an ameliorating effect on meniscus degeneration. Similar results have also been shown when MSCs are seeded on various types of scaffolds and implanted into meniscal defects. These scaffolds included devitalized meniscal allografts [207] and fibrin glue [208], as well as, hyaluronan/gelatin [209] and hyaluronan/collagen [210] composite matrices. Clearly, MSCs can play a role in the reparative process of the meniscus, although the mode of their administration and the nature of the carrier materials require further optimization. Interestingly, multipotent mesenchymal cells have also been identified and isolated from bovine meniscus [211], suggesting that an endogenous reparative mechanism may reside in the meniscus.

Gene therapy in musculoskeletal tissue engineering

Gene therapy is a promising technique for disease treatment, in which genetically modified cells are transferred into or generated within individuals for therapeutic purpose. The cells with modified genes can be induced to differentiate into desired cell types or produce proteins needed for tissue repair. Although safety issues still remain, a likely future scenario is the merging of gene therapy, cell therapy, and tissue engineering for the treatment of musculoskeletal diseases.

There are two strategies that are currently used in gene therapy, viral transduction and non-viral transfection, and both strategies can be conducted *in vivo* and *ex vivo* [212]. *In vivo* gene therapy is simple but often lacks controlled tracking of the gene-modified cells and target site specificity. An attractive alternative is to combine gene therapy with tissue-engineering approaches to transduce or transfect cells of interest *ex vivo* and then use a carrier or scaffold to deliver these genetically modified cells to the target site. This approach offers the advantages of flexibility of target cell type and retainment of gene-modified cells at the site of interest. Gene transduction using viral vectors, such as retrovirus, adenovirus, and lentivirus, effectively modifies the host chromosome but raises concerns about mutagenesis and possible immune reactions. In contrast, a non-viral approach is safer but the efficiency of transduction is lower. More research efforts are needed to overcome the limitations associated with each approach. The ultimate goal is to develop a simple, safe, and effective means to transfer genes into cells of interest.

Currently, the most frequently applied example of gene therapy in bone and cartilage tissue engineering is the transduction/transfection of MSCs with BMP genes. BMPs play important roles in the regulation of osteogenic differentiation of MSCs and the production of bone matrix during bone formation. To overcome the short half-life of growth factors, MSCs are transduced or transfected with BMP genes for continuous protein expression *in vitro* and *in vivo*. BMP2 transduced MSCs were induced to differentiate into osteoblasts producing bone matrix and synthesize BMP2, thus attracting host cells to migrate and differentiate. Compared to control MSCs, the BMP2-producing MSCs effectively enhance bone formation, even at large defect sites like segmental femoral defects [213]. Similar results have also been reported

when BMP2 or BMP4 transduced MSCs were delivered to bone defects using different biomaterial scaffolds, such as demineralized bone matrix [213], gelatin [214], β-TCP [215], and calcium phosphate cement [216]. Currently several research groups are testing the effects of overexpressing a combination of factors such as BMP with VEGF, a potent inducer of angiogenesis [217]. The potential benefits of cartilage repair using gene therapy are also gaining recognition. For example, BMP7 transduced MSCs delivered by PGA scaffolds successfully regenerated cartilage, whereas the control non-transduced group did poorly [218], and BMP4 transduction induced chondrogenesis and enhanced cartilage repair [219].

CONCLUSIONS AND FUTURE PERSPECTIVES

Due to their ease of isolation, their capacity for undergoing *in vitro* proliferation to achieve a large number of cells for cell therapy, their ability to undergo lineage specific differentiation into musculoskeletal cells, and their potential immuno-modulatory advantages, MSCs present significant potential in musculoskeletal skeletal tissue engineering, which promises to bring hope to patients and surgeons alike for the generation of functional tissue substitutes. As discussed in this chapter, MSCs have been used in tissue engineering of a number of musculoskeletal tissues, including cartilage, bone, osteochondral constructs, ligament, and tendon. Currently, tissue-engineered constructs have not been readily accepted for clinical use to treat skeletal tissue defects. There clearly is a need for further research that combines concerted efforts of biologists, engineers, and clinicians. Critical to the success of these approaches is a better understanding of MSC biology. So far, there has not been a marker that can be used to unequivocally identify and prospectively select MSCs from various tissues. The factors that regulate phenotype transition, i.e., uncommitted *versus* differentiated phenotype, remain to be identified and studied. Additionally, the various growth factors, signaling pathways, and transcription factors that can influence MSCs to differentiate completely and stably into a desirable lineage require further elucidation. Despite our incomplete understanding of MSC biology, their use in musculoskeletal tissue engineering is of high potential. Current and future efforts in this area should focus on the fabrication of optimized scaffolds, systematic studies of the interplay of MSCs, scaffold and environmental factors, and the development of quantitative outcome measurements for tissue-engineered constructs. *In vivo* testing of the engineered construct serves as the gold standard for the long-term survival of the constructs, and warrants much greater attention. The immunosuppressive and anti-inflammatory effects of MSCs present a potentially powerful biological target for their use in allogeneic transplantation in the inflammatory environment of an injury site. With further research and development, the use of MSCs in tissue engineering is expected to bring to fruition a tissue substitute suitable for implantation to improve the quality of life of patients with debilitating musculoskeletal injuries.

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CHAPTER 55

Bone Regeneration

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INTRODUCTION

Bone regeneration is a marvelous *pas de deux* of biology and biomechanics wrapped in discrete temporal events nurtured by a lush vascular cascade. The outcome of the highly complex process of regeneration is the *restoration of form and function*, ideally, enduring, to a bone insufficiency. Broadly, such an insufficiency may be stated as *any discontinuity in bone integrity, spanning microfractures, through macrofractures to developmental-congenital defects to avulsive trauma and surgical resection.*

An epochal question: How to harness the intrinsic regenerative capacity of bone? Bone regenerative tacticians have proposed a *deconstructive approach,* that is: What are the compositional elemental components of bone beginning with cells, extracellular matrices (organic and inorganic) and soluble signaling molecules? Further posing the questions: How do these compositional elements inspire the multi-tiered temporal osteogenic regenerative cascade? Is there a trigger factor? Are there co-factors?

The prototypic and conventional approach to bone regeneration for this chapter is to highlight bone regeneration by exploiting contemporary, albeit pedestrian approaches of autografts, allografts and xenografts. First, we will define these modalities and emphasize what is good about them and what is lacking. We will further offer consensus definitions of the biological and biomechanical properties of regeneration, stating specific performance parameters that define the temporal road map for the regenerative cascade. As such, we will exploit a *fracture healing model* as a prototype for the regenerative process. We will introduce key directional concepts that must be acknowledged for the rational design and development of a regenerative therapy. These concepts will include the *osteogenic biohemodynamic cascade* and for bone regenerative therapies, the importance of a *4D environmental blastema matrix* where embryogenesis is recapitulated. And lastly, we will offer stringent performance criteria with objective data to provide a framework for the rational design and fielding of a master tool kit for bone regenerative therapeutics.

CURRENT CLINICAL PRACTICES

We will begin with an explanation of the most commonly employed techniques for bone regeneration. At the current time, this practice revolves around natural (as opposed to synthetic) bone and its derivatives.

We will first establish a broad criterion for the generic term *graft.* We will carefully partition the term *graft* and *implant*; then abandon this rigor in favor of clinical jargon.

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Grafts contain cells; *implants* do not. Therefore, an *autograft* (recipient and donor being the same individual), contains *cells*. An *allograft* (recipient and donor being genetically matched individuals), contains *cells*. However, traditional tissue banking procedures effectively and efficiently remove cells from allo-stock bone, rendering the allo-stock product *acellular* and *non-immunogenic*. Consequently, such a product should be defined scientifically as an *alloimplant*. Nevertheless, clinical jargon has made an exception to the stringent scientific definition, and while incorrect, *allograft* has evolved as a clinically acceptable term for an acellular *allo-stock* product. Therefore, in this chapter, we will use the term *allograft* (despite the fact that the term *alloimplant* is scientifically correct).

In order to understand the rationale for the use of bone-derived products for regeneration, it is important to define the criteria by which we measure successful bone regeneration. Bone healing and subsequent new bone formation after the implantation of a graft occur through the processes of osteogenesis, osteoinduction and/or osteoconduction [1]. *Osteogenesis* is the process by which osteoblasts at the defect site express osteoid that subsequently mineralizes, yielding new bone. *Osteoinduction* is the induction of osteoprogenitor cells (or other non-differentiated cells) to differentiate down an osteoblast lineage. *Osteoconduction* is the property by which a graft supports the attachment of new osteoblasts and osteoprogenitor cells. In this situation, the graft must provide an interconnected structure through which new cells migrate and blood vessels form (i.e., angiogenesis). An ideal bone graft is characterized by all three of these phenomena and clinically will promote regeneration of bone that is physiologically and functionally indistinguishable from the pre-injury defect site.

Bone autografts are harvested from a *donor* site in a patient (usually the iliac crest) and implanted in that same patient at the bone deficient locale (i.e., *recipient* site) [2]. Autografts are considered to be the gold standard for bone grafting applications for several reasons. Firstly, autografts satisfy all three of the previously mentioned bone regeneration criteria — that is, they are osteoconductive, osteoinductive and promote osteogenesis. The autograft is intrinsically vascularized and may be co-harvested with a vascular pedicle. Consequently, the autograft provides an ideal combination of biological signals for integration of the new bone to the recipient site. Additionally, autograft is the patient's own tissue, thus mitigating the risk of immunological sequelae. However, there are drawbacks associated with the use of autografts: they are limited in quantity, shape restrictions often require extensive intra-operative modifications, and donor-recipient procedures require a 'harvesting' site on the same patient [3]. As with any surgery, surgical complications may include inflammation, infection, chronic pain and donor site morbidity [4].

Allografts are tissues harvested from healthy, prescreened human donors and are processed and preserved for implantation in a patient. The benefits of allografts include eliminating the need for a second surgical site on the patient requiring the graft. However, allogeneic bone (i.e., the *allograft* bone donated by the same species: human-to human) is not necessarily immunopriviledged and may activate an immune response in the new host [5]. Additionally, during tissue bank processing, allografts go through freeze-drying, 'washing' (to render the product cell-free), demineralization (partial or complete — residual mineral may be up to approximately 4–6%), and gamma-irradiation or ethylene oxide sterilization. While these techniques lessen the risk of disease transmission and immunological responses, they also reduce the osteogenic potential of the graft.

An alternative to auto- and allografts are *xenografts*: bone from non-human species. Xenografts are processed to ensure sterility and biocompatibility and this processing must mitigate disease transmission [6]. Xenogeneic bone is administered as a *bone void filler*. Examples of xenogeneic bone products include BioOss [7], an inorganic matrix from cows and XCM™ (Synthes), porcine organic bone matrix. While xenografts may have their uses in bone regeneration, the risks of disease transmission (however minimal they may be) and

ethical issues have limited their clinical appeal and thus, they have not really 'caught on' in the clinic.

Autografts and allografts have many clinical benefits and thus constitute a majority of bone graft procedures. However, recognized limitations have necessitated the search for alternatives. Therefore, laboratory inspired bone tissue-engineering approaches have been assiduously pursued. We emphasize that a rational approach to regenerative bone tissue-engineered products must be based on fundamental osteobiology. It is thus imperative to understand mechanisms, concepts and the guiding principles of the osteogenic cascade.

CONCEPTS AND DEFINITIONS

Our bodies have evolved sophisticated mechanisms to heal wounds. When injured, there are two distinct responses that can be initiated: *repair* and *regeneration*. *Repair* is the restoration of the continuity of tissues at the injury site, but not necessarily by the same cells and tissues that existed prior to the injury. *Regeneration*, however, is a series of biologically- and biomechanically-inspired events that produce restoration of form and function of the injured tissues to a state that is biologically and functionally indistinguishable from the pre-injury wound site [8].

The molecular-level biological agents involved in wound healing and regeneration are slowly being elucidated. Our collective understanding of these processes has increased. One area, however, that still causes confusion is the nomenclature in literature of biological factors, specifically between *growth factors* and *cytokines*. In this chapter, *growth factors* are defined as biologic agents whose actions are exerted on cells of mesenchymal lineage and *cytokines* are defined as agents acting on cells of hematopoietic lineages, including immune cells.

Biological factors are crucial for regenerative processes and can be classified into three categories – *autocrine*, *paracrine* and *endocrine*. *Autocrine* signaling refers to a cell releasing an agent that acts on the same cell type. *Paracrine* signals affect those cells that are in the same area as the cell from which the signal originated. *Endocrine* signals are those that must travel through the bloodstream in order to reach their target.

Biology is characterized by redundancy, which results in many similarities among responses of tissues to injury, particularly with respect to the biological agents [9,10]. One key difference between osseous and non-osseous (aka 'soft') tissues is their respective regenerative potential. Soft tissues *heal* exclusively by scar (i.e., fibroblast generated collagen) tissue formation, resulting in the restoration of tissue continuity at the injury site at the expense of the original tissue function. Osseous tissues, however, have the potential to regenerate to a state that is biologically and biomechanically indistinguishable from that derived from embryogenesis.

If bone would *always spontaneously regenerate* and overcome any manner of insufficiency/defect, this chapter on bone tissue regeneration would not be necessary. There are limitations to the regenerative potential of osseous tissue that renders defects (i.e., deficiencies: congenital, surgical, avulsive) greater than a certain size unlikely to regenerate [11]. This size has been defined as a *critical sized defect* (CSD), which is:

> 'the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal' [12,13].

The central question is: how do we extend our regenerative capabilities for sub-critical size defects to bone insufficiencies larger than the critical size?

The answer lies in elucidating, defining and understanding the differences in each of these pathways. Specifically, what is the osteogenic pathway for a critical sized defect? For a non-critical sized defect? Consequently, a prototypic pathway is an obligatory first step. Consequently, the next section introduces a *fracture healing model*. This model is defined by systematic processes of meticulously choreographed events that lead to bone regeneration. Bone tissue

engineers must exploit and recapitulate the complex process of fracture healing, capture the biological and biomechanical nuances in its evolution, and thus, define performance criteria for achieving bone regeneration in critical size defects with tissue-engineered products.

FRACTURE HEALING MODEL

In developing novel bone tissue-engineering tools, we seek to extend our bodies natural regenerative capabilities to include bone defects larger than the critical size. Fracture healing is the prototypic physiological model for bone regeneration. It is a multistage process that is characterized by complex, yet well-orchestrated, predictable steps in response to an injury. Fracture healing begins immediately following the injury and ends following the *remodeling* of the newly formed bone into mature bone. This process has components that recapitulate the processes of *de novo* bone formation during embryogenesis [14].

The process of fracture healing is a multi-phase, multi-tiered series of events segmented into four main steps:

1) The formation of a hematoma,
2) The migration and mitosis of mesenchymal cells,
3) Cartilage formation and substitution of cartilage by bone, and
4) Remodeling [15].

Immediately after a bone is fractured, the damage to local vasculature at the fracture site is responsible for producing a *hematoma*, or a blood clot. A hematoma is a localized collection of blood products including platelets, leukocytes, macrophages, fibrin and soluble biological growth factors and cytokines. This first phase of fracture healing, termed the *destructive phase*, lasts for about three days and is characterized by inflammation and local hypoxia [9].

The constructive phase of regeneration follows the destructive phase (Fig. 55.2) and begins roughly three days after the injury. It is characterized by new vasculature formation due to the migration and subsequent capillary formation of endothelial cells. Local hypoxia during the destructive phase is a stimulant for the formation of new blood vessels (i.e., angiogenesis). The formation of new vasculature allows for the recruitment of mesenchymal stem cells

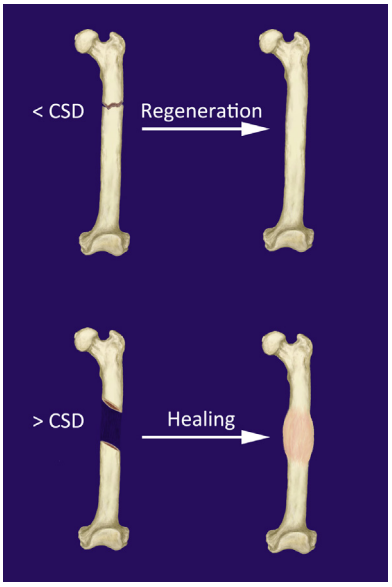

FIGURE 55.1
Bone will regenerate 'gaps' smaller than a critical size but when a 'gap' exceeds a certain volumetric dimension, the outcome of the healing process is fibrosis rather than osteogenesis.

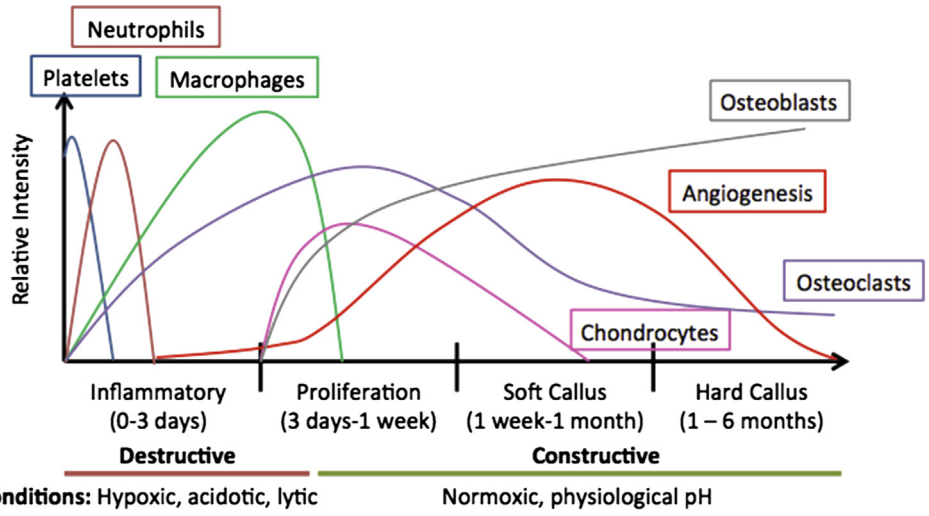

FIGURE 55.2
The temporal activity of key cell phenotypes and events during fracture healing.

(or pericytes: fusiform cells of the mesenchymal lineage that line blood vessels) that differentiate into chondrocytes and osteoblasts as well as providing the conduits for monocytic-derived *osteoclasts* [16,17]. The *chondrocytes* are responsible for cartilage matrix formation and the *osteoblasts* produce the new bone and the osteoclast-osteoblast coupling remodel that bone.

Granulation tissue condenses into a 'soft' callus that acts as a stabilizing buttress, supporting the bone fragments at the distal and proximal bone fragments. The elegance of this soft callus formation is that it provides the bone fracture site with a cartilaginous scaffold that acts as both a fixation and stabilization structure and a template for subsequent mineralization [18]. Chondrocytes in the soft callus undergo a programmed cell death (apoptosis); concurrently, osteoblasts deposit bone matrix (osteoid) to replace the soft callus. At the fracture locale, over the course of months to years, the bone is remodeled to a physiological status indistinguishable from the pre-fractured condition [19].

The processes driving the biology and biomechanics of bone regeneration at a fracture remain in large part, a mystery. There are many highly complex interactions among multiple cell types and mediated by soluble and non-soluble signaling agents that have not been sufficiently characterized and elucidated. *The profoundly compelling challenge for biomedical tissue engineers is to deconstruct the regenerative process of fracture healing and subsequently stitch those components together to produce osteo-regenerative therapies for applications in bone defects exceeding the 'critical size'.*

PERFORMANCE CRITERIA FOR BONE REGENERATION

We establish a goal for bone regeneration: Recapitulate the *Biohemodynamic Cascade* of fracture healing on a larger scale, one that may be applied to osseous regeneration of the critical sized defect. We then ask the following: How can we mimic fracture healing to develop therapies that consistently regenerate bone in gap defects? What must bone tissue engineering develop as a therapy to regenerate bone defects that exceed the ability of the body to spontaneously promote regeneration, that is, to regenerate bone in critically sized defects? The clinical requirements for bone regeneration demand that we develop products that outperform the existing standards set by autografts and allografts. We must develop a biologically- and biomechanically-based therapy that is osteo-angiogenic, that is: fulfills the parameters of the *biohemodynamic cascade* of fracture healing.

Our approach for achieving this is two-pronged: Since bones have an intrinsic ability for regeneration, our first mission is to design therapeutics that *do not interfere* with the natural regenerative processes. The body executes a network of biological and biomechanical

events that are highly complex; as such, we must not interfere with the intrinsic processes but rather, supplement them. The second tactic to our approach is that bone regeneration therapeutics must work *synergistically* with endogenously-driven healing cues. Therefore, we must carefully engineer therapeutics to match the biology and biomechanics of natural bone regenerative processes.

Safety

A fundamental requirement of any bone regeneration therapeutic is that it must do no harm to the patient. This is a basic ethical principle for scientists, clinicians and the corporate sector. Doing no harm to the patient includes ensuring the biocompatibility of bone regenerative therapeutics. When a bone therapeutic is implanted at the site of a bone insufficiency, the first interactions are with host blood and blood proteins. Therapeutics must not elicit either an acute or a chronic immunological response [20].

An additional factor affecting biocompatibility, and therefore the safety of a therapy, is the degradation properties of its materials. Therapeutics based on materials such as metals, ceramics and polymers may degrade in the body. Polymeric systems such as the polyalpha hydroxy acids degrade by hydrolysis or enzymatic processes. While we have already stressed the importance of biocompatibility *upon implantation*, of further importance is the biocompatibility *of degradation products*. Polyalpha hydroxy acids such as polylactide and polyglycolide degrade into lactic and glycolic acids, producing an acidotic environment. As such, it is crucial that therapeutic bone grafts degrade in a fashion that does not hinder bone regenerative activities.

Mechanical properties

We have discussed the importance of avoiding immunologic responses, but we have not yet mentioned the biomechanical requirements for a bone regeneration therapeutic. Initially, a bone defect site is in a state of dynamic instability, where structural integrity has been compromised but may still be subjected to loads. Consequently, the biomaterial must provide sufficient mechanical strength and be able to accommodate to tensile, compressive and shear forces. Moreover, the material must degrade in a coordinated temporal manner with the new bone formation as well as support the new vasculature. If a bone graft material is degraded too quickly (i.e., before the infiltration of bone begins — roughly four weeks after the injury), the area will fail to provide the requisite support. Conversely, if the material remains at the bone recipient site for longer than this four week window, it will impede regeneration. Furthermore, implanted materials in bone must support physiological loads, for which there is a biomechanical threshold. A material that exceeds this threshold will cause *stress shielding* of the bone and promote bone resorption. Many of the terms and end-stage properties stated above are well-known to bone tissue engineers. However, what is neither well-known nor precisely defined, are the *quantitative performance criteria* that match to 'sufficent strength', 'degradation' and 'how slowly or rapidly' a bone regenerative material should either be retained or degraded. An inadequate product will be engineered when specific performance criteria are lacking. Consequently, a focused effort must be made by bone tissue engineers to accurately and precisely define, with rigorous science, performance criteria for bone tissue-engineered product. Based on the timeline of fracture healing, an implanted material should be fully degraded by the formation of the soft callus, providing a target of roughly two to four weeks for full degradation [18].

Bone regenerative properties

General performance criteria for bone graft therapeutics must include properties of osteoconductivity, osteoinductivity and osteogenicity.

An osteoconductive graft will support the attachment and differentiation of the many different cell types that migrate to the defect site. One method for influencing osteoconductivity in synthetic grafts is by tailoring the hydrophilicity and hydrophobicity of the graft surfaces. These surface properties enable cell attachment and protein adsorption; however, they alone are not sufficient for a suitable therapeutic [21].

Another strategy for improving cellular adhesion to bone graft materials is to engineer peptide-based adhesion sequences, such as the arginine-glycine-aspartic acid (RGD) sequence and extracellular matrix components such as collagen, fibronectin [22], and vitronectin. Moreover, the inclusion of angiogenic growth factors in the composition, such as vascular endothelial growth factor and basic fibroblast growth factor, can contribute to the osteoinductive ability of a bone biomaterial [4]. This approach is akin to *compositional engineering*. Compositional engineering of bone regeneration therapies must comply with the temporal and dosing profile of the *osteogenic cascade* (Fig. 55.3). Specific biological factors are expressed at certain, designated time(s) during osteogenesis and bone regeneration. Moreover, there may be a waxing and waning of biological agents. These agents evoke an outcome driven by cell phenotypes that craft extracellular matrices resulting in tissue regeneration. We posit that bone 'gaps' less than a certain size will regenerate as a consequence of sufficient biological cues and cellular craftsmen; exceeding that certain size, that is, being critically sized, these 'gaps' will undergo fibrogenesis rather than osteogenesis. Consequently, only by following the *biological roadmap* with its meticulously timed and dosed boundaries that define the osteogenic cascade (Fig. 55.3), will the bone tissue engineer be able to produce a predictable and safe clinical outcome: bone regeneration of a critically sized defect.

Another approach to bone regeneration materials exploits *architectural engineering*. We define this as the physical properties that may be adjusted in a material to inspire bone regeneration. An example of architectural engineering is *pore structure* (i.e., *void volume*) [23]. Macroporosity (pore size >50 μm) has been shown to promote cell ingrowth as well as support angiogenesis. Microporosity (pore size <10 μm), on the other hand, plays a role in cellular signaling via transport of biologically active signaling agents [24]. A combination of the macro- and microporosity may sustain osteogenesis and angiogenesis [25] throughout the material, assuming those pores are *interconnected*. Further, pore size range, pore distribution and volumetric porosity comprise the three parameters available to the bone tissue engineer for modulating the design of a product that matches the intended clinical use. The design of a porous structure for bone regeneration is a delicate art as volumetric void volume is inversely related to overall strength of the material.

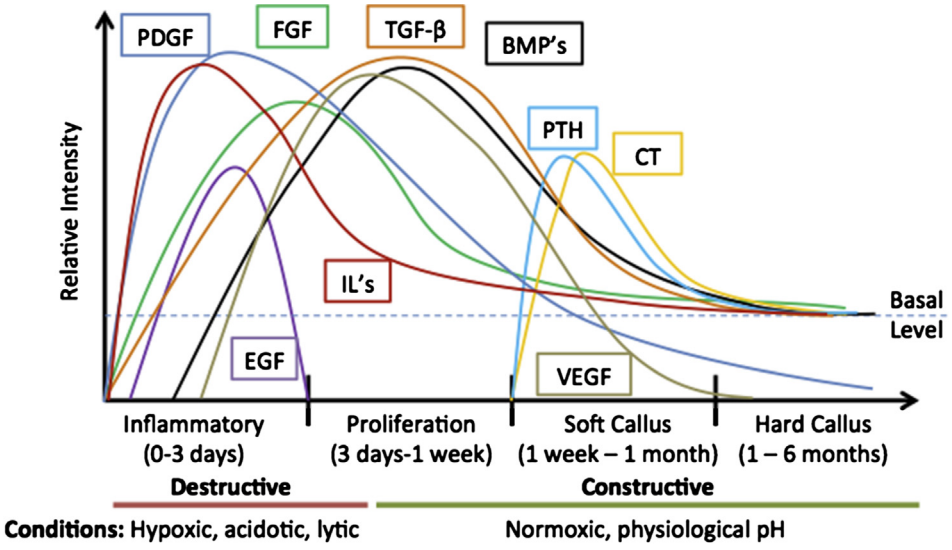

FIGURE 55.3
Relative levels of key biological factors in the bone regeneration cascade, compiled from multiple sources [9,15,18,26–28].

TABLE 55.1 Recommended temporal windows (given as days post injury) in which to deliver fracture healing biologicals based on extensive literature reviews [14,16,28–35]

Growth factor	Proposed delivery period
PDGF	0 – 3 days
VEGF	14 – 21 days, though required earlier as well
BMP	BMP-4: 3 – 5 days BMP-2: 3 – 21 days
PRP	0 – 5 days

Recapitulating osteogenesis within bone defect requires precise, dose-dependent external contributions. Therefore, we are obligated to pay attention to the specific spatial, dosing and temporal events that occur in fracture healing and embryogenic bone formation. By delivering cells and biological factors to a bone defect site, we may overcome the limitations in bone regenerative potential caused by an insufficiency. However, cells and biologicals must be delivered at the appropriate dose, times and with release profiles mimicking the osteogenic cascade (see Fig. 55.3, above). For example, the delivery of pro-inflammatory cytokines must occur within the first 72 hours after injury. If delivery of these cytokines is delayed, constructive phase events of bone regeneration may be impeded. Table 55.1 summarizes our recommended delivery times for several growth factors based on their consensus appearance in bone regeneration.

CLASSICAL RESEARCH APPROACHES

Now that we have highlighted the performance criteria for bone regeneration approaches, we will discuss current tactics in bone regeneration research. It is worth noting that in spite of the criteria provided above, there are many approaches to bone tissue regeneration. Moreover, owing to the tremendous complexity (and diversity of opinions) about the process of bone regeneration, it is naïve to assume a single approach either will fit all clinical indications or provide solutions in all circumstances. However, we underscore that contemporary efforts to promote bone regeneration focus on cells, biological factors and materials.

Cell-based approaches for bone tissue engineering

The antecedents for the cell phenotypes in bone regeneration trace their pedigree to hematopoietic and mesenchymal lineages. Cells that resorb bone, debris, and implant material – such as macrophages and osteoclasts – are derived from hematopoietic monocytes. Monocytes may diverge to either macrophages or osteoclasts depending on the biological environmental cues. Macrophages release cytokines to recruit mesenchymal cells for neovascularization, as well as orchestrate an anti-inflammatory, pro-healing environment [36]. Macrophages and monocytes, as well as the osteoclasts are marquis players during the *destructive phase* of bone regeneration.

During the *constructive phase,* mesenchymal cells differentiate to pre-osteoblasts. The periosteum and endosteum, as well as vascular lining pericytes [31], are the putative sources for the preosteoblast lineage. Biological cues for the progression of the differentiation of the precursor cells to pre-osteoblasts are under the aegis of bone morphogenetic proteins.

The regeneration of bone in a bone deficient site is a time-sensitive process; as such, if bone formation by osteoblasts does not occur in register with *soft callus* remodeling, the outcome will be fibrogenesis rather than osteogenesis. Moreover, mechano-transductive signals matched to bone will not occur: fibrous tissue is not biofunctionally equivalent to osseous tissue. Consequently, a 'pool' of programmable osteoblastic precursors must be localized to

TABLE 55.2 The advantages and disadvantages of commonly used biomaterials for bone regeneration

Material	Advantages	Disadvantages	References
Alginate	Can be used for drug delivery, cell encapsulation, wound dressing, anti-adhesion applications; biodegradable	Anti-adhesive, mechanically weak	[86–89]
Bone Mineral Matrix	Low immunogenicity, well documented success	Potential for disease transmission, decreased mechanical strength, limited supply	[88,90,91]
Chitosan Sponge	Can be used as a hemostat or to deliver soluble signaling molecules, biodegradable	Mechanically weak	[88,92,93]
Collagen Sponge	Biocompatible and osteocompatible, antigenicity can be weakened by removing telopeptides, biodegradable, adhesive, highly porous, can be incorporated with new tissue matrix or combined with other materials	Lack of rigidity (mechanically weak), potentially antigenic	[88,94,95]
Collagen-Ceramic composite	Biocompatible, biodegradable, degradation profile better than collagen alone, spatially adaptable, can control shape, higher stiffness than collagen sponge, increased particle and defect wall adhesion	Mechanically weak	[88,96–98]
Hyaluronic Acid	Can be used as a coating or to deliver cells or signaling molecules, low immunogenicity, hydrophilic, injectable	Low cell adhesion, mechanically weak	[88,99,100]
Hydroxyapatite	Moldable, can be used for coatings, high degree of tissue integration, creates undistinguishable unions with bone, low immune response	Risk of disease transmission, requires increased time for bony restoration	[88,101,102]
Polycaprolactone (PCL)	Can be used as delivery system, mechanical properties can be adjusted	Hydrophobic, degrades slowly	[88,103–105]
Poly(lactice-co-glycolic acid) (PLGA)	Can be used as a delivery system, biodegradable, mechanical properties and biodegradation kinetics are suited for bone applications	Acidic Degradation products	[88,106–108]
Polyphosphazenes	Fine-tunable degradation profile, thermosensitive, injectable, supports cell attachment	Acidic degradation products	[88,107,109]
Polyurethane	Biostable, biocompatible, adjustable mechanical properties (durability, toughness), cost effective	Risk of toxic degradation products	[88,107,110]
Tyrosine-derived Polycarbonates	Biodegradable, cell-impermeable, low immunogenicity, can be used as a delivery system, mechanical properties (stiffness and strength) suited for bone applications, cost effective	Hydrophobic	[88,111–113]

the healing bone regenerative domain (i.e., by chemoattraction), expanded in quantity (the process of mitogenesis) and modulated to an end-stage phenotype (i.e., undergo differentiation) to produce the desired outcome: restoration of form and function. The timing of this event is but one of many 'key' movements in the dynamics of osteogenesis. Sources for osteoblastic precursor cells include mesenchymal stem cells (MSCs), adipocytes and perivascular pericytes populations contiguous to the bone wound. The tempo of regeneration must be orchestrated by a meticulous sequence between these inducible cell populations and the cueing biological signals that will produce osteoblasts. Exploiting osteoblast precursor populations is the inspiration for dedicated cell-based therapies for bone regeneration.

The bone-marrow-derived mesenchymal stem cells (BMSCs) are commonly used considered the 'gold standard' [37–39]. Bone-marrow-derived mesenchymal stem cells have been used for gap bone defect treatments and purportedly improve short-term and long-term healing [40,41]. However, there are limitations to BMSCs for bone regeneration that include the postoperative painful sequelae from the 'collection' procedure, the uncertainty about the quantity of cells necessary for a predictable outcome, variability of cell quantity that may be harvested from patients and ambiguity regarding the actual quantity of viable cells that have been implanted in the recipient site. Moreover, the lack of standardization and rigor associated with the reports on bone-marrow-derived stem cell treatments have led to controversy and vocal opposition for the clinical merit of the procedure. Despite significant clinical and scientific deficiencies of the procedure, aspirated BMSCs remain the 'gold standard', albeit somewhat lusterless.

The research on induced pluripotent stem (iPS) cells has made progress over the last five years. It is exciting that an individual's epithelial cells could be secured, for example, and de-differentiated to stem cells and subsequently modulated to an osteogenic differentiation pathway. Reports suggest iPS cells will differentiate into osteoblasts and produce bone either by being cultured in differentiation media or by viral induction of the *Runx2* gene [42,43]. With iPS cells, however, a significant drawback to their therapeutic potential is their tendency to form teratomas, even after being differentiated down an osteogenic pathway; in the future this may be solved by irradiation of the iPS cells or by a refinement of iPS production [44].

Adipose derived stem cells offer another source for autogenous MSCs. While the procedure to collect the stem cells is more invasive than needed for iPS cells, the harvested tissue is one that most patients would be more willing to endure. The adipose derived mesenchymal stem cells (ADSCs) have the advantage of undergoing less processing steps before becoming osteoblasts and – since they are already differentiated into a mesenchymal lineage – have less of a risk of accidentally becoming non-osseous cells. Studies have reported that ADSCs can undergo osteogenic differentiation, but do not produce mineralized matrix [45,46]. A more sophisticated understanding of ADSCs will be needed before this type of therapy advances into the clinic.

Human umbilical cord derived MSCs (UCMSCs) are readily available, can be harvested inexpensively with no donor site morbidity and can differentiate to osteoblasts [47]. UCMSCs may be harvested from the Wharton's jelly post-partem. The UCMSCs expand easier in culture than BMSCs [48] and have been increased 300-fold in culture without loss of differentiation potential [49]. Furthermore, UCMSCs have the advantage of being *immunopriviledged* due to immunosuppressive isoforms of HLA, as well as the ability to suppress splenocytes and T cells [50,51]. This biological phenomenon allows these UCMSCs to be matched with a wider range of tissue-types, providing allografts to patients with rare immunotypes. Moreover, studies suggest hUCMSCs may differentiate into osteoblasts and produce mineralized matrix, however, the outcome is less robust than that produced by hBMSCs [49,52,53].

Biological therapies for bone tissue engineering

Earlier in this chapter we provided an overview of the fracture healing process that operated on a macroscopic scale. The emphasis was placed on the 'big-picture' events at the bone defect site. Therefore, we will now emphasize selective molecular cues that play a role in the osteogenic cascade. We will note the harmonized release of biologicals among multiple cell phenotypes that direct the osteogenic process and lead to bone regeneration

The dynamic fate of biological signaling molecules at a bone defect underscores the four-dimensional nature of bone regeneration. Consensus abounds on the three spatial dimensions (X, Y and Z) of *volumetric bone* regeneration'; however, there also exists the crucial temporal aspect. Consequently, we emphasize that bone regeneration must be viewed as a *dynamic four dimensional process*. Biological signaling molecules function effectively for a limited window of time to elicit an appropriate outcome on a dedicated target cell. Consequently, it is important to engineer the delivery of biological signaling agents in a bone regeneration therapy with a precise understanding of their temporal pathways during natural bone regeneration [18].

The biological signaling agents we examine in this chapter can be broadly classified into the following categories:

1) Pro-inflammatory cytokines,
2) Growth and differentiation factors, and
3) Angiogenic factors.

Pro-inflammatory cytokines are active following bone injury and establish and maintain the initial *destructive environment*. Growth and differentiation factors function during the *destructive and constructive phases* while angiogenic factors are focal points during the re-vascularization of the injury site. The focus of this section will be on growth and differentiation factors along with angiogenic factors. There are many growth factors that belong in this group and in an effort to provide a more meaningful summary of the most likely factors that will be encountered in the clinic, we will down-select to specific factors based on known effects and published data regarding the bone regenerative processes.

We begin with the *destructive phase* of fracture healing that is characterized by an acidotic, hypoxic environment. The primary cell types in this environment are platelets, lymphocytes and macrophages, which release fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF).

Members of the FGF family are present in the wound site for up to three weeks, and as such, have a broad range of activities [35]. Chief among these activities is the stimulation of endothelial cell migration and subsequent angiogenesis. FGFs affect the migration and proliferation of chondrocytes during the *constructive phase* of bone regeneration [54]. TGF-β growth factors functions as a mitogen for fibroblasts, marrow stem cells and pre-osteoblasts. Both FGF and non-bone morphogenetic protein (BMP) members of the TGF-β super family of proteins propel bone regeneration up to several weeks post injury.

PLATELET-DERIVED GROWTH FACTOR

PDGF is a dimeric growth factor composed of monomers linked by a disulphide linkage. Four individual chains (A, B, C, and D) combine to make five dimers (AA, AB, BB, CC and DD) of which one dimer in particular — BB — plays a key role in bone regeneration [30]. During embryogenesis, PDGF promotes formation and differentiation of somites, which are mesodermal structures that eventually mature into precursors for bone, muscle and skin [55]. Following an injury to bone, PDGF is released from macrophages and the α-granules of platelets and is a potent chemoattractant and mitogenic factor for cells of the mesenchymal lineage. At the site of injury, PDGF will recruit fibroblasts, endothelial cells, osteoblasts as well

as cells of the immune system. PDGF is active for the first 72 hours after injury and as such, is an exemplary candidate for delivery early in the regenerative process [35]. Additionally, as a promoter of angiogenesis, PDGF plays a role in the re-vascularization of the bone defect site [29].

PDGF is secreted by platelets, macrophages, osteoblasts and fibroblasts. In addition to its mitogenic and chemoattractive properties, PDGF will increase osteoprotegerin expression in vascular smooth muscle cells (an inhibitor of osteoclasts) and functions during embryonic development [56]. As a modulator of early-stage wound and fracture healing, PDGF has a significant impact on recruiting (i.e., functioning as a *chemoattractant)* cells to the fracture site. As such, it is a strong candidate for delivery within 72 hours of bone injury.

PLATELET-RICH PLASMA

Platelet-rich plasma (PRP) provides an interesting alternative to the delivery of PDGF alone. PRP consists of a centrifugated blood fraction that contains a concentration of platelets that is often several fold greater than physiological platelet concentrations at wound sites [57]. This concentrated solution is achieved through a multi-step centrifugation to separate platelets from other cells found in blood. In the fracture healing model, the recruitment of immune cells as well as cells of the mesenchymal lineage begin with the release of PDGF from platelets. Consequently, the logic for the clinical application of PRP is that while one cannot precisely mimic the composition of the growth factors endogenously released by platelets in an osseous wound, increasing the concentration of platelets at the site may augment the healing process [58]. That is, there will be an increase in the presence of downstream growth factors such as Angiopoietin-2, endodermal growth factor (EGF), TGF-β1, FGF-2, and predominantly, PDGF and Vascular endothelial growth factor (VEGF). It is generally accepted that many of these factors are necessary for bone regeneration. Purportedly the administration of PRP will assure the appropriate healing milieu composition is available and suitable for the regenerative process [58]. Moreover, there is the argument that PRP is autogenous, patients, as their own donor-recipient pair, consequently, will benefit. Regrettably, the notion of PRP effectiveness has been met with skepticism. An absence of rigorously designed scientific and clinical studies and a proliferation of anecdotal 'evidence' have significantly diminished possible benefits. Controversy over PRP has mitigated against clinical acceptance where successful outcomes seem to be segregated to localized clinical practices.

Nevertheless, given the strong biological rationale for the use of PRP in bone regeneration therapies, it is somewhat surprising to see that its use has resulted in controversial results in literature [57,58]. There are a few potential explanations for the range of reported results. Firstly, the method of preparation of the PRP may differ in many of these studies, leading to invalid comparisons among results. Another explanation for the discrepancies could be variations in the administered dose of PRP. Like any 'drug', PRP functions optimally when present within a certain therapeutic range [59] and studies report using different concentrations [60,61]. In spite of this controversy, PRP remains an intriguing option for growth factor-based augmentation of bone regeneration, particularly when administered immediately following injury.

VASCULAR ENDOTHELIAL GROWTH FACTOR

VEGF is angiogenic and includes agents of the angiopoietin pathway. Of these major angiogenic regulators, VEGF is particularly important for bone regeneration [62]. As a major regulator of both *vasculogenesis* (i.e., spontaneous formation of blood vessels) and *angiogenesis* (i.e., the sprouting of new blood vessels from existing vessels), VEGF plays an important role in bone tissue regeneration [63]. Vasculogenesis, the *de novo* formation of vascular networks, speaks to the similarities that exist between fracture healing and embryonic bone development. However, with respect to bone regeneration, the angiogenic capabilities of VEGF are of

more interest in this application – that is, there is a heightened need for the creation of blood vessels from pre-existing vasculature during bone regeneration.

VEGF is expressed in hypertrophic chondrocytes, but not in resting or proliferating ones [19]. During osteogenic healing, the soft callus is invaded by blood vessels, resulting in the transformation of a predominantly cartilaginous matrix into osseous tissue. This underscores the importance of vascularization of the soft callus as it allows for it to be gradually replaced by woven bone. With regards to temporal expression, VEGF is expressed predominantly 14 to 21 days following injury [35]. As such, it is a viable candidate for delivery to the bone wound site during the early remodeling and mineralization phases of bone regeneration.

Upregulation and delivery of VEGF inhibitors prevent osteogenesis [64]. Additionally, the inhibition of VEGF receptors impairs the healing of bone defects in mice [32]. VEGF has been shown to function in a synergistic fashion with another group of proteins called the bone morphogenetic proteins [31]. That is, VEGF alone does not promote bone regeneration but rather, it acts in a coordinated way with BMPs to increase the recruitment of mesenchymal stem cells to the defect site and promote their differentiate into active osteoblasts. Studies report that the *dose* of VEGF administered to the injury site needs to be finely tuned in accordance with BMP delivery to avoid adverse effects [65]. With respect to promoting *osteoinduction*, VEGF is an excellent candidate for inclusion in porous scaffolds. Its local release in a programmed profile titrated to bone regeneration may be an effective strategy in treating gap bone defects [65].

BONE MORPHOGENETIC PROTEINS

No chapter on bone regeneration would be complete without inclusion of the BMP family. Marshal Urist discovered bone morphogenetic proteins and determined that demineralized bone matrix induced bone formation as a consequence of its endogenous BMP [66]. Urist's epochal work opened a therapeutic paradise; however, it is a paradise that includes peril for the unwary. We will in this section, underscore the *good, bad and ugly* regarding the therapeutic applications of recombinant human (rh)BMP.

While the actions of BMPs have been known for decades, its specific mechanisms of action were elucidated only just recently [67]. BMPs function by binding to type I and I serine/threonine kinase receptors and activating intracellular signal transduction through SMAD proteins 1, 5 and 8. These signals, when translocated into the nucleus, lead to an upregulation of osteoblast transcription factors such as RUNX2 (or core binding factor 1), COL1A1 (Collagen Type I), and OSX (Osterix) among others (see Fig. 55.4).

The most potent members of the BMP family involved in bone regeneration appear to be BMP-2, -4, and -7 homodimers [68,69]. BMPs are active 'early' in the osteogenic cascade. BMP-4 is predominantly active from zero to five days following injury, with its peak closer to day 5. BMP-2 is active throughout the entire bone regeneration process, culminating in the remodeling of the woven bone to lamellar and Haversian bone and BMP-7 is active following day 14.

The cellular targets of BMPs include pluripotent mesenchymal cells, bone marrow cells, pre-osteoblasts, myoblasts, fibroblasts and neural cells. Key markers of osteoblast upregulation include alkaline phosphatase, osteocalcin, osteopontin and osteonectin – the presence of these markers can be used as indicators of cell differentiation from precursors to the osteoblastic lineage [70]. The effects of BMPs on precursor cells are dose-dependent. It is well documented that appropriate doses of BP can induce differentiation of precursor cells into osteoblasts, as well as stimulate cartilage formation and alkaline phosphatase activity [33]. However, 'low' BMP concentrations *in vitro* may promote differentiation into adipocytes. The general effects of BMP on osteoblasts and periosteal cells, however, involve an increase in DNA synthesis activity and the transcription of genes involved in the synthesis of bone matrix proteins.

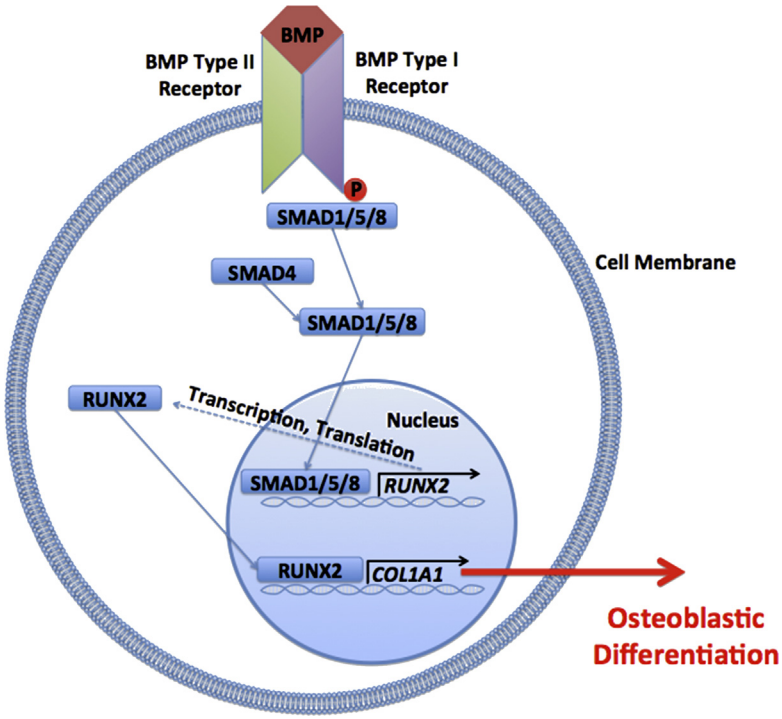

FIGURE 55.4

Key components of the BMP signaling pathway, ultimately leading to bone formation. The *COL1A1* gene is just one of many genes activated downstream of *RUNX2*. Other key markers include Osterix, Alkaline Phosphatase, Osteocalcin, Osteonectin and Bone Sialoprotein though the exact mechanisms by which each of these becomes activated is not yet understood.

The evidence for the role BMPs in bone regeneration is overwhelming. There is a plethora of examples where BMP delivery to bone deficient sites promotes regeneration in a variety of animal models [71–75]. Such overwhelming evidence inspired an industry to focus on clinical applications for BMP though perhaps the rush to the clinic affected judgment. Specifically, the compelling clinical utility of BMP must be countered with caution for potential sinister outcomes. For example, BMPs have been detected in osteosarcomas [76]. In osteosarcomas, the question is whether BMP expression is an effect or a cause of the tumor. Further, a concern with rhBMP's in the clinic has been the supraphysiological dose for clinical effect. The therapeutic effectiveness of rhBMP requires milligram dosing. Consequently, off target effects have been reported with supraphysiological dosing, including calcification of heart valves, heterotopic bone formation, airway obstruction, neuropathies. These outcomes underscore the ineffectiveness of the rhBMP delivery system as well as the potency of rhBMP to promote uncontrolled clinical sequelae.

Biomaterial therapies for bone tissue engineering

One method for the precise, controlled delivery of biologicals and cells to a bone defect site is through their sequestration within synthetic bone graft materials. Synthetic biomaterials allow for the incorporation of cells and biological factors that will have release kinetic profiles and dosing levels matching the physiological profiles of the osteogenic cascade. The following is a focused summary of frequently used biomaterials for bone regeneration.

BIOACTIVE INORGANIC MATERIALS

Currently, there is a wide range of inorganic materials available, with a similar composition to the inorganic matrix of bone, that have gained clinical interest. Among these materials are hydroxyapatite, tricalcium phosphate and bioactive glasses [77,78]. Bioactive inorganic materials, certain ceramics and bioglasses can undergo a time-dependent kinetic modification to their surfaces [79,80]. However, the brittle nature of these materials cannot match bone and, therefore, they are not suitable for load-bearing applications.

POLYMERS

Polymers, whether biological or synthetic, have also attracted clinical enthusiasm. The ability to modify the physical, mechanical and compositional properties of polymers allows for a design with specific clinical targets. Among the most commonly used biological polymeric materials are collagen-based polymers and hyaluronic acid [81]. However, there are concerns regarding the potential risk of disease transmission, immunogenicity, sourcing and relatively weak mechanical properties of these materials [82]. Commonly used synthetic polymers include polycaprolactone (PCL), polyurethane, poly(lactic-co-glycolic acid) (PLGA), and tyrosine-derived polycarbonates. These polymers may be processed using clever synthesis and manufacturing techniques exploiting porogen leaching, phase separation, fiber meshing and microsphere sintering to make 3D-engineered constructs [82–85].

VISION FOR BONE REGENERATION

When designing practical therapeutics for bone regeneration, it is necessary to be cognizant of the regulations set forth by the United States Food and Drug Administration (FDA). The design and validation of therapeutics targeted for the clinic begins on a lab bench but ends with the treatment of patients in hospital operating rooms. As such, we must anticipate regulatory oversight.

However, this is a delicate art; components of the contemporary bone regenerative tool kit are clearly not the answer, but radical new concepts often invoke vociferous and contrary responses from the FDA. In essence, we must strive to achieve a more effective treatment than is currently available but at the same time, be mindful of the eventual reaction from the FDA. This section highlights some novel approaches to bone regeneration that we embrace as steps in the right direction.

The approach for bone tissue regeneration should focus on the complex phenomenon of wound healing, which requires a 4D structure (time, is the 4th element), competent bone-forming cells, and biological stimulants [114]. Contemporary approaches emphasize materials that may deliver a signaling molecule in synchrony with the wound healing and bone regeneration cascades [115]. The most commonly used medical device for recombinant human bone morphogenetic protein-2 (rhBMP-2) delivery is Medtronic's InFuse™, which is essentially a collagen sponge. InFuse™ is currently FDA-approved for maxillary alveolar bone augmentation (i.e., sinus lift procedures) and single level posterior lateral spinal fusion and is an example of a therapy that combines a growth factor-based approach with biomaterial delivery. Another FDA-approved therapy is GEM21S™, which is a rhPDGF-BB and a beta-tricalcium phosphate matrix. GEM21S™ is FDA-approved for periodontal regenerative procedures. In contrast to a single growth factor approach, is a strategy to deliver multiple biological cues to promote osteogenic differentiation as well as the incorporation of growth factor binding peptides, proteins and glycosaminoglycans into scaffolds [82]. However, the daunting challenge with this approach, in addition to achieving the appropriate temporal release profiles, is a regulatory obstacle.

An alternative to the signaling molecule delivery approach is the acceleration of bone healing via a construct made from extracellular matrix (ECM) components, which naturally deliver osteoinductive proteins and signaling cues. Two promising strategies for accomplishing this are to fabricate a scaffold from demineralized bone matrix [116] or to deliver *in vitro* synthesized ECM from marrow stromal cells (MSCs), osteoblasts, or fibroblasts to the fracture site [117]. Materials may be coated with ECM from MSCs or with an adhesive peptide such as fibronectin to promote osteogenic differentiation upon implantation [117,118].

A unique approach to bone regeneration is the use of microparticles to deliver DNA plasmids or siRNAs into cells to induce (or prevent) osteogenic differentiation. Just as upregulating RUNX2 and OSX — two transcriptional regulators for bone formation — may incite osteoblast

lineage progression and in turn, bone formation, silencing RUNX2 and OSX may have the opposite effect. Therefore, siRNAs have been used against these two transcription factors to prevent ectopic ossification [119–121]. A more relevant tactic for promoting bone regeneration is delivering siRNAs against known inhibitors of osteogenic differentiation such as TWIST1. Alternative pathways for promoting bone regeneration are parathyroid hormone (PTH) 1–34 amino acid sequence and BMP-2 signaling. However the advantage of delivering siRNAs and plasmids over other signaling molecules is their stability and a lower risk of eliciting off target effects.

Gene therapy, which was originally proposed for correction of genetic defects, has been reported to induce the expression of molecules that can promote a regenerative response [122]. The most common gene therapies use vectors to enhance the expression of a particular gene [123]. Viruses have been explored as gene delivery vectors, with retroviruses, adenovirus, lentiviruses and adeno-associated viruses being the most promising vectors [124–128]. However, the immunogenic potential of these vectors, as well as the risk of dysregulating normal gene function has kept them from advancing to the clinic [129,130]. Regardless, the natural abilities of viruses to bypass cellular defenses and modify host DNA make them a very powerful tool for guiding the fate of cells at the defect site.

Hydrogels, another bone regeneration therapy option, can be used to deliver cells with the potential to differentiate into bone or vasculature. In order to prepare these implants, cells are seeded in hydrogel constructs and cultured in bioreactors. After the appropriate maturation period, the construct is implanted into a bone defect. Natural hydrogels, such as collagen and hyaluronic acid, have the intrinsic physical and biological characteristics of ECM; they are able to direct cellular function and interact with osteoprogenitors at the implant site [131]. However, the limitations of working with natural hydrogels include difficulty in processing and tailoring them for specific purposes. Additionally, hydrogel degradation products may invoke an immune response. On the other hand, synthetic materials, such as poly-N-isopropylacrylamide (poly-NIPAM), can be manufactured into hydrogels with consistent, reproducible properties. The downside of synthetic hydrogels, however, is minimal cell interaction, and the requirement of tailoring them to direct cellular function [131].

CONCLUSION

Bone is complex tissue-organ system that presents many challenges in the quest to augment its regenerative capabilities. In spite of its natural ability to regenerate, there are limitations to the endogenous regenerative potential. We must harness the intrinsic regenerative capacity of bone and extend it to bone insufficiencies that do not spontaneously regenerate. At this point, we have begun to slowly elucidate the molecular-level biological signals involved in bone regeneration. However, in order to advance bone regeneration therapeutics with twenty-first century science, there is a crucial need to aim for more ambitious, radical lines of research. The *status quo* of autografts, allografts and xenografts are no longer adequate and necessitate the search for transforming alternatives. The majority of current research into bone therapeutics focuses on individual cell-, growth factor- and materials-based approaches that are rather pedestrian and there is a need for radical, innovative strategies.

We offer some starting points: namely, to design therapeutics that work *synergistically* with natural bone regenerative mechanisms. A key component of this approach involves minimizing the interference with these processes that often occurs as a result of implants lacking biocompatibility. Both the implant materials and its degradation products must not extend the destructive phase of bone regeneration. At the same time, it must support the physiological loads associated with bone tissue, though there is a fine threshold for achieving this. The implant must degrade in a coordinated temporal manner with the formation of new bone at

the defect site. Finally, the implant must be osteoconductive, osteoinductive and osteogenic to enhance bone regeneration at the defect site.

In conclusion, in this chapter we highlighted the advantages and disadvantages of contemporary bone regenerative methods. We discussed the importance of the fundamentals of basic osteobiology as a requisite road map for the design and development of bone therapies. We provided our vision for bone regeneration and identified clinical performance criteria that must be defined and met. Finally, we shared some visionary approaches to bone regeneration. It is our hope that this chapter provides some guidance towards improving the regeneration capabilities of the structural *tour de force* that is bone.

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CHAPTER 56

Tissue Engineering for Regeneration and Replacement of the Intervertebral Disc

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INTRODUCTION

The intervertebral disc (IVD) is the fibrocartilaginous part of a 'three-joint complex' that governs motion, flexibility and weight-bearing in the spine (Fig. 56.1). As part of this complex, the disc undergoes a lifetime of 'wear and tear' that contributes to multiple IVD disorders of enormous consequence for human disability and suffering. These IVD disorders are poorly understood musculoskeletal pathologies characterized by multiple anatomic features including internal disc disruption, loss of IVD height, IVD tears, IVD dehydration, and the generation of herniated disc fragments [1–3]. These anatomic features may associate with nerve root compression or irritation, spinal canal narrowing (stenosis or spondylolisthesis), or facet joint impingement that contribute to symptoms of low back pain, neurological deficits and disability that affect between 4 to 33% of the US population annually and has a mean global lifetime prevalence of 38.9% [4–8]. Like most cartilaginous tissues, the IVD is an avascular and alymphatic structure that exhibits little to no capacity for repair following injury, and experiences aging-related cell density losses that may further limit biologically-mediated repair [9]. The extreme mechanical demands on the IVD may also contribute to tissue failure and degeneration, due to the high magnitudes of compressive, tensile and shear stresses and strains that result from joint loading, muscle activation and spinal flexibility. As a result, strategies to intervene in the progression of IVD disorders are met with significant biological and mechanical challenges that frustrate success.

Numerous surgical procedures have been developed to treat IVD disorders that almost completely rely upon reducing motions across the disc space to restore stability during weight-bearing. A very large number of devices have been developed that promote bony fusion

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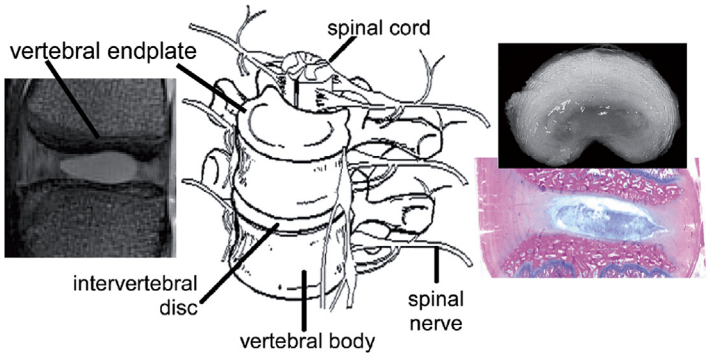

FIGURE 56.1

Schema of spinal motion segment illustrating location of intervertebral disc between superior and inferior vertebral bodies. Image at left MRI appearance of immature lumbar disc with characteristic intense nucleus pulposus region. Images at right illustrate (top) gross appearance of non-degenerate lumbar disc and (bottom) histological appearance of immature disc in a stained section. *(Modified schema reprinted with permission from Columbia-Presbyterian Neurosurgery at www.cumc.columbia.edu/dept/nsg)*

processes across the disc space, with lesser attention being given to mobile-bearing artificial disc replacements and dynamic stabilization devices that allow but limit motions [10]. In cases where the pathology permits, removal of extruded IVD fragments may be performed in a procedure termed a discectomy. Together these procedures comprise more than 300,000 in-patient hospitalizations annually in the USA alone [11]. The clinical practitioner would not typically entertain the idea of fusing or plating most pathological joints in the body, such as the hip, knee, or shoulder, although this is our twenty first century standard of care for the pathological IVD. There exists an obvious need to develop alternative strategies to not only treat the consequences of IVD disorders, but also to detect and limit the progression of symptomatic IVD pathology.

Success with cellular therapies for articular cartilage regeneration, gene therapy, and *in vitro* regeneration of cartilaginous tissue, has raised hope for tissue-engineered treatments for IVD disorders. Tissue-engineered approaches to IVD regeneration have been focused around implantation of cell-supplemented or acellular biomaterials that may partially or fully replace the IVD structure, as well as delivery of cells or bioactive factors designed to promote the natural repair process. In this chapter, a review of these tissue-engineering strategies will be provided along with evaluations of their adaptation and implementation for treatment of IVD disorders.

IVD STRUCTURE AND FUNCTION

In all structures of the IVD, the extracellular matrix provides physical and biochemical cues that regulate cell-mediated repair or breakdown in mature or aging tissues [12,13]. The native matrix organization and interaction with the local IVD cell population will be important considerations in the design of any tissue-engineered regeneration strategy. The IVD is composed of a centrally situated and gelatinous tissue, the nucleus pulposus, that differs substantially from the more fibrocartilaginous anulus fibrosus, on the radial periphery (Fig. 56.2). On both superior and inferior faces is a cartilaginous endplate that provides an intimate mechanical and biophysical connection between the vascularized vertebral bone and the avascular IVD. Both the anulus fibrosus, with a vascularized periphery, and the cartilaginous endplates are believed to be important routes of nutrient transport to all cells of the IVD [14,15]. Given the very low cell density of the IVD, maintenance of both cellularity and a generous nutrient supply are often held to be critical to a successful biologically-based regenerative strategy.

The immature nucleus pulposus is highly hydrated (>80% water) with extracellular matrix components that include randomly organized type II collagen fibers and multiple forms of

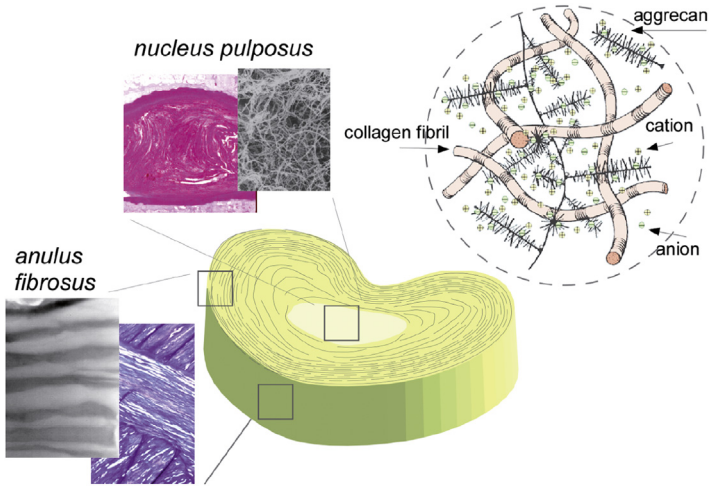

FIGURE 56.2

Schema of different zones and microstructures comprising the intervertebral disc. Anulus fibrosus insets of macroscopic appearance and stained section illustrate the lamellar structure of the tissue. The lamella are comprised of aligned collagen fiber bundles that are oriented with alternating angles of ±60°. Nucleus pulposus insets of a stained section and scanning electron micrograph illustrate the randomly organized network of fine collagen fibers and gelatinous nature of the tissue. Circular inset contains a schema of building blocks for these cartilaginous tissues that include banded type I and type II collagen fibrils, aggrecan and smaller proteoglycans, water and multiple ionic species.

negatively charged proteoglycans (Table 56.1 [16]). A population of large and highly vacuolated cells is present in the nucleus pulposus during development and growth, with a shift towards a more chondrocyte-like cell population by age seven [17–19]. Like all IVD regions, the nucleus pulposus contains multiple collagenous and non-collagenous proteins including types III, V, VI, and IX collagens, elastin, fibronectin and laminin [20,21,22,23–25]. The nucleus pulposus is largely loaded in compression (Fig. 56.3) and experiences high interstitial swelling and fluid pressures, that arise from joint loading and a high density of osmotically active, proteoglycan-associated negative charges [26,27]. Nachemson and co-workers showed, as early as the 1960s, that this interstitial fluid pressure is greater than 0.5MPa (or ~5 times atmospheric pressure) in the nucleus pulposus region [28–30]. An early loss of hydration or tearing in the nucleus pulposus [3], often detected as a loss of MR signal [31], is believed to contribute to a loss of fluid pressurization in the IVD that may lead to herniation or stenosis with aging [32–34]. With loss of fluid pressurization, the load distribution to the anulus

TABLE 56.1 IVD composition and mechanical properties

	Water (% wt)	Collagen (% dry wt)	Proteoglycan (% dry wt)	Other proteins (% dry wt)	Compressive modulus (MPa)	Shear modulus (MPa)	Tensile modulus (MPa)	Interstitial pressure (MPa)[a]
nucleus pulposus	70–90	15–35	25–60	20–45	0.5–1.5	0.005–0.01	*NA*	0.5–3.0
anulus fibrosus	65–80	10–65	10–35	15–40	0.5–1.5	0.08–0.40	20–50 - ∥ circ	0.1–1.0
notes		types I, II, VI, IX, XI	aggrecan, decorin, biglycan, fibromodulin, versican and more				0.5–5.0 - ⊥ circ	

Ranges reported for compositional features and mechanical properties for nucleus pulposus and anulus fibrosus tissue regions of the non-degenerate intervertebral disc. Both composition and mechanical properties of the disc vary substantially with region and with degeneration. Additional mechanical features important to tissue function, such as failure strength, are not shown here.

[a]*Reported also as peak hydrostatic pressures, or swelling pressures.*

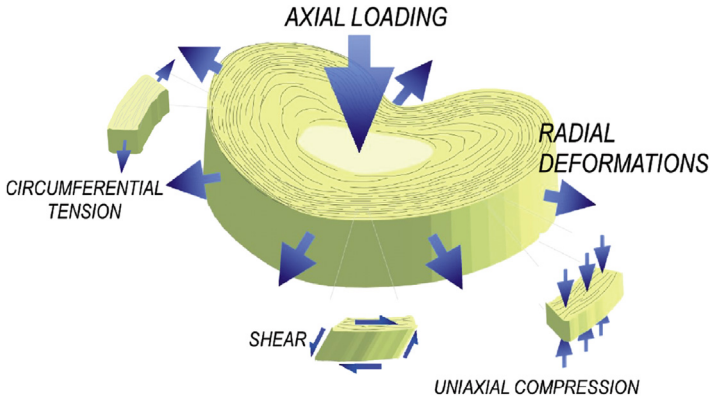

FIGURE 56.3

Axial compressive loading of the intervertebral disc gives rise to a radial deformation, or 'outward bulge', as the disc deforms in response to the compressive load. The high tensile stiffness of the healthy anulus fibrosus in the circumferential direction acts to restrict this outwardly directed deformation. Tissues of the disc will be variably loaded and experience a combination of compression, tension and shear, as shown. Pressurization of the central and gelatinous nucleus pulposus is an important mechanism for load support and load transfer to the anulus fibrosus, and contributes to maintenance of disc height.

fibrosus will shift from a characteristic outward 'bulging' of the anulus to one of inward displacement [29,35–37]. Partial or complete removal of the nucleus pulposus, occurring in some discectomy procedures, may lead to a loss of disc pressurization and disc height that will transfer loads to facet joints of the spine, increase segmental range of motion and impact overall spinal stability. Restoration of this interstitial swelling pressure in the nucleus pulposus, or restoration of MR signal intensity, is an oft-cited criterion for restoration of a healthy functioning disc.

The anulus fibrosus is a lamellar, fibrocartilaginous structure that is highly organized into distinct lamellae [38,39] of highly oriented, and largely type I collagen containing fiber bundles [40,41]. Type II collagen concentration increases towards the innermost region of the anulus fibrosus, as the concentration of type I collagen is diminished. As with the nucleus pulposus, the anulus fibrosus contains proteoglycans within the collagenous extracellular matrix, although at lesser concentrations that vary from outer to inner regions of the tissue. The collagen reinforcement within the anulus fibrosus resists the tensile loads that arise during physiological joint motions, and the swelling effects, that give rise to significant anular bulging and deformation. Consequently, the anulus fibrosus has a very high stiffness in tension, with moduli that vary with the angle of orientation along the principal collagen fiber direction (Table 56.1 [42–47]). Cells of the anulus fibrosus originate from the mesenchyme and exhibit many characteristics of fibroblasts and chondrocytes [13,48–51]. These cells are sparsely distributed in the mature IVD and exhibit very little intrinsic ability for self-repair. Disorders of the IVD that involve displacement or herniation of an IVD fragment are believed to arise from tears in the anulus fibrosus region, and discectomy procedures frequently involve removing a portion of this anulus tissue. Some tissue-engineering strategies are being developed around restoration of healthy anulus fibrosus function or composition (see Anulus fibrosus (AF) Cell-Biomaterial Implants section) in part motivated by a need to repair damage subsequent to intra-discal cell or biologics delivery (see Nucleus pulposus (NP) Cell-Biomaterial Implants section).

The hyaline cartilage endplates of the IVD are important structures that transmit and distribute loads of the spinal column to the discs. Because of their direct contact with the both the anulus fibrosus and the nucleus pulposus, the endplates are believed to be an important route of nutrient transport, particularly to cells of the nucleus pulposus [15,52–55]. With aging, the cartilage endplate will thin, as it undergoes mineralization and eventual replacement by bone. This mineralization of the endplate is thought to impede diffusion and nutrient flow to the

disc, principally the nucleus pulposus that is lacking in an alternate short diffusion pathway. Endplate changes, such as sclerosis, fracture or modified vascularity may be detected by magnetic resonance imaging (MRI) changes [56], and are believed to contribute to symptomatic IVD degeneration [2,57,58]. Thus, tissue-engineering strategies that preserve the health of the endplate without inducing additional damage are believed to be critical to restoring IVD function.

BIOMATERIALS FOR NUCLEUS PULPOSUS REPLACEMENT

In situ hydrating polymers

The complexity of the IVD with its three distinct sub-structures and multiple pathologies, together with very harsh loading conditions and mechanical requirements, has led to challenges for engineering tissue replacements. The concept that nucleus pulposus changes are an important contributor to IVD disorders has led to an initial focus on use of acellular biomaterials for restoration of the nucleus pulposus tissue or function [59–61]. In this section, attention will be given to strategies developed around the concept of using '*in situ* hydrating', synthetic polymers to restore nucleus pulposus hydration and consequently, IVD disc pressure and disc height. The device with the longest clinical history is based on a co-polymeric hydrogel encased in a polyethylene fiber jacket (polyacrylonitrile and polyacrylamide, PDN™, Raymedica Inc., Fig. 56.4). When implanted in a dessicated state, the polymers absorb water while the polyethylene jacket restricts excessive swelling of the polymer. Similar concepts have been developed based on implantation of pre-formed devices constructed from semi-hydrated poly(vinyl) alcohol (Aquarelle™, Stryker Spine Inc.), a co-polymer of poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP) [62], or modified poly(acrylonitrile) reinforced by a Dacron mesh [63] (NeuDisc™, Replication Medical). The design goal is to exploit implant swelling pressure to restore the high compressive stiffness of the IVD, which is lost upon dehydration or denucleation of the nucleus pulposus [64]. Controlling interstitial hydration of the polymer is important for this class of polymers, however, as excessive swelling can cause implant stiffness and endplate overloading. The relevant stiffness is that measured after placement of the implant, with stiffness values reflecting both the material behaviors of the implant as well as the integration with the containing anulus fibrosus and endplates. An additional concept that has promoted development of these devices is an ability to maintain disc height.

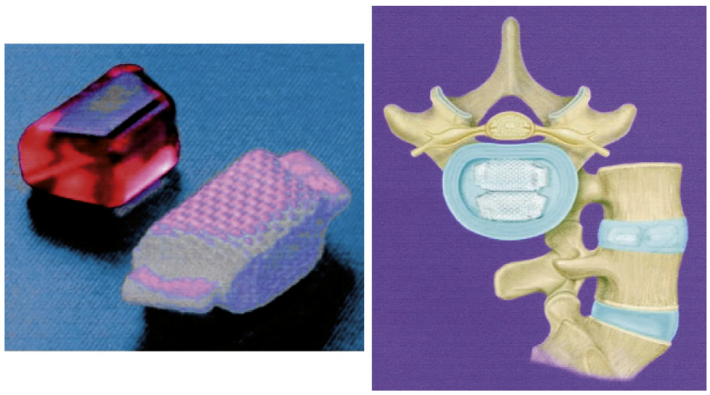

FIGURE 56.4
(Left) Prosthetic disc nucleus (PDN, Raymedica) shown on left composed of an *in situ* swelling synthetic polymer. The pellet is encased in a polyethylene jacket that restricts water imbibition, shown adjacent to the pellet. (Right) The PDN is designed to fit into either the anterior portion of the vacated disc nucleus or the posterior portion. Shown is a schematic of placement of both anterior and posterior components within the nucleus pulposus disc space. *(Modified from Klara and Ray, Spine 2002)*

As with many tissue replacements, there is a long list of requirements that must be satisfied for biomaterials to be used in this application, including the need to achieve:

1) 'Favorable mechanical stiffness' or mechanical properties matched to that of the native structure; in particular, the compressive stresses generated must not exceed the failure strength of the adjacent endplate, in order to avoid endplate fracture or subsidence of the device, IVD height loss, and associated problems;
2) Integration with adjacent structures in order to promote load transfer, minimize device migration or extrusion, and restore stability for the motion segment;
3) Durability, or an ability to maintain physical support over millions of cycles of loading;
4) Minimal generation of wear debris, if appropriate;
5) Standards of biocompatibility; it must not elicit systemic, cellular or immuno-toxicity.

Some of the polymeric devices for nucleus pulposus replacement have experienced device extrusion, endplate failure and endplate sclerosis after implantation, and clinical evaluation has been discontinued. These observations are thought to relate to a mismatch in mechanical stiffness that leads to excessive endplate loading, and poor integration associated with device migration [65,66].

In situ forming polymers

Injectable polymer systems, such as polymers that will undergo a physical transition to a gel-like or solid-like form via crosslinking or thermal or pH-induced transitioning, have been evaluated for placement into a vacant nucleus pulposus space [62,67–70]. Many of the requirements for success of this strategy are similar to that described above, with additional requirement that the polymers must provide the benefit of minimally invasive insertion into the disc space. While many *in situ* forming polymers have been developed for preclinical and clinical evaluation based on varying chemistry platforms, the descriptions below will reveal a tale of failure at the clinical trial stage. Nevertheless, the lessons learned from these diverse strategies provide great insights into the challenges associated with nucleus pulposus replacements.

In situ curing polyurethane is one widely studied *in situ* curing polymer that has been delivered to the disc space through an inflatable polyurethane 'balloon' in order to contain excessive swelling. Despite promising clinical results in Europe, failure to obtain Food and Drug Administration (FDA) approval resulted in the bankruptcy of Disc Dynamics. Despite such failures, a number of *in situ* forming polymers are still under investigation. DiscCell™ (Gentis) is a polymerized water in oil emulsion composite that is capable of curing in 5–7 minutes after delivery to the disc space and reproducing the viscoelastic behavior of the nucleus pulposus (NP). An alternative approach is based on use of chemically crosslinked biopolymers that can form following injection into the IVD space. Nucore™ (SpineWave Inc.) is a protein hydrogel developed from a silk and elastin peptide containing a sequence that is chemically crosslinked at the time of injection [65]. This crosslinking confers an extra stiffness to protein polymers that is necessary to achieve satisfactory stiffness values for a disc implant. This implant system has been able to maintain disc stiffness and to restore disc height when implanted, providing evidence that successful integration is being achieved upon injection. Furthermore, systems composed of native IVD polymers, such as the elastin peptide sequences, may confer some additional benefit as recognized components of the body.

Virtually all of the above-described strategies are focused on approaches to reconstruct the nucleus pulposus following the observation that loss of tissue hydration and structure is a dominant contributor to intervertebral disc degeneration. However, introduction of these biomaterials into the nucleus pulposus via needle puncture necessarily disrupts the structurally intact anulus fibrosus, which can be a significant contributor to disc degeneration [71,72]. For this reason, recent attention has focused on the development of biomaterials

strategies for anulus repair, using sutures [73], anchored sutures [74], polyethylene annular closure devices [75], mesh [76], and PMMA injections [77]. These approaches are promising as they move through clinical trials of implant feasibility in the current period, but their ability to promote repair long term may be limited and they may require tissue-engineering solutions to repair the AF.

CELL-BIOMATERIAL CONSTRUCTS FOR IVD REGENERATION

A persistent limitation of acellular, materials-based replacements of the IVD is their biologically-mediated, or mechanically-induced failure due to the harsh loading conditions and bioactivities of cells within the disc space. These challenges are rooted in the fact that the materials used for such applications have no capacity for self-renewal nor self-repair. This has led to increasing interest in tissue-engineering methods to regenerate new IVD *in situ* or to transplant IVD tissue that has been generated *ex vivo*. Such strategies have been employed to augment repair of other types of cartilage, most notably articular cartilage, and meniscus, which share some features of the harsh biologic and mechanical loading environment within the IVD.

NP cell-biomaterial implants

As in other cartilage tissue-engineering applications, a main strategy for IVD regeneration has been the inclusion of cells with biomaterials to enable production and long-term maintenance of newly generated tissue. Biomaterials that enable appropriate cellular phenotypes and matrix biosynthesis, and that sometimes enable polymeric degradation or resorption, have been proposed as alternative implantable biomaterials and have been studied largely *in vitro*. The goals for use of these scaffolds are similar to that for other biomaterial implants, with the added requirements that the biomaterial must generate no cytotoxic or immunogenic degradation or breakdown fragments and that new matrix formation is enabled. Studies of cell-biomaterial constructs cultured *in vitro* have demonstrated potential for many materials (Table 56.2), including thermosensitive gels such as chitosan, modified chitosans and elastin-like polypeptides [78–81], self-associating gels composed of agarose, collagen and fibrin [82–84] or modified forms of these same materials, native tissue constructs such as intestinal submucosa [85], crosslinkable alginates, polyethylene glycol, poly(glycolic acids) and more [86–91]. *In vitro* studies with these materials are based on evaluating new matrix formation and sometimes degradation characteristics, through culturing native disc or other cell types within these matrices. Hydrogels, such as alginate and gelatin, have been used most commonly for engineering nucleus pulposus tissue, likely due to the fact that such materials reasonably approximate the gel-like properties of the native tissue. Cells of different origin, including native IVD cells, stem cells and chondrocytes, are capable of synthesizing and depositing collagen and glycosaminoglycans within these hydrogels although there is little agreement upon the targeted composition necessary to achieve a satisfactory tissue construct. This is a particularly challenging determination for the intervertebral disc as the matrix contains varying amounts of both types I and II collagen, so that the exclusive presence of type II collagen does not serve as a phenotypic matrix marker as is the case for articular cartilage.

Anulus fibrosus (AF) cell-biomaterial implants

Efforts to regenerate anulus fibrosus have also involved gels such as alginate, agarose, gelatin and collagen as well as fibers or sponges made from materials such as poly(glycolic) acid, polylactic acid, poly(ε-caprolactone), collagen, silk, hyaluronic acid and/or glycosaminoglycans. Oftentimes, the same scaffolds evaluated for nucleus pulposus cells are also studied with cells of the anulus fibrosus, with findings that generally illustrate the importance of cell origin in determining the resultant extracellular matrix synthesis. A common observation, however, is that cells of either origin that are maintained in a rounded morphology tend to generate more

TABLE 56.2 Representative IVD tissue engineering studies

Material	Cell type	Cell source	Cell density	*In vitro/in vivo*	Assessment
PCL [1]	AF, NP	bovine	5 x 10^3 /cm^2	*in vitro*	Histology, SEM, gene expression
Alginate [2]	AF, NP	porcine	4 x 10^6/ml	*in vitro*	DNA, ECM analysis
Gelatin/C6S/HA [3,4]	NP	human	20 x 10^6/ml	*in vitro*	Histology, DNA, ECM analysis, gene expression
CPP [5,6]	NP	bovine	16 x 10^6/cm^2	*in vitro*	Histology, mechanical analysis
Agarose, collagen [7]	AF	human	0.2 x 10^6/ml	*in vitro*	Histology, ECM analysis
Gelatin, PLA [8]	NP	porcine	5 x 10^6/ml	*in vitro*	Histology, ECM analysis, gene expression
Collagen/GAG [9,10]	AF	canine	40 x 10^6/ml	*in vitro*	Histology, ECM analysis
Collagen/HA [11]	AF,NP	bovine	13 x 10^6/ml	*in vitro*	Histology DNA, ECM analysis, gene expression
Alginate [12,13]	AF,NP	porcine	1–10 x 10^6/ml	*in vitro*	Histology, gene expression, mechanical analysis
Collagen [14]	AF	lapine	10 x 10^6/ml	*in vivo*	Histology
Electrospun PLLA (AF), HA (NP) [15]	MSC	human	20 x 10^6/ml	*in vitro*	Histology, SEM, gene expression, ECM analysis
Electrospun PCL (AF), agarose (NP) [16]	MSC	bovine	25 x 10^6/ml (NP), 3 x 10^6/lamella	*in vitro*	Histology, SEM, DNA, ECM analysis, mechanical analysis
Contracted Collagen Gel (AF), alginate (NP) [17,18]	AF, NP	ovine	25 x 10^6/ml (NP), 1 x 10^6/ml (AF)	*in vivo*	Histology, DNA, ECM analysis, mechanical analysis, MRI

Representative overview of studies involving cell-scaffold based tissue engineering of IVD using cells obtained from native tissues only.
Abbreviations: PCL (polycaprolactone); C6S (chondroitin-6-sulfate); HA (hyaluronan); CPP (calcium polyphosphate); PLA (polylactic acid); GAG (glycosaminoglycan); PGA (polyglycolic acid); AF (anulus fibrosus); NP (nucleus pulposus); SEM (scanning electron microscopy); DNA (deoxyribonucleic acid); ECM (extracellular matrix).
1) Johnson et al, Eur Spine J, 2006. 2) Akeda et al, Spine, 2006. 3) Yang et al, Artific Organ, 2005. 4) Yang et al, J Biomed Mat Res B, 2005. 5) Hamilton et al., Biomaterials, 2005. 6) Seguin et al, Spine, 2004. 7) Gruber et al, Biomaterials, 2006. 8) Brown et al, J Biomed Mat Res A, 2005. 9) Saad and Spector, J Biomed Mat Res A, 2004. 10) Rong et al, Tissue Eng, 2002. 11) Alini et al, Spine, 2003. 12) Baer et al, J Orthop Res, 2001. 13) Wang et al, Spine, 2001. 14) Sato et al, Spine, 2003. 15) Nesti et al, Tissue Eng Part A, 2008. 16) Nerurkar et al, Spine, 2010. 17) Bowles et al, PNAS, 2011. 18) Bowles et al, NMR in Biomedicine, 2012.

type II collagen, characteristic of hyaline cartilage, whereas those that are cultured in an elongated morphology generate more type I collagen (Fig. 56.5).

A main challenge has been reproducing the intricate lamellar arrangement of collagen fibers that give the anulus fibrosus its unique mechanical properties and the cells their unique morphology. For this reason, anulus tissue-engineered materials have moved toward scaffolds that mimic the apparent collagen architecture. Some investigators have developed synthetic and natural polymeric scaffolds with anisotropic features such as an oriented honeycomb structure [92], aligned collagen gel fibers [84], oriented electrospun fibers [93–96], and oriented silk fibers [97]. One approach is based on engineering AF to contain oriented and lamellar electrospun PCL fibers with tensile mechanical properties on the order of native AF [98] (Fig. 56.6). These results are indeed suggestive of the potential to engineer anisotropic collagenous tissues.

Efforts to tissue engineer AF have been constructed in combination with NP tissue-engineering techniques to produce composite TE-IVD implants (see next section), as well as with the hope

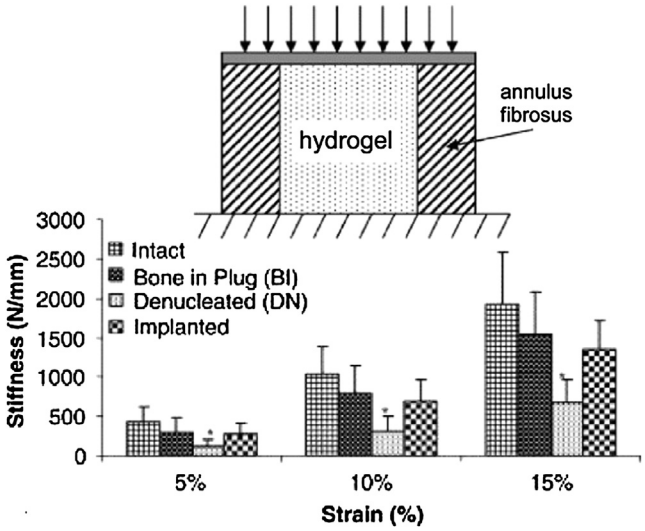

FIGURE 56.5
Top: (Left) Anulus fibrosus cells are highly elongated in the outermost regions of the tissue, but adopt a more rounded morphology in the inner tissue regions, as shown (Middle). (Right) When anulus fibrosus cells are embedded in an alginate hydrogel, they assume a rounded morphology and express matrix proteins associated with chondrocytes such as type II collagen and aggrecan (Bottom). *(bottom figure modified from Le Maitre et al., Arth Res Therapy 2005 with permission)*

to repair degenerated or disrupted AF. One such study demonstrated the usefulness of using a tissue based strategy to repair an AF defect by patching AF defects with small intestinal submucosa (SIS) anchored by titanium bone screws [99]. The SIS patch produced new integrated tissue at the defect site and increased the capacity of the IVD to pressurize post anulotomy and increased disc hydration compared to anulotomy-only levels after 24 weeks in an ovine model. In another study aimed at producing a tissue-engineering strategy to repair AF defects, genipin crosslinked fibrin hydrogels were developed that demonstrated mechanical properties on the order of AF, maintained cell viability, and could maintain adhesion to native AF tissue under physiological load [100]. Many of the AF tissue-engineering strategies discussed previously are being considered for AF defect repair. However, most of these promising studies have been performed *in vitro* and will need to demonstrate effective repair of annular defects *in vivo* before being advanced to the clinic.

Composite cell-biomaterial IVD implants

Cell-based regeneration of the IVD *ex vivo* is complicated by the inherent multi-component structure of the IVD, which includes two distinct regions, the anulus fibrosus and the nucleus pulposus. Given this added complication, it is not surprising that there are fewer examples of efforts to engineer in integration of the multiple components of the IVD *ex vivo*. This can prove to be a critical limitation, as integration is needed to insure proper load transfer and to limit damaging motions during disc loading. However, seminal studies in humans have demonstrated the successful transplantation of IVD allografts in place of herniated cervical IVDs [101]. At a five year follow up, the IVD allografts had maintained stability and mobility while the patients showed no signs of significant cervical pain. This suggests that composite cell-biomaterial IVD implants are a worthwhile endeavor. In studies conducted by Bonassar and co-workers, IVD regeneration was attempted in the native disc space of an athymic rat with a fully integrated scaffold combining contracted collagen gels as a scaffold for anulus fibrosus, and a crosslinked alginate hydrogel as a scaffold for nucleus pulposus tissue [102,103] (Fig. 56.7). Primary cells for culture within each scaffold region were derived from the corresponding native IVD tissues, and the resultant cell-laden scaffolds were implanted in place of the native disc in athymic rats for a period of six months. Results illustrate spatially-directed matrix regeneration with extracellular matrix that exhibited distinct morphologies, contained native levels of collagen and glycosaminoglycans, and was functionally integrated with native tissue. In biomechanical tests, the motion segments containing the composite tissue-engineered discs were found to have a compressive modulus and permeability to flow similar to the native motion segments. Thus, this approach illustrated an ability for cell-laden

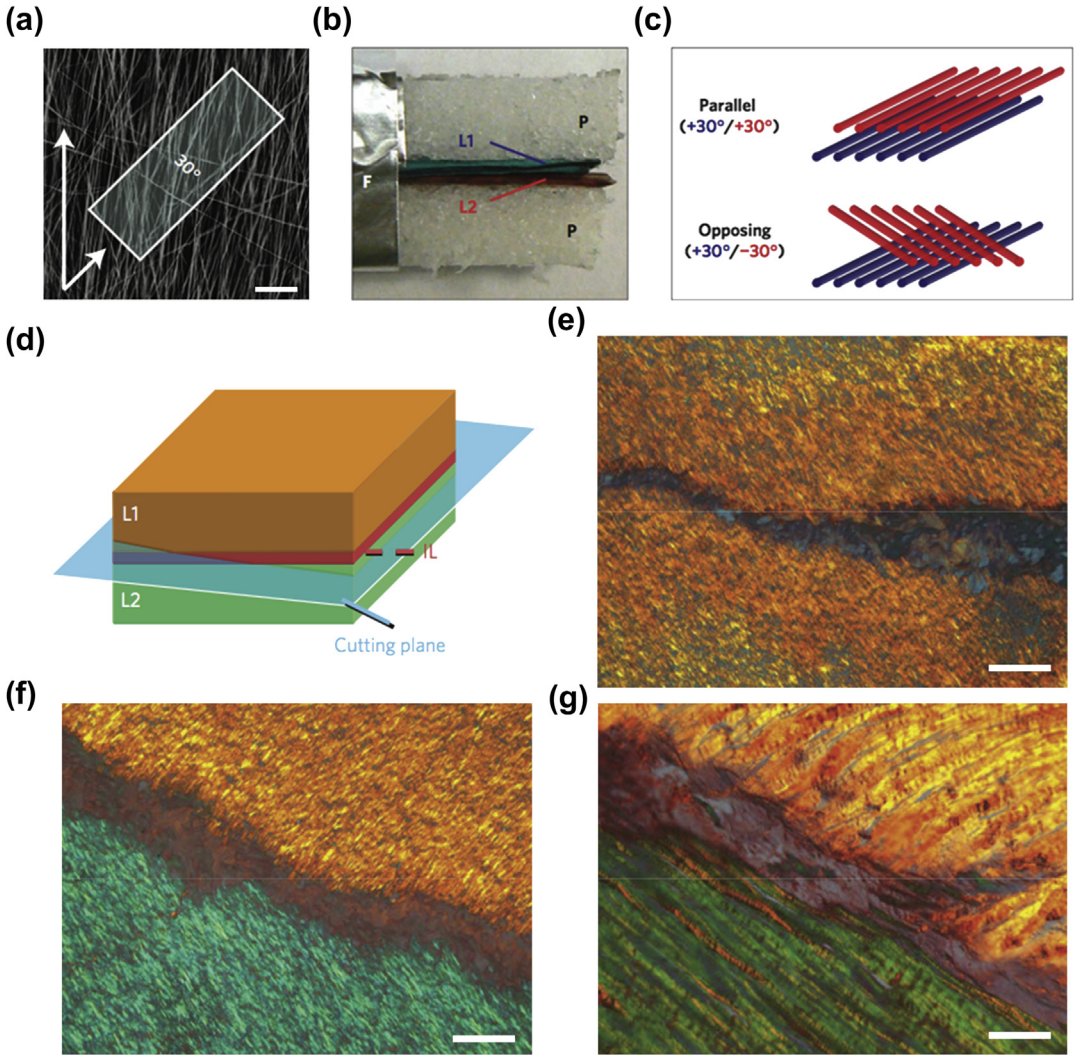

FIGURE 56.6

Fabrication of fiber oriented AF tissue constructs. (a) Scaffolds were excised 30° from the prevailing fiber direction of electrospun nanofibrous mats to replicate the oblique collagen orientation within a single lamella of the annulus fibrosus. Scale bar: 25 μm. (b) At zero weeks, MSC-seeded scaffolds were formed into bilayers between pieces of porous polypropylene and wrapped with a foil sleeve. P: porous polypropylene; F: foil; L1/2: lamella 1/2. (c) Bilayers were oriented with either parallel (+30°/+30°) or opposing (+30°/−30°) fiber alignment relative to the long axis of the scaffold. (d) Sections were collected obliquely across lamellae, stained with Picrosirius Red, and viewed under polarized light microscopy to visualize collagen organization. When viewed under crossed polarizers, birefringent intensity indicates the degree of alignment of the specimen, and the hue of birefringence indicates the direction of alignment. L1/2: lamella 1/2; IL: inter-lamellar space. (e) After 10 weeks of *in vitro* culture, parallel bilayers contained co-aligned intra-lamellar collagen within each lamella. (f, g) Opposing bilayers contained intra-lamellar collagen aligned along two opposing directions (f), successfully replicating the gross fiber orientation of native bovine annulus fibrosus. (g) In engineered bilayers, as well as the native annulus fibrosus, a thin layer of disorganized (non-birefringent) collagen was observed at the lamellar interface. The distribution of collagen fiber orientations was determined by quantitative polarized light analysis [45]. Scale bars: 200 μm (b,c), 100 μm (d). *(Figure modified with permission from Nerurkar et al. Nature Materials, 2009)*

scaffolds to regenerate integrated functional extracellular matrix with some of the functional and compositional features of the native tissue in the native disc space.

Recent studies have further explored development of composite IVDs using a variety of materials and techniques. Composite IVDs have been produced with anulus fibrosus composed of polyglycolic acid/polylactic acid (PGA/PLA), electrospun poly-L-lactic acid (PLLA), contracted collagen gel, silk, electrospun polycaprolactone (PCL), and demineralized bone matrix gelatin and nucleus pulposus composed of alginate, hyaluronic acid, hyaluronic acid/fibrin, agarose, silk hydrogel, and collagen II/hyaluronate/chondroitin-6-sulfate

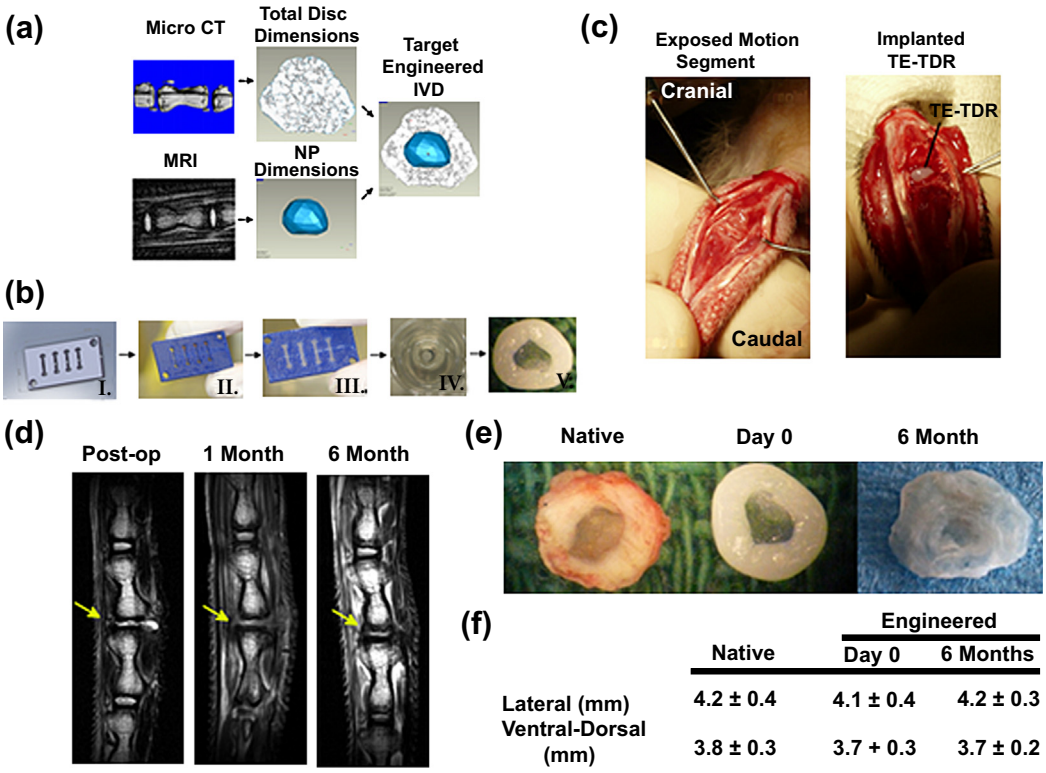

FIGURE 56.7

Anatomical composite tissue-engineered IVD (TE-IVD), designed from MRI and CT, survives in disc space for six months. (a) CT and MRI design procedure for obtaining TE-IVD dimensions. (b) Fabrication of TE-IVD. (i) NP dimensions used to design injection molds via computer-aided design. (ii) Injection mold 3-D printed out of acrylonitrile butadiene styrene plastic. (iii) Cell-seeded alginate was injected into mold, removed, (iv) placed in center of 24 well plate, and cell-seeded collagen was poured around alginate NP. (v) After 2 wk of culture, cell-seeded collagen contracts around the NP to form composite TE-IVD. (c) Intraoperative images showing exposed caudal 3/4 disc space and implanted TE-IVD. (d) T2-weighted MRI of implanted disc space (marked by yellow arrows) and adjacent native levels immediately postoperative, at 1 month, and 6 month after implantation. (e) History of TE-IVD in native disc space. Intraoperative photo showing explanted native IVD next to the TE-IVD (day 0) that was implanted in its place and TE-IVD after being implanted into native disc space for 6 mo. (f) Size of engineered IVD compared to native IVD. Measurements were taken along the lateral and ventral-dorsal planes of the engineered and native IVD. Engineered IVD measurements were taken at day zero prior to implantation (n =12) and compared to explanted native discs (n = 12). Engineered IVD measurements were also taken after 6 months of implantation (n = 12). *(Reprinted with permission from Bowles et al. PNAS, 2011).*

[97,102,104–108]. In one of the earliest examples of an integrative tissue-engineering approach, investigators generated nucleus pulposus tissue *in vitro* by culturing primary bovine nucleus pulposus cells at high density upon a calcium polyphosphate substrate, in order to mimic the natural integration of the nucleus pulposus against the vertebral endplate (Fig. 56.8 [109]). The nucleus pulposus cells formed tissue with a proteoglycan, but not collagen content matched to that of the native nucleus pulposus. Importantly, functional properties in some testing configurations approached that of the native tissue. Additional work will be required in adapting these integrative tissue-engineering approaches to insure that mechanical integration with adjacent tissues is adequate, but these studies focused on generating integrated nucleus-bone or nucleus-anulus are an important step in illustrating feasibility for this approach.

Assessment of the success of IVD tissue-engineering efforts is critical to moving this technology toward clinical application. A majority of studies have focused on generating new IVD *in vitro*, with fewer documenting tissue formation and integration in preclinical evaluations *in vivo*. To date, the most common tool for assessment of newly generated IVD tissue has been histology, although analysis of gene expression and extracellular matrix composition has been frequently employed to confirm the appropriate phenotypic behavior in engineered IVD. *In vitro* studies have laid the foundation for necessary and/or sufficient characteristics of a successful

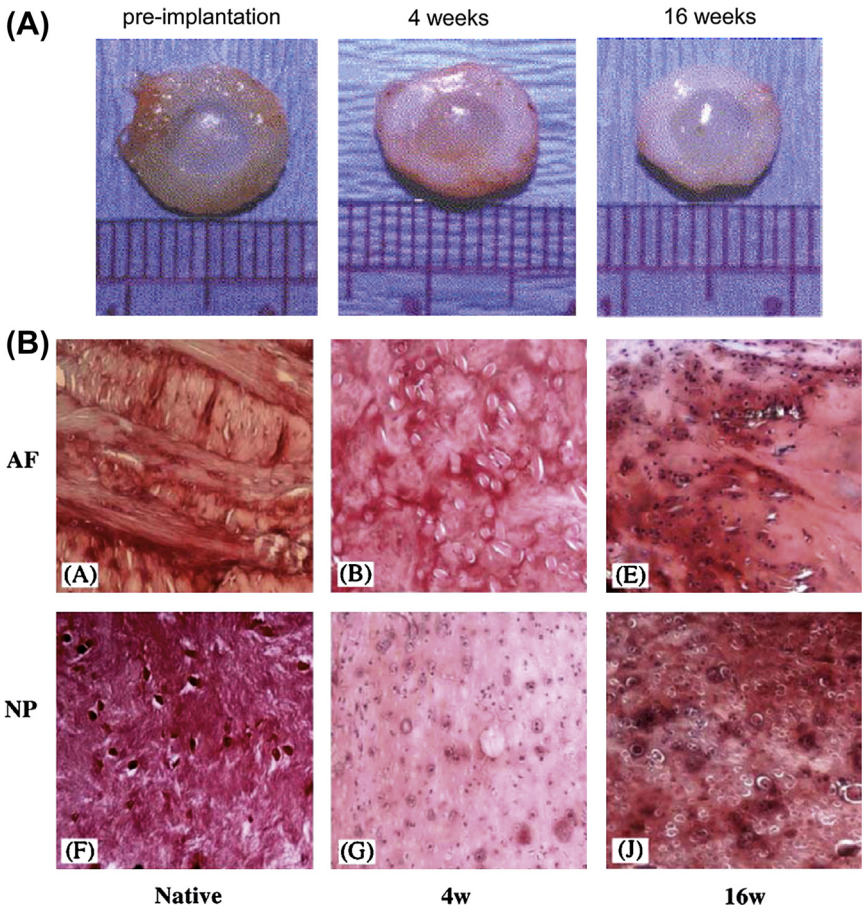

FIGURE 56.8
Histological appearance of *in vitro*-formed tissues showing newly generated nucleus pulposus (NP) tissue. Histological appearance of (a) *in vitro*-formed cartilage at two weeks (time at which the NP cells would be seeded); (b) *in vitro*-formed NP-cartilage—calcium pyrophosphate composite (triphasic construct) at eight weeks following seeding of chondrocytes; and (c) *in vitro*-formed cartilage tissue alone (biphasic construct) at eight weeks. Arrowheads indicate tissue growing within the pores of the CPP; arrow indicates interface between cartilage and NP tissue. (toluidine blue stain; original magnification ×50) (from Hamilton DJ et al. Biomaterials V27, 2006, pp. 397–405).

scaffold for anulus fibrosus and nucleus pulposus replacement. From these studies, for example, it is evident that a high starting cell density, and a high degree of initial matrix stability is essential for promoting long term construct stability and matrix accumulation to eventually restore mechanical function and swelling pressure [110]. Mechanical analysis of engineered IVD tissue is essential in assessing the formation of functional IVD tissue, however, both for nucleus pulposus implants that must restore spinal stability, as well as anular repair strategies that must withstand repeated cycles of nucleus pressure. *In vivo* studies may also evaluate an ability to restore disc height as a common outcome variable, as well as clinical MRI imaging to access *in vivo* tissue hydration. *In vivo*, largely preclinical work has begun to verify that engineered IVD tissues may eventually be capable of restoring disc height, maintaining spinal stability and flexibility, and retarding disc degeneration in pathological tissues.

CELLULAR ENGINEERING FOR INTERVERTEBRAL DISC REGENERATION

Given the relatively small numbers of studies in the area of IVD tissue engineering, there is a surprising amount of breadth to not only the biomaterials, but also the cell sources

utilized for regeneration. The question of cell source is of particular note for IVD tissue engineering, given that the availability of autologous disc cells is extremely low in the adult, and that the phenotype of cells varies so substantially with both spatial position and with age. In animals studied for IVD tissue engineering *ex vivo*, the origin of cells in the nucleus pulposus may be partly notochordal or mesenchymal, depending on the age of the animal in question. As such, the choice of species used as a source of cells may be quite important. Due to the ease of availability, porcine and bovine cells are the most commonly used, with other efforts reporting the use of cells of canine, lapine, and ovine origin as well as human. However, cells derived from bovine or ovine tissues may be exclusively mesenchymal in origin while those derived from porcine, lapine or rodent tissues may be largely notochordal. These phenotypic differences add an additional and unique complicating factor for investigators studying preclinical models for IVD tissue regeneration.

Given the very limited availability of native IVD cells that can be effectively harvested for tissue engineering, there has been interest in using other cells as sources for these efforts. The primary target for other sources has been stem cells derived from sources such as bone marrow [111], embryonic cell lines [112] and adipose tissue [113]. A major challenge in this approach has been the development of methods to guide the development of stem cells toward phenotypes found in the IVD (see next section). This has been attempted through manipulation of the culture medium and gas conditions [114], as well as co-culture with primary cells from the IVD [115–117]. The more recent development of induced pluripotent stem cells (IPSC) has also provided the possibility for an additional cell source for cell delivery to treat musculoskeletal disorders [118,119]. In comparison to use of adult primary disc cells derived from often pathological or degenerated IVDs, the use of autologous mesenchymal stem cells or progenitor cells may be most promising to the future of *ex vivo* tissue-engineering strategies that rely upon cell supplementation.

In addition to origin, cell density is also known to have a profound effect on the efficacy of the tissue-engineering process. Here there has been a great deal of variability in protocols, with studies reporting densities of delivered cells ranging from $0.2–50 \times 10$[6] cells/ml. While the lower end of this scale is likely more reflective of the actual cell density in nucleus pulposus tissue, the densities at the higher end of the scale are more in line with those known to be effective in generating other types of cartilage [120]. A critical concern for disc tissue regeneration, particularly in the case of strategies that employ high cell densities, is the issue of nutrient and gas supply necessary to maintain cell viability and health. The IVD is both avascular and alymphatic, meaning that the transport of nutrients and oxygen is driven largely by diffusion from the vascularized periphery and through the vertebral endplates [14,15,121–123]. It is noteworthy that calcification and endplate changes in the degenerating IVD can lead to impaired nutrient transport, which is linked to decreased cell viability [53,54,124]. Thus, supplementation of scaffolds with very high cell densities may not be optimized for long term survival in the largely hypoxic and glucose-poor, lactate-rich environment within the IVD (Fig. 56.9). This concern has been expressed for cell-laden IVD scaffolds, but has not been directly addressed nor investigated as an issue in IVD tissue regeneration.

Cellular supplementation in the IVD

If the local environment within the IVD is conducive to the survival of cells, direct cell supplementation without biomaterial scaffolds may hold promise for IVD repair. This strategy has been pursued by several groups in preclinical studies, using either IVD cells, chondrocyte-like cells, or progenitor cells. In the first reported work, nucleus pulposus cells from the rabbit were inserted following removal of nucleus tissue, and showed some beneficial effects in inhibiting the degenerative IVD changes of nucleotomy [125]. Similar procedures have also shown the effectiveness of autologous disc cell implantation in both a sand rat

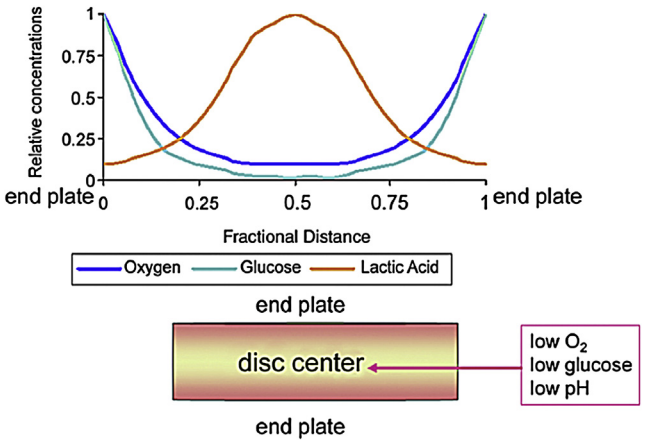

FIGURE 56.9
Schematic showing the concentrations of oxygen, glucose, and lactic acid across the disc from end plate to end plate. *(Reprinted with permission from Grunhagen et al. JBJS, 2006).*

model of spontaneous disc degeneration [126] as well as a canine model of disc degeneration [127]. Furthermore, work by Nomura and co-workers has shown that supplementation with allogeneic nucleus pulposus cells did not induce any appreciable host-versus-graft rejection response, and also retarded disc degeneration in a rabbit nucleotomy model [128].

Limitations will always exist in obtaining sufficient numbers of autologous or allogeneic disc cells from a single site, as well as concerns about impaired cellular activity for the native cells. Some studies have thus focused on using co-culture of nucleus pulposus and anulus fibrosus cells to stimulate cell metabolism prior to re-insertion [129]. These approaches were shown to be effective in delaying some degenerative features such as the loss of disc architecture following re-insertion of the 'activated' cells in a rabbit model. Still, other studies have focused on delivery of cells through delivery of allograft tissues based on the concept that preservation of extracellular matrix is an equally important criteria for regeneration [109,130,131]. Indeed, nucleus pulposus cell insertion has been shown to result in a slightly poorer outcome than insertion of the allograft nucleus pulposus tissue itself, indicating that inclusion of the extracellular matrix in an allograft may be as or more important than the absolute number of cells inserted [128]. These findings have led to the development and investigation of matrix-based cell carriers derived from hyaluronan, laminins, collagen and engineered polypeptides [132,133].

Beginning in 2002, a prospective, controlled, multicenter study was undertaken to compare autologous disc cell transplantation plus discectomy against discectomy alone [134]. The interim analysis of the first 28 patients at two years showed a clinically significant reduction of low back pain in the transplantation group compared to the discectomy group, suggesting a potential benefit of the cell transplantation strategies described above. Little is understood about the mechanism by which the cell supplementation provides this benefit, although disc hydration but not disc height was found to be higher in the patients receiving the cell transplantation compared to the discectomy group. This clinical study led to the development of at least one product termed ADCT, or autologous disc chondrocyte transplantion with or without a hydrogel carrier (NOVOCART), that has been widely used and approved in Germany since 2008. The advancement of these new therapeutic modalities underscores the role of autologous cell-mediated biological factors in regulating symptoms with IVD pathology, and illustrates a potentially important role for sustaining cell viability of the IVD in inhibiting this pathology.

Clinically, the autologous re-insertion of the nucleus pulposus cells into the degenerative disc remains challenging [135]. As a source of cells for transplantation, mesenchymal stem cells (MSCs) that can be harvested from a patient's own bone marrow or adipose tissue are a possible candidate [114,136,137]. *In vitro*, the differentiation of MSCs into nucleus pulposus-

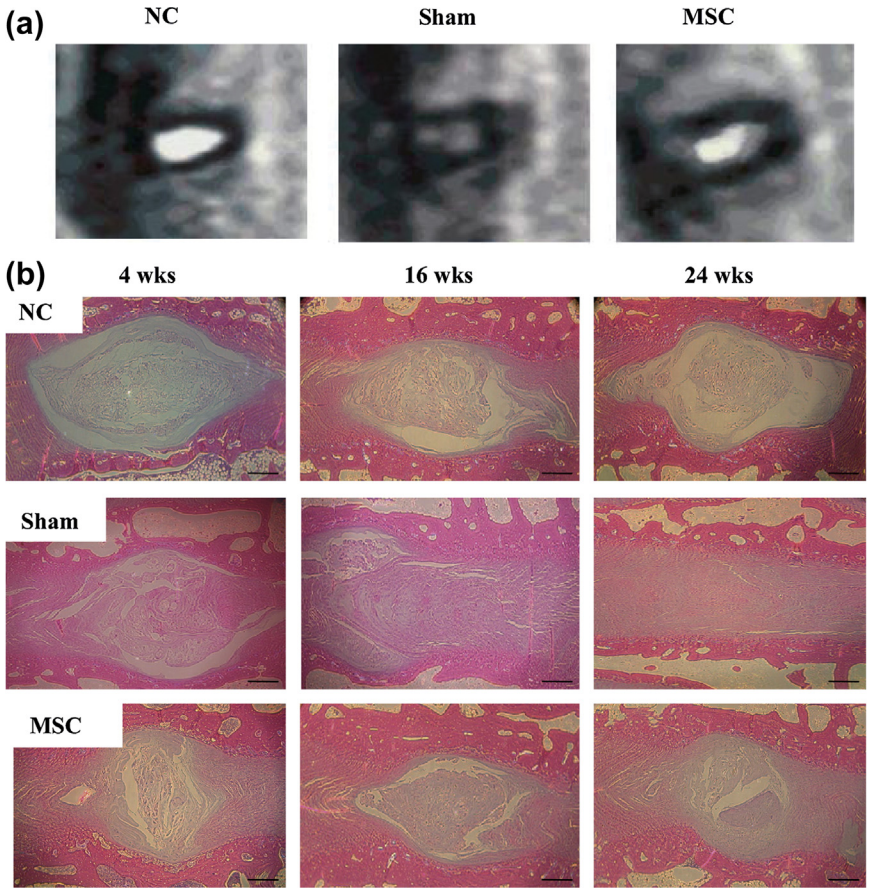

FIGURE 56.10
MRI image of normal control, sham (disc degeneration model) and MSC transplanted rabbits taken 26 weeks post induction of degeneration (24 weeks after MSC transplantation in MSC transplanted animals). Images of sham and MSC transplanted animals are among the best model obtained by means of degeneration and restoration. Significant recovery of T2-weighted signal intensity is seen in discs of MSC transplanted discs compared to very low signal intensity in sham. The arrows indicate the garlic oil supplement capsules used for standardizing control. (graciously provided by Dr. Sakai) Fig. 56.11. Histological changes seen over after MSC transplantation in discs. Left = 4 weeks and right = 24 weeks after transplantation. Control group discs show oval shaped nucleus with no collapse of the inner annular structure. Sham operated disc show collapse of the inner annulus morphology from four weeks (six weeks after induction of degeneration). Fibrotic change in the nucleus due to cell invasion from the surrounding region is completed at 24 weeks. MSC group discs showed relatively preserved inner annulus structure with minimal fibrosis in the nucleus region. Bar = 200μm. *(Graciously provided by Dr. Sakai)*

like or chondrocyte-like cells has been shown under hypoxic and high osmotic conditions, [114] and also with TGF-β stimulation [138]. Preclinical studies have followed injection of autologous MSCs embedded in atellocollagen gel as well as direct injection of MSCs into rabbit or rat models of IVD degeneration, and observed an ability for these cells to differentiate or regenerate matrix [137,139,140]. In one set of studies, transplantation of MSCs with an atelocollagen carrier into the rabbit discs effectively maintained disc height, MR signal intensity and the histological appearance of the nucleus pulposus and anulus fibrosus regions at 24 weeks after transplantation (Fig. 56.10) [139]. Some of the positive outcomes observed for tissue regeneration in the animal models may arise from factors released from MSCs, or direct contact with MSCs, which may enhance metabolism of native disc cells [141,115]. These findings have supported expansion of the use of MSCs into clinical trials, including one by Mesoblast Limited that has been cleared for Phase 2 clinical trials in the United States to test the delivery of adult mesenchymal precursor cells to the degenerated disc. We will have much to learn from these early studies of clinical outcomes following MSC delivery to the pathological disc, with findings and protocols that are likely to advance future research.

GROWTH FACTORS AND OTHER BIOLOGICS FOR INTERVERTEBRAL DISC REGENERATION

In vitro studies

Disc cells modulate their activity by a variety of substances, including cytokines, growth factors, enzymes and enzyme inhibitors in a paracrine and/or autocrine fashion [142]. Tissue-engineering approaches to disc regeneration have been based on attempts to upregulate important matrix proteins (e.g., aggrecan), or to downregulate pro-inflammatory cytokines

[e.g., interleukin-1 (IL-1); tumor necrosis factor-α (TNF-α)] [143–150] and matrix-degrading enzymes [e.g., matrix metalloproteinases (MMPs); aggrecanases] [135,151–153]. The delivery of these modulating biologic agents, with and without scaffolds and/or through cell transplantation, has been the subject of many years of efforts in tissue engineering. *In vitro* studies have shown that the rate of matrix synthesis or gene expression for matrix proteins, principally proteoglycan or collagen, can be increased several-fold in IVD cells in the presence of one or a combination of these growth factors: supplemental transforming growth factor-β (TGF-β) [154,155] and its sub-type TGF-β3 [156–158], osteogenic protein-1 (OP-1; otherwise known as bone morphogenetic protein-7, BMP-7) [90,159–161], BMP-2 [162,163], BMP-12 [164], growth and differentiation factor-5 (GDF-5) [165,166], epidermal growth factor (EGF) [155,167], or insulin-like growth factor-1 (IGF-1) [168]. Other studies have demonstrated the potential of these growth factors, as well as platelet-derived growth factor (PDGF), to reduce cell apoptosis [162,155,169] and to promote cell proliferation [155,170]. Several recent studies examined the effects of the *in vitro* co-stimulation of IVD disc cells by two or more growth factors. In micromass cultures of fetal outer anulus cells, TGF-β1 and IGF-1, both alone and in combination, stimulated the synthesis of sulfated glycosaminoglycans, and collagen types I and II [171]. While TGF-β1 and BMP-2 each had strong anabolic effects on nucleus pulposus cells cultured in an atelocollagen scaffold, there were no additive or synergistic effects on cell proliferation and matrix synthesis [172]. Autologous platelet-rich plasma (PRP), which contains a variable mixture of growth factors, including TGF-β1, PDGF, and IGF-1, has also been shown to be an effective stimulator of cell proliferation, proteoglycan and collagen synthesis, as well as proteoglycan accumulation, when added to porcine IVD cell cultures *in vitro* [173]. In human nucleus pulposus cells, a 'growth factor cocktail' of TGF-β1 at 1 ng/ml in PRP induced cell proliferation and differentiation and promoted the *in vitro* formation of tissue-engineered nucleus pulposus [174].

In a different approach, supplementation of IVD cell cultures with a naturally-occurring anti-inflammatory molecule, interleukin-1 receptor antagonist (rhIL-1Ra), has been shown to inhibit the downregulation of biosynthesis induced by the pro-inflammatory cytokine, IL-1 [173]. Pre-treatment of nucleus pulposus cells with both IL-1Ra and a fusion protein between IL-1Ra and an elastin-like polypeptide (ELP) reduced the expression of MMP-3 and the aggrecanase, ADAMTS-4, when the cells were subsequently treated with IL-1 [175]. The *in vitro* application of rhIL-1Ra to degenerated human disc tissues reduced levels of enzymes, MMP-3, -7, and -13, implicated in the degradation of the IVD [176]. The incubation of herniated human disc tissues with IL-1Ra or an antibody inhibitor of TNF both decreased levels of active MMP-3, indicating that these inhibitors may have an effect on the resorption of herniated discs [177]. The inhibition of p38 mitogen-activated protein kinase (MAPK) in IL-1-activated nucleus pulposus cells reduced the production of factors associated with the catabolic effects of IL-1 and TNF-α [178]. An extension of that study revealed that the IL-1 upregulation of gene expression of MMP-3, IL-1, and IL-6 was reduced, while the IL-1 downregulation of matrix protein gene expression and proteoglycan synthesis was reversed in nucleus pulposus cells [179].

These above *in vitro* studies illustrate that both stimulatory factors, as well as anti-inflammatory or anti-catabolic factors, may be considered for therapeutic purposes in IVD regeneration. Overall, the potential for biologics to assist in matrix regeneration through controlling both cell metabolism and cell number has been established and paved the way for more recent studies evaluating these biologics *in vivo*.

In vivo studies: growth factors

Protein injection into the disc space is relatively simple and practical and has been the most widely studied of all approaches for delivery of growth factors and biologics for IVD regeneration. Walsh and co-workers reported the *in vivo* effect of single or multiple injections of several growth factors, including basic fibroblast growth factor (bFGF), GDF-5, IGF-1 or TGF-β,

in mouse caudal discs with degeneration induced by static compression [180]. Fibrochondrocyte aggregates were observed at four weeks in the nucleus of discs that received a single injection of GDF-5 and multiple injections of TGF-β, while a single injection of IGF-1 may have elicited an early, transient response at one week and bFGF treatment had little effect [180]. A statistically significant increase in disc height four weeks after GDF-5 treatment was also reported and the authors suggested that GDF-5 and TGF-β are mitogens for anular chondrocytes [180].

A single intradiscal administration of recombinant human OP-1 (rhOP-1) into normal rabbit discs *in vivo* resulted in increased disc height and proteoglycan content in the nucleus pulposus regions in comparison to a saline injection control group [181]. In an animal model of disc degeneration caused by needle puncture of the anulus fibrosus, an injection of rhOP-1 (100 μg/disc) restored disc height, structural change, and mechanical properties (Fig. 56.11) [182,183]. In another rabbit model of disc degeneration, a single injection of recombinant human osteogenic protein-1 (rhOP-1) (100 μg/disc) significantly reversed the decrease in disc

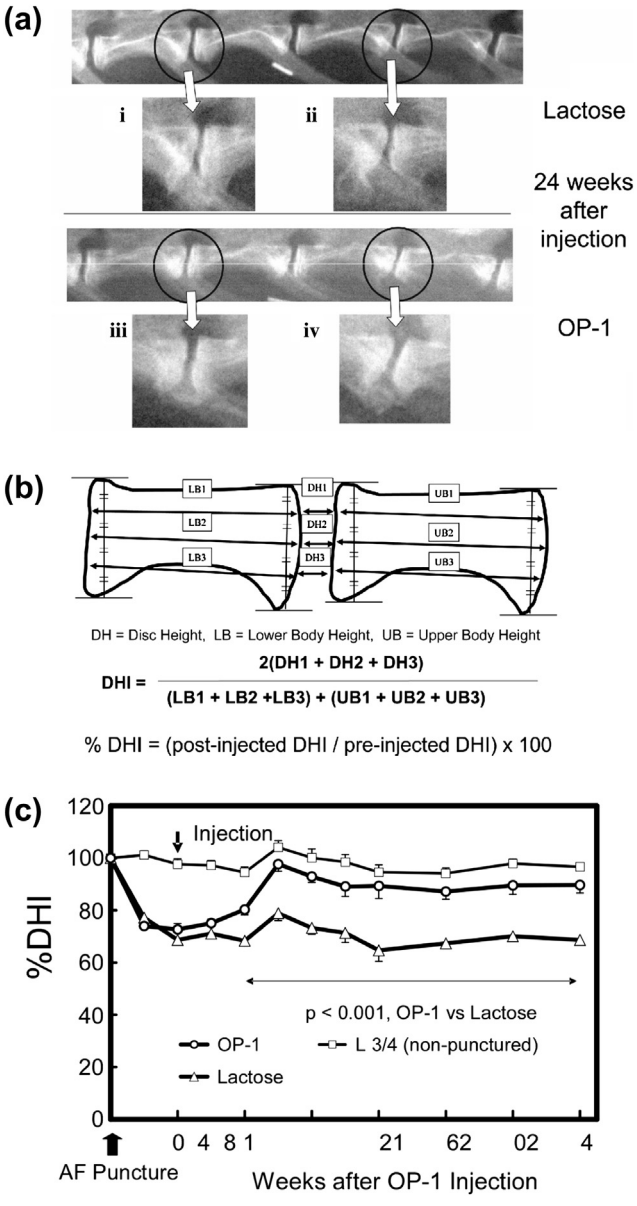

FIGURE 56.11

IVDs of rabbit lumbar spines were subjected to anular puncture by an 18-gauge needle to induce disc degeneration. Four weeks after the initial puncture, the vehicle (5% lactose, 10 μL) or OP-1 in 5% lactose (100 μg/10 μL at each level) was injected into the center of the nucleus pulposus. (a, top) Radiographs at the 24 week time after injection with lactose; (a, bottom) Radiographs of rabbit IVDs following injection with OP-1 in the experimental group, shown at 24 weeks after injection. (b) Method for radiographic measurement of disc height (DH). The average IVD height (DHI) was calculated by measurements obtained from the anterior, middle, and posterior portions and divided by the average of adjacent vertebral body heights. LBH indicates lower body height; UBH, upper body height. (c) Changes in the IVD height index (DHI) after the anular puncture and OP-1 injections. As shown, %DHI of injected discs in the OP-1 group was significantly higher than in the lactose injected control group by 4 weeks ($p < 0.001$, repeated ANOVA). This difference %DHI was maintained out to later periods.

height following chondroitinase ABC chemonucleolysis; the reversal was sustained for up to 16 weeks, and resulted in a significantly higher proteoglycan content compared to the vehicle control group [161]. The *in vivo* efficacy of rhOP-1 has been confirmed in a rat model of chronic compression of tail IVDs; under continuous compression load the injection of rhOP-1 resulted in an increase of the extracellular matrix, as observed by histology, in the degenerated discs [184]. An extension of that study confirmed that a direct injection of OP-1 into the chronically compressed rat tail IVDs induced anabolic and anti-catabolic activities, as documented by reduced immunostaining for aggrecanase, MMP-13, TNF-α, and IL-1β [185].

The effectiveness of direct protein delivery was also confirmed in experiments using rhGDF-5, where a single injection of rhGDF-5 resulted in restoration of disc height, and improvements in MRI and histological grading scores in the rabbit anular puncture model of disc degeneration [165]. In this model, a single injection of rhGDF-5 significantly suppressed the mRNA expression of cytokines (IL-1β, IL-6, TNF-α), catabolic enzymes [a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), cyclooxygenase-2 (COX-2)], and pain-related molecules [nerve growth factor (NGF), vascular endothelial growth factor (VEGF)] by punctured discs. In a thrombin injection (10 U) rabbit model of disc degeneration evaluated with quantitative T1ρ MRI, T1ρ values of the nucleus pulposus of rhGDF-5 treated discs were similar to values of the control level at 12 weeks [186].

In a rabbit anular stab model of disc degeneration, an intradiscal injection of BMP-2 resulted in degenerative changes that were more frequent and severe than with the saline control and promoted hypervascularity and fibroblast proliferation, but had no effect on new bone formation or fusion of the IVD after 12 weeks; the authors suggest that rhBMP-2 may be involved with a response to injury after an anular tear [187]. In an anular stab ovine large animal model of disc degeneration, the simultaneous injection of rhBMP-13, which plays a role in spinal column development and promotes the expression of chondrogenic marker genes in a variety of cell types, was protective against loss of disc height and cells from the disc, and resulted in increased levels of disc extracellular matrix proteins compared to anular stab alone [188]. The *in vivo* application of PRP-impregnated gelation hydrogel microspheres (PRP-GHMs) has been evaluated in a nucleotomy rabbit model of disc degeneration. Histological and immunohistochemical studies revealed that the structure of the IVD and the accumulation of proteoglycans were preserved by the injection of PRP-GHMs, but not in PRP alone and saline controls [189]. An extension of these studies in this model, reported that:

1) Disc height and water content were preserved in PRP-GHMs-treated IVDs in MR images,
2) The mRNA expression of proteoglycan core protein and type II collagen was significantly higher, and
3) The number of apoptotic cells in the nucleus pulposus were lower after PRP-GHM treatment compared with other treatment groups (PRP-saline, saline-GHM, puncture only) [190].

In vivo studies: other biologics

LinkN is an *in vivo* proteolytic degradation byproduct of proteoglycan aggregate stabilizing link protein. The effect of the intradiscal administration of the synthetic peptide of LinkN, which stimulates proteoglycan and collagen synthesis of IVD cells *in vitro*, was evaluated in the rabbit anular puncture model of disc degeneration and was shown to partially restore disc height, increase anabolic extracellular matrix gene expression, and reduce catabolic gene expression [170]. In a recent study in a large animal model of disc degeneration, the disc architecture and mechanical properties of surgically denucleated porcine (minipig) IVDs injected with a fibrin sealant, which promotes IVD cell proliferation and matrix synthesis, were preserved [191]. In addition, fibrin also inhibited fibrosis of the nucleus pulposus, increased proteoglycan synthesis, reduced secretion of pro-inflammatory cytokines and increased the synthesis of the pro-resolution factors, TGF-β and IL-4[191]. Nasto and co-

workers have recently demonstrated that inhibition of NFκB activity via intra-peritoneal injection of Nemo Binding Domain peptide reduced proteoglycan loss in disc degeneration observed in a mouse model of accelerated aging [192].

Summary

These studies are important for demonstrating that biologic manipulation of IVD cells *in vitro* may translate to an observed effect *in vivo*, and that direct protein delivery may be useful for promoting new matrix formation in the absence of cell delivery. It is noteworthy that the studies using rhOP-1 and rhGDF-5 also provide documentation of preclinical measures such as disc height, disc mechanics and MRI appearance that may be important for translating these technologies to non- or minimally-invasive clinical outcomes for patient treatment. Indeed, data for the *in vitro* studies have led to an investigational new drug study that has been initiated to test the safety and effect of injections of rhOP-1 into the disc space.

GENE THERAPY FOR INTERVERTEBRAL DISC REGENERATION

While the studies described above illustrate a range of proteins considered as possible therapies for IVD regeneration, it is important to consider the unavoidable limitations of protein delivery to the disc space. Issues such as protein half-life or solubility, the need for a proper carrier, need to preserve mechanical environment or cell numbers, and/or the presence of inhibitors are all factors that can be expected to affect the therapeutic efficacy of protein delivery *in vivo*. A consideration for the use of recombinant protein therapies is also cost, as some disc pathologies and the need to inhibit disc degeneration may be chronic in nature or require multiple treatments. Gene therapy has been advocated as a therapeutic alternative for the delivery of biologics in disc regeneration [193–196]. DNAs that encode specific proteins may be delivered into the cells by viral or non-viral transfection, with the result that these cells produce proteins to, theoretically, prolong the duration of action. The first successful attempt for *in vitro* gene transfer was reported for chondrocyte-like cells from the endplate of the IVD using a retro-viral mediated technique [193]. An *ex vivo* approach was used based on harvesting host cells, infecting these host cells *in vitro*, selecting and enriching infected cells, and finally returning these cells to the host. This approach avoids problems associated with low cell numbers and transfection efficiencies, but is both challenging and costly to perform. Nevertheless, because a decrease of cells by apoptosis or necrosis is considered to be associated with advanced disc degeneration, cell supplementation with genetically manipulated cells will continue to hold promise for disc regeneration.

Adenoviral vectors often possess high titers and infectivity and are able to infect non-dividing cells such as IVD cells. Adenoviral-mediated gene transfer to human IVD cells has been shown to be efficient and to produce transcripts across non-degenerative to degenerative cell types, using adenovirus carrying lacZ (Ad/CMV-lacZ) or luciferase 'marker' genes (Ad/CMV-luciferase), as well as Sox9 (Ad/Sox9-GFP), GDF-5 and TGF-β1 adenoviral constructs [197,198]. Yoon and co-workers also used adenoviral vector to transfer LIM mineralization protein-1 (LMP-1) to rat IVD cells *in vitro* and observed an increase of BMP-2 and BMP-7 gene expression and protein production, and proteoglycan synthesis [196]. The feasibility of using direct *in vivo* adenoviral-mediated gene transfer to disc cells has also been demonstrated using the lacZ, TGF-β, LMP-1 and Sox9 genes [196,197,199], finding transgene expression to be present or to exert a biological effect on biosynthesis, often for several weeks.

In addition to upregulation of anabolic factors, inhibition of catabolic processes has also been studied using gene therapy for IVD regeneration. Wallach and co-workers reported that gene transfer of the tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of catabolic enzymes, can increase proteoglycan accumulation within pellet cultures of human IVD cells [200]. LeMaitre reported that human disc cells infected with Ad-IL-1 receptor antagonist (Ad/IL-1Ra) were resistant to IL-1 [201]. When *in vitro* infected cells were injected into disc

explants *in vitro*, IL-1 receptor antagonist protein expression was also increased and maintained for the two week time period investigated. Recently, RNA interference has been demonstrated to downregulate gene expression *in vitro* in NP cells and *in vivo* in the rat coccygeal IVD [202] and degenerated rabbit IVD [203]. The use of RNAi technology is promising for the downregulation of deleterious catabolic gene expression in disc degeneration.

There are significant concerns about adenoviral vector use clinically, however, that may include significant toxicity when used in spinal applications [204,205]. These concerns have led investigators to begin consideration of adeno-associated viral vectors [206] and baculoviral vectors, the latter which may be non-toxic [207]. Both approaches may provide safe alternatives for future disc therapies, although much work remains. Also to avoid safety concerns found with viral gene transfer, several non-viral methods for direct gene transfer to cells have been proposed. Preliminary reports using microbubble-enhanced ultrasound gene therapy [208] and a 'gene gun' method [209,210] have shown that introduction of a marker or growth factor gene could be accomplished and provide sustained gene expression without need for viral vectors. Transfection efficacy with non-viral means is lower, however, than that in viral transfection and further investigation will be needed to apply these in a clinical setting. Nevertheless, both viral and non-viral transfection methods have pros and cons. Safety and immunological reactions, as well as the control of expression in viral-mediated gene therapy, are potential problems. The comparatively low immunologic exposure of the healthy disc and its low cellularity seem to suggest that safety with gene delivery of therapeutic agents is a lesser concern in the IVD. Cells are needed to transduce the biological effect, however, so that transplantation of *ex vivo* transfected cells may be an important part of the future potential for gene therapy in the IVD. Additionally, due to the proximity of the target to the spinal cord, significant harm can occur due to failure to control delivery, as seen with paralysis in rabbits due to accidental gene delivery in the intradural space [205]. During this time, work continues on identification of broader and diverse molecular targets that can be useful for gene delivery in the treatment of IVD regeneration.

CONCLUDING REMARKS

Efforts to regenerate and replace the tissues of the intervertebral disc have virtually exploded over the last two decades, although the field remains in its infancy. The complexity of the diverse degenerative and pathological processes that affect the IVD, as well as the intrinsic complexity of the heterogeneous disc structures, demands that multiple strategies be developed for treatment of the IVD. Partial IVD replacements using acellular, pre-formed or *in situ* formed biomaterials have the longest history of development and are important for defining procedural outcomes that will be relevant to long term functional success. Development of strategies use cells, biologics, or gene therapy is often focused upon restoration of a single tissue source, such as nucleus pulposus or anulus fibrosus, and with or without biomaterial scaffolds. However a growing number of tissue-engineering solutions have been proposed to integrate two dissimilar tissues in the repair process, and additional work to promote integration amongst native, neo-generated and implanted tissues will be critical to restoring IVD function. Many of the identified strategies derive largely from knowledge gained in cartilage tissue engineering, although the differing cellular, functional and structural requirements of the IVD suggest that custom approaches are needed. Advances in IVD cell biology are needed to enable the identification of novel therapeutic targets, to select for classes of biomaterials, and to suggest appropriate drug delivery strategies, as disc cell phenotype, cell-biomaterial interactions, and the biology of aging for these cells, are still poorly understood. While a diverse array of molecules, cell sources and materials are suggested as appropriate for IVD regeneration, additional work is needed to reveal some common and unique themes in human IVD cell responses that focus research on IVD specific strategies.

Currently underway clinical trials of autologous cell therapies or autologous protein products will pave the way for later generations of cellular and biologic-based therapies, as they are expected to illustrate the unique challenges of treating the pathologic and aged human IVD. The next decade promises great advances in the translation of basic and applied sciences to the clinical treatment of IVD regeneration and replacement.

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CHAPTER 57

Articular Cartilage Injury

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INTRODUCTION

In an effort to decrease the risk of post-traumatic osteoarthritis, surgeons and scientists have been seeking ways to prevent progressive joint degeneration following injury. Surgeons have used operative treatments, including penetrating subchondral bone, soft-tissue grafts, and cell transplants, to stimulate restoration of damaged articular surfaces, with variable results. This chapter covers recent advances in the use of artificial matrices, growth factors, and immature chondrocytes or stem cells to promote cartilage repair as well as work suggesting that several biologic agents, including capsase inhibitors, antioxidants, and anti-inflammatory drugs may minimize the effects of mechanical damage to chondrocytes.

Mechanical loading of articular surfaces which exceeds the tolerance of those surfaces damages chondrocytes and their matrix; damage that can cause joint degeneration leading to the clinical syndrome of post-traumatic osteoarthritis. The risk of post-traumatic osteoarthritis depends on the type and severity of the injury and also on the repair and remodeling of the damaged articular surfaces. Three classes of joint injuries can be identified, based on the type of articular surface damage:

1) Chondral damage and in some cases subchondral bone damage that does not cause visible disruption of the articular cartilage,
2) Mechanical disruption of the joint surface limited to articular cartilage (chondral ruptures or tears), and
3) Mechanical disruption of articular cartilage and subchondral bone (articular surface fractures).

In most instances, chondrocytes can repair damage that does not disrupt the articular surface if they are protected from further injury. Mechanical disruption of articular cartilage stimulates chondrocyte synthetic activity, but this response, with few if any exceptions, fails to repair the tissue damage. Disruption of articular cartilage and sub-chondral bone stimulates chondral and bony repair. The osteochondral repair response usually heals the bony injury, but the chondral repair tissue does not duplicate the properties of normal articular cartilage.

Normal pain-free movement depends on the unique properties of the articular cartilage that forms the bearing surfaces of synovial joints [1]. Degeneration of this remarkable tissue causes osteoarthritis: joint pain and dysfunction that limits mobility [2,3]. The mechanisms, frequency, and natural history of articular surface injuries are poorly understood, but it is clear that these injuries can lead to post-traumatic osteoarthritis [1,4–6]. Limited awareness of chondral and osteochondral injuries and difficulty in diagnosing these injuries makes it

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impossible to determine accurately their incidence, or their relationship to the development of joint degeneration [1,7,8]. However, arthroscopic examinations of injured knee joints suggest that closed articular surface injuries occur frequently [9,10]. One group of surgeons arthroscopically examined 85 knees with traumatic hemarthrosis but absent or negligible ligamentous instability [9]. Twenty percent of these knees had articular surface defects. In many patients cartilage injuries occur in association with injuries to other joint tissues, including menisci, ligaments, joint capsule and synovium. In these people the cartilage injury may be overlooked; even when it is identified it is difficult to distinguish the effects of the cartilage injury from the effects of the injuries to the other tissues. Damage to articular surfaces that does not result in visible disruption of articular cartilage or subchondral bone is not easily detected, although it probably occurs far more frequently than chondral and osteochondral fractures [4,6].

Recent advances in methods of diagnosing articular surface injuries, including arthroscopy and magnetic resonance imaging [11–13], combined with reports of new methods of stimulating cartilage repair or regeneration and osteochondral transplantation have increased interest in these injuries. Clinical evaluation of patients with articular surface damage and determining the appropriate role of these new treatments or the need for any treatment requires an understanding of the mechanisms of these injuries and their natural history. This chapter discusses the relationship between articular surface injury and joint degeneration, mechanisms of articular surface injuries, the responses of articular surfaces to injury and approaches to preventing joint degeneration following joint injury.

ARTICULAR CARTILAGE INJURY AND JOINT DEGENERATION

The end stage of post-traumatic osteoarthritis, the osteoarthritis that follows joint injury, is identical to that of primary osteoarthritis, but patients with post-traumatic osteoarthritis are often young or middle-aged adults and have a well-defined precipitating insult [14,15]. Clinical experience and epidemiologic studies show that meniscal, ligament, and joint-capsule tears, joint dislocations, and intra-articular fractures increase the risk of the progressive joint degeneration that causes post-traumatic osteoarthritis [14,16,17]. Participation in sports that expose joints to high levels of impact or torsional loading also increases the risk of joint degeneration [8,15].

The risk of osteoarthritis following joint injury varies with the type of injury: meniscal and ligamentous injuries have a lower risk than intra-articular fractures. Gelber et al. [14] found that 13.9% of those who had a knee injury (including meniscal, ligamentous or bone injuries) during adolescence or young adulthood developed knee osteoarthritis, as compared with 6.0% of those who did not have a knee injury. A study of patients who suffered ligamentous and meniscal injuries of the knee reported that they had a tenfold increased risk of osteoarthritis compared with patients who do not have joint injuries [18]. Intra-articular fractures have the greatest risk of osteoarthritis. Depending on the severity of the injury and on the joint, the risk ranges from about 25% to more than 50% of patients [15,19].

The time interval between joint injury and the development of osteoarthritis varies from less than a year in patients with severe intra-articular fractures to a decade or more in some patients with ligamentous or meniscal injuries [15,19]. Because many joint injuries occur in young adults, the population of patients with post-traumatic osteoarthritis includes many individuals under 50 years of age. But older individuals may have an increased risk of osteoarthritis after joint injuries. Studies of patients with intra-articular fractures of the knee show that patients older than 50 years of age have a two- to four-fold greater risk of developing osteoarthritis than younger patients; patients over 40 years who have acetabular fractures and patients over 50 years who have displaced ankle fractures may also have a greater risk of OA than younger patients who have similar injuries; and age increases the risk of knee-joint degeneration after anterior cruciate ligament injury [15,20].

MECHANISMS OF ARTICULAR CARTILAGE INJURIES

Understanding the mechanisms of articular surface injuries requires appreciation of how loads and rate of loading affect articular cartilage [6,21]. Slowly applied loads and suddenly applied loads differ considerably in their effects. The articular cartilage extracellular matrix consists of water and a macromolecular framework formed primarily by collagens and large aggregating proteoglycan [1]. The collagens give the tissue its form and tensile strength, and the interaction of aggregating proteoglycans with water gives the tissue its stiffness to compression, resilience, and probably its durability. Loading of articular surfaces causes movement of fluid within the articular cartilage matrix that dampens and distributes loads within the cartilage and to the subchondral bone [22]. When this occurs slowly, the fluid movement allows the cartilage to deform and decreases the force applied to the matrix macromolecular framework. When it occurs too rapidly for fluid movement through the matrix and deformation of the tissue, as with sudden impact or torsional joint loading of the joint surface, the matrix macromolecular framework sustains a greater share of the force. If this force is great enough, it ruptures the matrix macromolecular framework, damages cells, and exceeds the ability of articular cartilage to prevent subchondral bone damage by dampening and distributing loads.

In vivo, expected and unexpected and slow and sudden movements or impacts may differ in the amount of force transmitted to joint surfaces. Muscle contractions absorb much of the energy and stabilize joints during slow, expected movements or impacts. Sudden or unexpected movements or impacts may occur too rapidly for muscle contractions to stabilize joints and decrease the forces on the articular surfaces. For this reason, sudden and unexpected movements or impacts can transmit greater forces to joint surfaces.

Acute or repetitive blunt joint trauma can damage articular cartilage and the calcified cartilage zone-subchondral bone region while leaving the articular surface intact [4,23–26]. The intensity and type of joint loading that can cause chondral and subchondral damage without visible tissue disruption has not been well defined. Physiologic levels of joint loading do not appear to cause joint injury, but impact loading above that associated with normal activities but less than that necessary to produce cartilage disruption can cause alterations of the cartilage matrix and damage chondrocytes [4,23,24,26–28]. Experimental evidence shows that loss of proteoglycans or alteration of their organization (in particular, a decrease in proteoglycan aggregation) occurs before other signs of cartilage injury following impact loading. The loss of proteoglycans may be due either to increased degradation of the molecules or to decreased synthesis. Significant loss of matrix proteoglycans decreases cartilage stiffness and increases its permeability. These alterations may cause greater loading of the remaining macromolecular framework, including the collagen fibrils, increasing the vulnerability of the tissue to further damage from loading. These injuries may cause other matrix abnormalities besides loss of proteoglycans, such as distortions of the collagen fibril meshwork and disruptions of the collagen fibril proteoglycan relationships and swelling of the matrix), and they may injure chondrocytes [4,26].

Currently there is no clinically applicable method of detecting alterations in cartilage matrix composition such as decreased proteoglycan concentration and increased water concentration; however, new imaging techniques may provide methods of assessing articular cartilage composition. When probing the articular surface, surgeons sometimes find regions of apparent softening that may result from alterations in the matrix, and devices are being developed that will allow *in vivo* measurement of articular surface stiffness. Combined with information about cartilage composition, these measurements may make it possible to better define injuries to the articular surface that do not result in visible tissue disruption.

Disrupting a normal articular surface with a single impact requires substantial force, presumably because of the ability of articular cartilage and subchondral bone to dampen and distribute loads. A trans-articular load of 2170 newtons applied to canine patellofemoral joints

caused fractures in the zone of calcified cartilage visible by light microscopy and articular cartilage fissures that extended from the articular surface to the transitional or superficial radial zone of the articular cartilage [24]. A study of the response of human articular cartilage to blunt trauma showed that articular cartilage could withstand impact loads of up to 25 newtons per square millimeter (25 MPa) without apparent damage. Impact loads exceeding this level caused chondrocyte death and cartilage fissures [29]. The authors suggested that reaching a stress level that could cause cartilage damage required a force greater than that necessary to fracture the femur. Another study [30] measured the pressure on human patellofemoral articular cartilage during impact loading, and found that impact loads less than the level necessary to fracture bone caused stresses greater than 25 MPa in some regions of the articular surface. With the knee flexed 90°, 50% of the load necessary to cause a bone fracture produced joint pressures greater than 25 MPa for nearly 20% of the patellofemoral joint. At 70% of the bone fracture load, nearly 35% of the contact area of the patellofemoral joint pressures exceeded 25 MPa, and at 100% of the bone fracture load 60% of the patellofemoral joint pressures exceeded 25 MPa. These latter results show that impact loads can disrupt cartilage without fracturing bone.

Other experimental investigations show that repetitive impact loads split articular cartilage matrix and initiate progressive cartilage degeneration [31–33]. Cyclic loading of human cartilage samples *in vitro* caused surface fibrillation [32], and periodic impact loading of bovine metacarpal phalangeal joints *in vitro* combined with joint motion caused degeneration of articular cartilage [34]. Repeated overuse of rabbit joints *in vivo* combined with peak overloading caused articular cartilage damage, including formation of chondrocyte clusters, fibrillation of the matrix, thickening of subchondral bone, and penetration of subchondral capillaries into the calcified zone of articular cartilage [33]. The extent of cartilage damage appeared to increase with longer periods of repetitive overloading, and deterioration of the cartilage continued following cessation of excessive loading. This latter finding suggests that some cartilage damage is not immediately visible.

An investigation of cartilage plugs also showed that repetitive loading disrupted the tissue and that the severity of the damage increased with increasing load and increasing number of loading cycles [35]. Two hundred and fifty cycles of a 1,000 pounds per square inch compression load caused surface abrasions. Five hundred cycles produced primary fissures penetrating to calcified cartilage, and 1,000 cycles produced secondary fissures extending from the primary fissures. After 8,000 cycles, the fissures coalesced and undermined cartilage fragments. Higher loads caused similar changes with fewer cycles. The experiments suggested that repetitive loading can propagate vertical cartilage fissures from the joint surface to calcified cartilage and cause extension of oblique fissures into areas of intact cartilage.

Clinical studies have identified articular cartilage fissures, flaps, and free fragments and changes in subchondral bone similar to those produced experimentally by single and repetitive impact loads [4,36]. In at least some patients, acute impact loading of the articular surface or twisting movements of the joint apparently caused these injuries. In other patients, the cartilage damage may have resulted from repetitive loading. Magnetic resonance imaging of joints soon after an acute impact or torsional load occasionally shows changes in subchondral bone consistent with damage to the zone of calcified cartilage and subchondral bone, even when the articular surface is intact [11,12,13,37].

Clinical experience suggests that chondral ruptures or tears and osteochondral fractures result from similar impact and twisting-joint injuries, but they tend to occur in different age groups, and some individuals may have a greater risk of chondral tears. Chondral tears generally occur in skeletally mature people, while osteochondral fractures typically occur in skeletally immature people or young adults. This difference may result from age-related changes in the mechanical properties of the articular surface, including the uncalcified cartilage, the calcified cartilage zone, and the subchondral bone. That is, age-related alterations in the articular cartilage matrix decrease the tensile stiffness and strength of the superficial zone; and the calcified cartilage zone-subchondral bone region mineralizes fully following completion of

skeletal growth, presumably creating a marked difference in mechanical properties between the uncalcified cartilage and the calcified cartilage subchondral bone region. Taken together these changes probably increase the risk of ruptures of the superficial-cartilage matrix and of these ruptures extending to the calcified cartilage subchondral bone region. Genetically determined abnormalities of the articular cartilage may also increase the risk of chondral ruptures from a given impact or torsional load, but the relationships between known genetic abnormalities of articular cartilage and cartilage properties have not been well defined.

RESPONSE OF ARTICULAR CARTILAGE TO INJURY

Articular surface injuries can be classified based on the type of tissue damage and the repair response:

1) Cartilage matrix and cell injuries, that is, damage to the joint surface that does not cause visible mechanical disruption of the articular surface;
2) Chondral fissures; flap tears, or chondral defects, that is, visible mechanical disruption of articular cartilage limited to articular cartilage; and
3) Osteochondral injuries, that is, visible mechanical disruption of articular cartilage and bone [1, 4, 38–40]) (Table 57.1).

Matrix and cell injuries

Acute or repetitive blunt trauma, including excessive impact loading, can cause alterations in the articular cartilage matrix, including a decrease in proteoglycan concentration and possibly disruptions of the collagen fibril framework. Injuries that do not cause an apparent articular cartilage injury, including joint dislocations or ligament and joint capsule tears, may have associated damage to the articular cartilage cells and matrix [41]. The ability of chondrocytes to sense changes in matrix composition and to synthesize new molecules makes

TABLE 57.1 Chondral and osteochondral injuries

Injury	Clinical presentation	Repair response	Potential for healing
Damage to chondral matrix and/or cells and/or subchondral bone without visible disruption of the articular surface	No known symptoms, although subchondral- bone injury may cause pain Inspection of the articular surface and current clinical imaging methods for articular cartilage lesion may of injury Imaging of subchondral bone may show abnormalities	Synthesis of new matrix macromolecules Cell proliferation?	If the basic matrix structure remains intact and enough viable cells remain, the cells can restore the normal tissue composition If the matrix and/or cell population sustains significant damage or if the tissue sustains further cannot detect this type damage, the lesion may progress to cartilage degeneration
Cartilage disruption (chondral fractures or ruptures)	May cause mechanical symptoms, synovitis, pain, and joint effusions	No fibrin clot formation or inflammation Synthesis of new matrix macromolecules and cell proliferation, but new tissue does not fill the cartilage defect	Depending on the location and size of the lesion and the structural integrity, stability, and alignment of the joint, the lesion may or may not progress to cartilage degeneration
Cartilage and bone disruption (osteochondral fractures)	May cause mechanical symptoms, synovitis, pain, and joint effusions	Formation of a fibrin clot, inflammation, invasion of new cells, and production of new chondral and osseous tissue	Depending on the location and size of the lesion and the structural integrity, stability, and alignment of the joint, the lesion may or may not progress

it possible for them to repair damage to the macromolecular framework [42]. It is not clear at what point this type of injury becomes irreversible and leads to progressive loss of articular cartilage. Presumably, the chondrocytes can restore the matrix as long as the loss of matrix proteoglycan does not exceed what the cells can rapidly produce, if the fibrillar collagen meshwork remains intact, and if enough chondrocytes remain viable. When these conditions are not met, the cells cannot restore the matrix, the chondrocytes will be exposed to excessive loads, and the tissue will degenerate.

Chondral injuries

Acute or repetitive trauma can cause focal mechanical disruption of articular cartilage, including fissures, chondral flaps or tears, and loss of a segment of articular cartilage [4]. The lack of blood vessels and lack of cells that can repair significant tissue defects limit the response of cartilage to injury [39,43]. Chondrocytes respond to tissue injury by proliferating and increasing the synthesis of matrix macromolecules near the injury. But the newly synthesized matrix and proliferating cells do not fill the tissue defect, and soon after injury the increased proliferative and synthetic activity ceases.

Osteochondral injuries

Unlike injuries limited to cartilage, injuries that extend into subchondral bone cause hemorrhage and fibrin clot formation and activate the inflammatory response [39,40,43]. Soon after injury, blood escaping from the damaged-bone blood vessels forms a hematoma that temporarily fills the injury site. Fibrin forms within the hematoma and platelets bind to fibrillar collagen. A continuous fibrin clot fills the bone defect and extends for a variable distance into the cartilage defect. Platelets within the clot release vasoactive mediators and growth factors or cytokines (small proteins that influence multiple cell functions, including migration, proliferation, differentiation, and matrix synthesis), including transforming growth factor beta and platelet-derived growth factor. Bone matrix also contains growth factors, including transforming growth factor beta, bone morphogenic protein, platelet-derived growth factor, insulin-like growth factor I, insulin-like growth factor II, and possibly others. Release of these growth factors may have an important role in the repair of osteochondral defects. In particular, they probably stimulate vascular invasion and migration of undifferentiated cells into the clot and influence the proliferative and synthetic activities of the cells. Shortly after entering the tissue defect, the undifferentiated mesenchymal cells proliferate and synthesize a new matrix. Within two weeks of injury, some mesenchymal cells assume the rounded form of chondrocytes and begin to synthesize a matrix that contains type II collagen and a relatively high concentration of proteoglycans. These cells produce regions of hyaline-like cartilage in the chondral and bone portions of the defect. Six to eight weeks after injury, the repair tissue within the chondral region of osteochondral defects contains many chondrocyte-like cells in a matrix consisting of type II collagen, proteoglycans, some type I collagen, and noncollagenous proteins. Unlike the cells in the chondral portion of the defect, the cells in the bony portion of the defect produce immature bone, fibrous tissue, and hyaline-like cartilage.

The chondral repair tissue typically has a composition and structure intermediate between that of hyaline cartilage and fibrocartilage; and it rarely, if ever, replicates the elaborate structure of normal articular cartilage [1,38,44,45]. Occasionally, the cartilage repair tissue persists unchanged or progressively remodels to form a functional joint surface. But in most large osteochondral injuries, the chondral repair tissue begins to show evidence of depletion of matrix proteoglycans, fragmentation and fibrillation, increasing collagen content, and loss of cells with the appearance of chondrocytes within a year or less. The remaining cells often assume the appearance of fibroblasts as the surrounding matrix comes to consist primarily of densely packed collagen fibrils. This fibrous tissue usually fragments and often disintegrates, leaving areas of exposed bone. The inferior mechanical properties of chondral repair tissue may be responsible for its frequent deterioration [1,43]. Even repair tissue that successfully fills

osteochondral defects is less stiff and more permeable than normal articular cartilage and the orientation and organization of the collagen fibrils in even the most hyaline-like cartilage repair tissue does not follow the pattern seen in normal articular cartilage. In addition, the repair tissue cells may fail to establish the normal relationships between matrix macromolecules, in particular, the relationship between cartilage proteoglycans and the collagen fibril network. The decreased stiffness and increased permeability of repair cartilage matrix may increase loading of the macromolecular framework during joint use, resulting in progressive structural damage to the matrix collagen and proteoglycans, thereby exposing the repair chondrocytes to excessive loads and further compromising their ability to restore the matrix.

Clinical experience and experimental studies suggest that the success of chondral repair in osteochondral injuries may depend to some extent on the severity of the injury, as measured by the volume of tissue or surface area of cartilage injured and the age of the individual [46]. Smaller osteochondral defects that do not alter joint function heal more predictably than larger defects that may change the loading of the articular surface. Potential age-related differences in healing of chondral and osteochondral injuries have not been thoroughly investigated, but bone heals more rapidly in children than in adults, and the articular cartilage chondrocytes in skeletally immature animals show a better proliferative response to injury and synthesize larger proteoglycan molecules than those from mature animals [42,47–51]. Furthermore, a growing synovial joint has the potential to remodel the articular surface to decrease the mechanical abnormalities created by a chondral or osteochondral defect.

PREVENTING JOINT DEGENERATION FOLLOWING INJURY

Orthopedic surgeons routinely perform extensive surgical procedures, some having substantial complication rates, in an effort to restore the alignment and congruity of articular surfaces following intraarticular fractures [19]. The purpose of these procedures is to decrease residual joint incongruity and thereby to decrease focal elevations of contact stress, presumed to be responsible for post-traumatic osteoarthritis. These widely accepted practices are based largely on the assumption that joints are less likely to develop osteoarthritis if the peak stresses on focal areas of the articular surface are reduced. However, there is little evidence to guide surgeons in determining how much stress the articular surface can tolerate, in the form of either acute impact or chronically increased stress, and the potential for human joints to repair and remodel the articular surface after injury is poorly understood.

With better understanding of the role of mechanical forces in the pathogenesis of osteoarthritis, clinical and basic research could be focused on developing new, minimally invasive, and non-surgical treatments of joint injuries that have a high risk of post-traumatic osteoarthritis and on treatments that prevent or delay the development of joint degeneration in patients at high risk of post-traumatic osteoarthritis. There is increasing evidence that biologic interventions can decrease mechanical stress-induced chondrocyte damage. For example, the work of D'Lima and coworkers [52–54] shows that capsase inhibition can decrease mechanically induced chondrocyte and apoptosis, and Haut and colleagues have reported that P188 surfactant can limit chondrocyte necrosis following impact loading [55,56]. Other investigations show that antioxidants can prevent mechanically induced chondrocyte damage [57,58].

PROMOTING ARTICULAR SURFACE REPAIR

Better understanding of articular cartilage injuries and recognition of the limitations of the natural repair responses have contributed to the recent interest in cartilage repair and regeneration [4,39,43,59–61]. In the last three decades clinical and basic scientific investigations have shown that implantation of artificial matrices, growth factors, perichondrium,

periosteum, and transplanted chondrocytes and mesenchymal stem cells can stimulate formation of cartilaginous tissue in synovial joint osteochondral and chondral defects [1,59,61–65].

Penetration of subchondral bone

Experimental and clinical investigations show that penetration of subchondral bone leads to formation of fibrocartilagenous repair tissue on the articular surfaces of synovial joints [46,59,66–69]. In regions with full-thickness loss or advanced degeneration of articular cartilage, penetration of the exposed subchondral bone disrupts subchondral blood vessels, leading to formation of a fibrin clot that fills the bone defect and usually covers the exposed bone surface [46,59]. If the surface is protected from excessive loading, undifferentiated mesenchymal cells migrate into the clot, proliferate, and differentiate into cells with the morphologic features of chondrocytes [70]. In most instances, over a period of six to eight weeks they form bone in the osseous portion of the defect and fibrocartilagenous tissue in the chondral portion [39,71,72]. Initially, the chondral repair tissue can closely resemble articular cartilage in gross and light-microscopic appearance, but it fails to duplicate fully the composition (especially the types and concentrations of collagens and proteoglycans), structure, and mechanical properties of normal articular cartilage, and in many instances it deteriorates with time [1,38,39,43].

Surgeons currently use a variety of methods of penetrating subchondral bone to stimulate formation of a new cartilaginous surface, including arthroscopic drilling and abrasion of the articular surface and making multiple small-diameter defects or fractures with an awl or similar instrument, a method referred to as the microfracture technique [36,59,66,67,71,72,73]. Multiple authors report that these procedures can decrease the symptoms due to isolated articular cartilage defects of the knee in a majority of patients [36,66,67,71,73,74,75,76].

Periosteal and perichondrial grafts

The potential benefits of periosteal and perichondrial grafts include introduction of a new cell population along with an organic matrix and some protection of the graft or host cells from excessive loading. Animal experiments and clinical experience show that perichondrial and periosteal grafts placed in articular cartilage defects can produce new cartilage [59,76]. O'Driscoll has described the use of periosteal grafts for the treatment of isolated chondral and osteochondral defects and demonstrated that these grafts can produce a new articular surface [63,64,77,78]. Other investigators have reported encouraging results with perichondrial grafts [79,80]. However, one study suggests that increasing patient age adversely affects the results of soft-tissue grafts. Seradge et al. [81] studied the results of rib perichondrial arthroplasties in 16 metacarpophalangeal joints and 20 proximal interphalangeal joints at a minimum of three years following surgery. Patient age was directly related to the results. One hundred percent of the patients in their twenties and 75% of the patients in their thirties had good results following metacarpophalangeal joint arthroplasties. Seventy-five percent of the patients in their teens and 66% of the patients in their twenties had good results following proximal interphalangeal joint arthroplasties. None of the patients older than 40 years had a good result with either type of arthroplasty. The clinical observation that perichondrial grafts produced the best results in younger patients [81] agrees with the concept that age may adversely affect the ability of undifferentiated cells or chondrocytes to form an articular surface or that with age the population of cells that can form an articular surface declines [42,47,51].

Cell transplantation

Transplantation of cells grown in culture provides another method of introducing a new cell population into chondral and osteochondral defects. Experimental work has shown that both chondrocytes and undifferentiated mesenchymal cells placed in articular cartilage defects

survive and produce a new cartilage matrix [1,82–85,106]. In addition to these animal experiments, orthopedic surgeons have used autologous chondrocyte transplants for treatment of localized cartilage defects [68,86–90]. Proponents of this procedure report that it produces satisfactory results, including the ability to return to demanding physical activities, in as many as 90% of patients [68,89].

Artificial matrices

Treatment of chondral defects with growth factors or cell transplants requires a method of delivering and in most instances at least temporarily stabilizing the growth factors or cells in the defect. For these reasons, the success of these approaches often depends on an artificial matrix. In addition, artificial matrices may allow, and in some instances stimulate, ingrowth of host cells, matrix formation, and binding of new cells and matrix to host tissue [91,92]. Investigators have found that implants formed from a variety of biologic and nonbiologic materials, including treated cartilage and bone matrices, collagens, hyaluronan, chitosan, fibrin, carbon fiber, hydroxlyapatite, porous polylactic acid, polytetrafluoroethylene, polyester, and other synthetic polymers, facilitate restoration of an articular surface [1,59,92–94]. Lack of studies that directly compare different types of artificial matrices makes it difficult to evaluate their relative merits, but the available reports show that this approach may contribute to restoration of an articular surface.

Growth factors

Growth factors influence a variety of cell activities, including proliferation, migration, matrix synthesis, and differentiation. Many of these factors, including the fibroblast growth factors, insulin-like growth factors, and transforming growth factor betas, have been shown to affect chondrocyte metabolism and chondrogenesis [46,59,96,97]. Bone matrix contains a variety of these molecules, including transforming growth factor betas, insulin-like growth factors, bone morphogenic proteins, platelet-derived growth factors, and others [46,95]. In addition, mesenchymal cells, endothelial cells, and platelets produce many of these factors. Thus, osteochondral injuries and exposure of bone due to loss of articular cartilage may release these agents, which affect the formation of cartilage repair tissue, and they probably have an important role in the formation of new articular surfaces after currently used surgical procedures, including penetration of subchondral bone. Local treatment of chondral or osteochondral defects with growth factors has the potential to stimulate restoration of an articular surface [97–100]. Despite the promise of this approach, the wide variety of growth factors, their multiple effects, the interactions among them, and the possibility that the responsiveness of cells to growth factors may decline with age [47,49–51,101] have made it difficult to develop a simple strategy for using these agents to treat articular surface injuries. However, development of growth factor-based treatments for isolated chondral and osteochondral injuries in combination with other approaches, including use of artificial matrices and cell transplants, appears promising [61,102].

Anti-inflammatories

Severe post-traumatic synovitis is associated with joint injuries that cause OA [103]. Joint injury results in increases in pro-inflammatory cytokines in synovial fluids, which promote cartilage degeneration (ref). A recent clinical study indicated that anti-cytokine therapy relieved acute knee pain in patients with ACL tear [104], a result suggesting that such treatments could protect the joint from inflammatory damage. Why damage to joint tissues results in cytokine elevation and inflammation is unclear. However, results from studies of other organ systems show that inflammation is triggered by cell death resulting from tissue injury [105]. These observations strongly suggest that antioxidants, or drugs that downregulate immune responses to cell death, could block joint inflammation following a wide variety of joint injuries.

CONCLUSION

Articular surface injuries are a significant unsolved problem. They are common, the value of current treatments is uncertain, and many of these injuries initiate progressive joint degeneration, a condition recognized as post-traumatic osteoarthritis. Surgeons attempt to decrease the risk of post-traumatic osteoarthritis by restoring joint stability, congruity, and alignment following injury. In addition, to promote repair and remodeling of damaged articular surfaces, they penetrate subchondral bone and insert periosteal and perichondral grafts and autologous chondrocytes. The results of these procedures vary considerably among patients, and there is limited information concerning the long-term outcomes. However, the available studies of people who have suffered joint injuries indicate that even with optimal current treatment the risk of post-traumatic osteoarthritis is high following chondral tears and articular surface fractures. For this reason there is a clear need to improve the treatment of joint injuries. Clinical and experimental studies show that chondrocyte and mesenchymal stem cell transplantation, synthetic matrices, growth factors, and combinations of these treatments have the potential to restore articular surfaces. Other investigations suggest that biologic interventions may minimize chondrocyte damage due to mechanical forces. The most dramatic improvements in the treatment of articular surface injuries are likely to come from approaches that help maintain chondrocyte viability and function, restore joint stability and congruity, and promote articular surface repair and remodeling following joint injury.

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CHAPTER 58

Engineering Cartilage and Other Structural Tissues: Principles of Bone and Cartilage Reconstruction

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INTRODUCTION

According to legend, the first homotransplantation of an entire limb was performed by Saints Cosmas and Damian, as depicted by the artist, Fra Angelico [1]. Indeed, the concept was described in writing even earlier:

> 'The Lord God cast a deep sleep on the man, and while he was asleep, he took out one of his ribs and closed up its place with flesh. The Lord God then built up into a woman the rib that he had taken from the man' [2].

The number of research centers undertaking tissue engineering has waxed and waned, but overall, has steadily increased. While potential applications have broadened, the basic concept that the repair and regeneration of biological tissues can be guided through application and control of cells, materials, and chemoactive proteins has remained the same. As emphasis continues to shift toward the use of stem cells, and manipulation of the local environment to minimize inflammation, maximize cell survival and to direct development, tissue engineering continues to be an interdisciplinary field, requiring the interactions of physicians, and physical, chemical and biologic scientists, and engineers.

The ultimate goal is to utilize cells from a relatively small sample or from a universal donor source, expand them *in vitro*, and deliver them associated with a template, into an environment conducive to new tissue growth, and in a configuration that will generate a new functional tissue. The ability to achieve this goal requires a keen understanding of extracellular matrix, structural framework and regulation of cell development and chemofactors in the local environment.

In 1908, Lexer [3] described attempts at tissue engineering of structural tissue in his report of the use of freshly amputated or cadaver allografts for joint reconstruction. Several decades later, Huggins published his results concerning the search for bone morphogenic proteins.

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In 1965, Urist demonstrated the generation of one structural tissue; that is, bone by means of auto induction [4]. He described 'wandering histiocytes, foreign body giant cells, and inflammatory connective cells' being 'stimulated by degradation products of dead matrix to grow in and repopulate the area of an implant of decalcified bone'. The process, as described by Urist, is followed immediately by auto-induction, in which both the inductor cells and the induced cells are derived from ingrowing cells of the host bed. Differentiation of the osteoprogenitor cell was felt to be elicited by local alterations in cell metabolic cycles.

Later, in the 1970s, Dr. W.T. Green [5] described a series of experiments devoted to the generation of new cartilage. In one study, chondrocytes, seeded onto sterile bone spicules, were implanted into nude mice. Although the experiments were not entirely successful, Green correctly postulated that the advent of new biocompatible materials might enable cells to be seeded onto synthetic scaffolding and implanted into animals to generate new functional tissue.

Over three decades a number of reports involving the generation replacement of bone and cartilage have appeared in the literature. Unfortunately, many of the studies were performed in immune compromised animal models. Living cells were delivered on alloplastic implants to produce bone [6]. Cartilage growth, whether achieved by use of cell suspensions alone [7–10], cell attachment to naturally occurring matrices [11,12] from perichondrium [13] or peptide stimulation [14] had been described only in minute quantities until the early 90s. Prior to 1990, new cartilage was generated only in association with, and/or confined to, a defect in underlying bone or cartilage, and was devoid of specific shapes. Guided growth of new hyaline cartilage in significant amounts, not associated with endogenous bone or cartilage [15], was then demonstrated in immune compromised animal models [16,17]. During the next decade, the principles of tissue engineering were applied to many organ systems, with a great deal of attention being focused on orthopedic and maxillofacial applications as well as engineering of bone [18] new cartilage [19], tendon [20] and ligament [21].

At the turn of the millennium, new reports related to manipulating the local environment to achieve better cells survival and engraftment appeared. The use of mesenchymal stem cells (MSCs) in combination with tissue specific mature cells was reported with much success [22]. The MSCs were shown to not necessarily develop into the tissue, but to modulate the local environment to reduce inflammation and improve cell survival. Studies related to the mechanisms of inflammation, and how to modulate them have arisen in the last decade [23]. It is this author's opinion that the use of 'resolvins' to minimize inflammation will be tremendously helpful in advancing the field.

At the same time, descriptions of many stem cell types have arisen, including embryonic stem cells, (ESCs); much attention has been given to marrow derived stem cells, fat derived stem cells and MSCs. Reports of induced pluripotent stem cells (iPSCs) [24] seemed to give new promise to the fields of tissue engineering and regenerative medicine. Unfortunately, they have met with significant obstacles, as their potential was explored. By 2012, the biology of stem cells was re-evaluated. New reports challenged 'dogma' of the last decade. The ability of mature cells to revert to a stem cell state via a natural process following injury gives a new direction for the challenges faced in tissue engineering [25]. 'Stress altered cells' (SACs) appear to have the same, or better potential than ESCs for tissue-engineering applications, in that they are autologous, and have been shown to have the potential to generate cells representative of all three germ layers.

MATERIALS DEVELOPMENT

The development of matrices to serve as templates for cell attachment/suspension and delivery continues to progress. While initial efforts focused on the use of collagen as a natural matrix for cell delivery, a large degree of its early success was reported in the development of skin

equivalents, primarily the dermal component [26]. Ultimately, the generation of skin products proved to be one of the more difficult challenges.

Cartilage, bone and ligament studies first also focused on using natural extracellular matrix (ECM) proteins such as collagen as structural support for cell transplantation.

Poor mechanical properties and variable physical properties with different sources of the protein matrices have hampered progress with these approaches. Concerns arose regarding immunogenic problems associated with the introduction of foreign collagen. Also, there were inherent biophysical constraints in collagen used as scaffolding. For example, cartilage grown from cells seeded on collagen had to be confined in a rigid 'well' such as underlying bone or cartilage, because of collagen's non-moldability.

Xenografts or animal-derived collagen devices have also been studied. Although they have advantages related to availability, decreased risk of transmission of infectious diseases, and avoiding damage to the patient's healthy tissues, the preparation of the grafts apparently affects significantly their biomechanical properties, and the possibility of rejection can still limit their use. Tendon autografts and allografts are currently the most commonly used substitutes to reconstruct injured ligaments or tendon. Allografts, however, still face the risk of potential transmission of infectious diseases [27,28], and some of the techniques used to sterilize them present the possible risk of affecting the biomechanical characteristics of the grafts. Tendon autografts are perhaps the most commonly transplanted tissue structures. Their biological and biomechanical characteristics serve their purpose relatively well, considering that usually they rely on creeping substitution by the host's cells of a frequently inert organic scaffold from which most cells have usually disappeared. Autografts however, present with the disadvantage of creating a secondary morbid site during harvesting, and the fact that a functioning structure has to be removed from its original site for use in a nearby or remote site. Synthetic devices to replace or reinforce biologic materials were also studied as scaffolds, with the belief that fibrous tissue formation could be induced to form ligamentous and tendinous tissues. One hypothesis theorized that, in the presence of a tensile force, immature fibrous tissue so formed will gradually mature, with its fibers oriented functionally. However, this is disputed by reports [29,30] that the induced tissue is characterized by a chronic foreign body reaction and by disorderly collagen fibers. The use of carbon fiber-derived scaffolds that are eventually replaced by fibrous tissue to ultimately replace the lost function of a tendon or ligament was extensively studied in the 1970s and 1980s [31,32]. Nevertheless, the fact that carbon particles did not disappear completely and were eventually found in regional lymph nodes even several years later their use discouraged its use [33]. Later, carbon fibers were shown to somehow inhibit tendon fibroblast growth [34] concluding that collagen fibers do not significantly contribute to the tensile strength of carbon fiber composite ligaments and tendons. Entirely synthetic materials have also been used to replace or augment tendinous grafts in the treatment of injuries to the ligaments in the knee. Histological and biochemical have demonstrated that Dacron-induced fibrous tissue more closely resembles scar [35] or granulomatous tissue than to neoligament and has been shown to elicit a foreign body inflammatory reaction.

By the 1990s, the use of synthetic naturally occurring polymers as matrices for cell transplantation exploded. It was believed that synthetic scaffoldings had the advantages of being biocompatible as well as biodegradable. Synthetic polymer scaffolds provide intrinsic structure to the transplanted composite. This allows engineering of matrix configuration to meet the biophysical limitations of mass transfer. Synthetic matrices would in theory give one the flexibility to alter physical properties and potentially facilitates reproducibility and scale-up. The configuration of the synthetic matrix could be manipulated to vary the surface area available for cell attachment as well as to optimize the exposure of the attached cells to nutrients. The surface area to mass ratio could be altered or the porosity of differing configurations can be changed. The size of the pores of polymers of the same porosity can be altered to increase or decrease the intrinsic strength and elasticity of the polymer matrix, as well as,

compressibility or creep recovery. The chemical environment surrounding a synthetic polymer could be manipulated in a controlled fashion as the polymer is broken down. The potential exists to continuously deliver nutrients and hormones that can be incorporated into the polymer structure. The rate of degradation of the polymer matrices and the environment into which the cells are implanted could be manipulated by systematically altering the surface chemistry of the polymers, creating an acidic or basic environment as they degrade. For instance, polyaminocarbonates cause a local basic environment, while polyglycolic acid and polyanhydrides cause a local acidic environment. Polyanhydrides and polyorthoesters show surface erosion, other polymers show bulk erosion. Synthetic polymers also offer the advantage of being able to be consistently reproduced, and thus varying quality is not a problem.

It was possible to match the mechanical properties of the material with that of the tissue. Consequently, scaffolds for bone often contain ceramic hydroxyapatite, which has high stiffness like bone, while scaffolds for cartilage and tendon tend to be made from more compliant polymers.

Synthetic polymers are also useful, due to their good processing characteristics. They have a range degradation times from very short (days) to long (several months). Typically, they are configured in the form of fibrous meshes, porous sponges or foams, or hydrogels. The more common polymers used in fibrous meshes and foams include: the linear polyesters including polyglycolic acid (PGA), polylactic acid (PLA), and polycarpolactone (PCL); polyethylene glycol (PEG); and natural polymers such as collagen and hyaluronic acid (HA). Polymeric hydrogels have the distinct advantage of being injectable, which allows the delivery of the construct to be less invasive and thereby reducing surgical risks. Employment of these types of polymers also ensures delivery of an even distribution of a precise number of cells. They can be configured to provide mechanical support the cells to maintain their specific phenotype, without inhibiting migration. Common hydrogel substrates include the copolymers of polyethylene oxide and polypropylene oxide known as pluronics and natural polymers including alginate and agarose. The delivery of a known concentration of cells is simplified when using a hydrogel, where 100% of the cells are encapsulated within the delivery system, as compared to the fibers, where cell delivery is dependent on cell attachment. The hydrogels also allow the suspended cells to be uniformly distributed throughout the volume of polymer delivered. In contrast, the distribution of cells in the polymer fiber or foam scaffold is not uniform and difficult to predict.

Some materials were designed to play a more active role in guiding tissue development. Instead of merely holding cells in place, these bioactive matrices were designed to encourage cell attachment to the polymer through cell surface adhesion proteins. Studies have been undertaken, designed to identify features of a substrate to which different cell lines could be anchored and that were important for maintaining cell function. Identification of these properties allowed scientists to incorporate them into appropriate polymer scaffolds to be used for transplantation. For example, when some cell types are cultured under conventional conditions, such as on plastic or collagen coated dishes, gene transcription is depressed and cell specific mRNA declines, while the mRNA of structure related genes increases many fold. By contrast, when cultured on ECM, rich in laminin, type IV collagen some cell types exhibit increased longevity and maintenance of several cell specific functions. The capability of gene transcription persists when cells are cultured on extracellular gel matrix. Cell shape and function are altered by varying the coating density of any of a variety of different naturally occurring ECM substrates on bacteriologic plates. Some cell types, exhibit a rounded morphology when cultured on low density ECM, while an increase in ECM coating density results in more spreading of the cells and epithelial like morphology [36] as well as affecting the cells ability to enter the synthetic phase and continue through the cell cycle. Some cell types could be switched between

programs of growth and differentiation simply by modulating the ECM coating density, thereby altering the substratum's ability to resist cell-generated mechanical load. These and other studies also suggest that cell shape, which might be altered by manipulation of the physicochemical properties of the polymer, may be important in determining cell function.

These principles in combination with our studies led us to conclude that the matrix to which specific cell types cells are attached *in vitro* is one of the most important variables in the engineering of new functional tissue. Polymers can be synthesized to have an integrin polypeptide sequence (RGD) in the backbone [37–39] or which are constructed entirely of polypeptide sequences [40]. This allows the scaffold to effectively mimic the extracellular matrix and induce attachment of cells directly to the material. This may be particularly important in tissues which bear mechanical loads, since it would allow physical stimuli to be sensed by the cells in the developing tissue in a more physiologic manner.

Unfortunately, while all these concepts are valid, synthetic scaffolds may not possess all of the necessary factors to drive cells to the tissues desired. Efforts to provide growth factors and the hormonal environment normally associated with the tissue to be generated have increased by providing decellularized ECM in combination with synthetic scaffolds [41]. I believe that the optimal scaffold will ultimately prove to be a combination of decellularized ECM and synthetic scaffolds, both solid and as hydrogels.

The process of assembling an engineered structured tissue centers on the identification of an optimal cell source, and the function desired.

Expansion of the most effective cell population *in vitro* is critical in the process of building a construct. In the process, it is important to ensure that the expanded cell population expresses its phenotypic function. This is of great concern for chondrocytes, which dedifferentiate upon repeated passaging [42].

New approaches to expanding populations of chondrocytes continue to be explored. One new, very promising approach is to alter mature chondrocytes to a stem cell state, and then expand those *in vitro* [25]. They can later be induced to develop into mature chondrocytes for use in the construct to be implanted.

The formation of tissue is also greatly influenced by the scaffolds to which the cells are attached, as evidenced by the re-expression of the chondrocyte phenotype in agarose after dedifferentiation during monolayer culture [43]. As the tissue develops, the new ECM alleviates the need for a synthetic template.

It is known that periosteum multiply well *in vitro*. Implants seeded with periosteal cells initially generate what appears to be cartilage, both grossly and histologically, within the first few weeks after implantation. In time, such implants mature to form new bone containing the cellular elements of bone marrow. The rate of the morphogenesis from cartilage to bone correlated with the site into which the cell-polymer construct has been implanted, and seems to be related to the vascularity of the site. In contrast, chondrocyte seeded implants generate cartilage that does not undergo a morphogenesis to bone; regardless of the site and the duration of time in which they had been implanted.

When bone defects are surgically created in both weight bearing and non-weight bearing bones and are filled with polymers seeded with chondrocytes, periosteal cells, polymer alone, or nothing at all, either cartilage, bone, or scar tissue is generated.

Bone is composed of donor cells embedded within recipient interstitium and blood vessels. Scaffolds to deliver cells and grow new bone have included ceramics and polymers. Ceramic materials are useful because they have long degradation times *in vivo*, often of the order of years. One of the first synthetic scaffolds used in tissue engineering was a fiber based scaffold

composed of non-woven mesh of fibers of PGA, 15 microns in diameter, with average inter-fiber interspaces of 150–200 microns. This was a polymer construct that had worked very well for the generation of tissue-engineered cartilage. Two other degradable scaffolds, with which there is a large body of data, are injectable hydrogel systems, one being calcium alginate, and the other, a reverse thermosenstive co-polymer, referred to as pluronics, composed of polyethylene and polypropylene oxide.

The quality of new tissue-engineered cartilage is not only a function of optimal matching of the cell type to the polymer matrix employed, but also of the animal species studied. In nude recipients for example, the fiber system is an excellent scaffold for the generation of cartilage, while injectable hydrogels seem to be superior when engineering the same type of cartilage in immune competent animals using autologous cells.

In immune compromised animals little inflammation is seen, and very good structural tissues is generated when chondrocytes are delivered in pluronics. This is in contrast to the use of calcium alginate to deliver the cells. When transplanted in alginic acid, the chondrocytes are encapsulated in the vehicle which seems to impair cell replication, and takes a long period of time to degrade. When chondrocytes are delivered in pluronics, the polymer degrades much more rapidly, allows for cell replication with minimal inflammatory response, and generates excellent cartilage in higher species using autologous cells. The generation of bone is optimized by the use of calcium alginate. The engineered tissue appears to extract the calcium from the hydrogel as it undergoes morphogenesis to bone. By using calcium alginate as a vehicle for delivery of periosteal cells, the ability to make scaffolds in specific shapes, such as the distal femur of a rat, which resultant generation of tissue-engineered bone of the same shape has been demonstrated. A vascularized pedicle of tissue-engineered bone has been successfully generated in athymic animals by molding synthetic scaffolding, seeded with periosteal cells, around a vascular bundle. This vascularized pedicle of bone can then be transplanted into a large bone defect. Note that the histologic specimen is very similar to normal bone, to that point that it appears to contain a Haversian system.

Some studies have focused on the generation of new bone and cartilage in immune competent animals using autologous cells, as well as the ability to generate composite structures of bone and cartilage. In such models, there is a relatively large degree of inflammation associated with the procedure that results in extensive cell death and poor tissue formation. The use of MSCs in combination mature autologous bone or cartilage cells may modulate the local environment into which mature tissue specific cells are delivered, and increase cell engraftment and tissue formation [44]. Alternatively, other studies target the use of pharmacologic agents to reduce inflammation during the perioperative period of the implant, to decrease cell death and increase engraftment.

CONCLUSION

The field of tissue engineering is growing, albeit, as a somewhat slower pace than previously predicted. While the number of scientific studies is tremendous, the number of translational studies that will lead to human applications are far fewer. Acquisition and application of new knowledge in the fields of stem cell biology, and inflammation will help overcome these hurdles. Manipulations of the local environment in combination with taking advantage of the natural ability of the cells to develop in such environments to reconstitute as functional tissue, optimizing intercellular signaling mechanisms and a directed expression of genes may ultimately driving the principles of tissue engineering toward human applications. It is our hope to optimize cell development, scaffolds and the local environment to generate functional replacement tissue. Advances in an ever growing number of related fields will ultimately allow us to fully utilize this ability.

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CHAPTER 59

Tendons and Ligament Tissue Engineering

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INTRODUCTION

People are progressively aware of the positive impact of physical activities for the maintenance of their long-term quality of life. However, thousands of women and men experience joint injuries, including the loss of a ligament or tendon while practicing a sport or after giving an intense physical effort. The severity of a ligament and tendon injury will dictate the surgical approach to apply for optimal tissue repair and functional recovery. Tissue engineering has led to the development of new connective tissue repair strategies to reduce the morbidity associated with the use of healthy ligaments and tendons as replacement parts [1]. Synthetic, semi-synthetic, or natural scaffolds are under development to establish optimal conditions for permanent replacement of torn ligaments and tendons. In the case of Achilles tendon injury, the type of surgical repair depends on the size of the gap between the tendon ends. Small gaps will involve stitching both ends together, but a larger gap will require the use of an autograft – called tendon transfer, notably the flexor hallucis longus, or a graft taken from a cadaver [2–3]. The anterior cruciate ligament (ACL) of the knee joint, one of the strongest ligaments of the body, is also the target of traumatic injuries. Unfortunately, its healing potential is apparently limited, and the surgical options for its replacement are frequently associated with knee instability, pain, or muscular weakness. Rupture of the ACL affects over 175,000 patients per year worldwide [4]. Unfortunately, the torn ACL is most frequently replaced by an autologous healthy knee tendon; using the central portion of the patellar tendon or using gracilis and semitendinous tendon grafts. Tissue engineering could provide a potential solution to this issue. However, the reconstruction of connective tissues involves particular technological challenges. A tissue-engineered ligament or tendon would be an ideal graft, but only if they have some essential features. First, biocompatibility of the bioengineered graft will determine its level of integration at the graft site. Second, the ultrastucture of its scaffold is critical for cell colonization, before and/or after implantation. Third, the biomechanical features of the tissue-engineered graft must be adequate to sustain the physiological stresses that will be applied on its structure after grafting. Finally, the regeneration and remodeling of the graft is expected to occur following *in situ* post-implantation, leading to functional recovery, and therefore, this may require prevention of the material becoming a scar tissue with inferior properties due to a host response. Our research group has developed a bioengineered ACL (bACL) that seems to possess these advantageous characteristics and may

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eventually be used for torn ACL replacement in humans. The tissue-engineering approach and concepts to produce ligament and tendon substitutes or promote their healing are presented in this chapter.

HISTOLOGICAL DESCRIPTION OF TENDONS AND LIGAMENTS

Tendons connect bones and muscles, while ligaments join one bone to another. The literature used to describe the histological structures of fascia, tendons, and ligaments is similar. They are dense connective tissues consisting of fibroblasts surrounded by type I collagen bundles [5,6]. This may explain why patellar tendon and tensor fascia have frequently been used to replace a torn ACL of the knee joint [7]. The central quadriceps tendon autograft was reported as a promising alternative for ACL reconstruction. However, some pain, loss of motion, knee instability, and other problems can be associated with this approach [8], according to the type of trauma and depending on variables unique to each individual [9]. The success of such grafts depends on the revascularization of the transplanted tissues progressively surrounded by a synovial membrane, rich in vessels [7]. The tendon would finally gain some ligament properties, and the word ligamentization was applied by Amiel et al. [10], to describe this physiological phenomenon studied *in vivo* post-grafting. However, this term is likely not correct as the transplanted tendon gradually becomes more of a scar (initial cell death, infiltration of exogenous cells and blood vessels, altered matrix molecule composition) rather than becoming a ligament. Both ligaments and tendons normally consist mainly of closely packed and thick collagen fibers (predominantly type I, with a small proportion of type III collagen, except where a tendon goes around a boney prominence [11], but also include small quantities of glycosaminoglycans (dermatan sulfate and hyaluronic acid) [12]. Tendon collagen fibrillogenesis is initiated early in development by fibroblasts [13]. Fibrils are embedded in an organized, hydrated proteoglycan matrix and crosslinked through aldol or Schiff base adducts between aldehydes on one or more of the α-chains of the collagen molecules and aldehydes or amino groups on adjacent chains or molecules [14]. Such crosslinks contribute to the tensile strength of the fibrils and, thus, to the tensile properties of the whole tendon [14]. The aldehyde-derived crosslinks are found in two forms: Some are unstable in dilute acids and others are stable [14]. The ratio of one form to the other varies in different tissues, generally increasing with aging. Data reported by Amiel et al. [5] suggested that ligaments are more metabolically active than tendons, having higher DNA content, larger amounts of reducible crosslinks, and more type III collagen, as compared with tendons. They also contain slightly less total collagen than tendons and more glycosaminoglycans, particularly close to the joint [5]. Extracellular matrix fibers in ligaments are oriented parallel to the longitudinal axis of both tissues [5]. For example, the ACL is composed of multiple fascicles, the basic unit of which is type I collagen [15]. The collagen fibrils are non-parallel but are themselves arranged into fibers oriented roughly along the long axis of the ligament in a wavy, undulating pattern ('crimp'), which slowly straightens out as small loads are applied to the ligament [15]. To give an example of the forces applied on such tissues *in vivo*, the ACL usually supports loads of about 169 newtons and ruptures around 1,730 newtons [15]. An elongation of only 6% (about 2 mm) of human ACL (about 32 mm long) is reported to be the limit beyond which damage must be expected [16]. Tendons and ligaments are innervated for proprioceptive functions [17–19]. Thus, the main challenge associated with the production of bioengineered tendons and ligaments is to obtain strong and functional tissues.

This challenge is further complicated by the fact that not all tendons or ligaments are identical [20]. That is, they are uniquely designed to operate within specific loading environments and thus, the Achilles tendon, patellar tendon and those of the shoulder are quite different in some respects. Similarly, the medial collateral ligament and the ACL operate in quite different biological and mechanical environments. An additional aspect of ligaments and tendons that is relevant is the fact that ligaments are mainly passive structures involved in joint

stabilization, and thus many of them operate in the low load (~5%) 'toe' region of their stress-strain curves. In contrast, tendons are active structures responsible for joint motion as part of the muscle-tendon-bone complex and operate in more of the 'linear' region (~50–80%) of their stress-strain curves. Finally, another variable which needs to be factored into the tissue engineering approach is related to the fact that following injury to a ligament or tendon, the remaining joint tissues can adapt (e.g., the joint as an organ concept) [21], leading to a new set point for the joint and thus, the requirements for the tissue-engineered construct.

BIOENGINEERED TENDON AND LIGAMENT SUBSTITUTES

Damaged ligaments are graded on a severity scale. The grade 1 sprain indicates that the ligament is mildly damaged. It has been slightly stretched, but remains functional and able to help keep the knee joint stable (likely due to the fact that it is a passive structure operating at the low toe region of the stress-strain curve). A grade 2 sprain stretches the ligament and it becomes loose, and some function may be lost. This is often referred to as a partial tear of the ligament. The grade 3 sprain is most commonly referred to as a complete tear of the ligament. The ligament has been split into two pieces, resulting in an unstable knee joint. Similarly, acute tendon injury has been classified into three grades. The primary grade describes tendinitis that includes inflammation and swelling in the tendon. The secondary grade involves a partial tear of the tendon causing pain and weakness. Finally, the tertiary grade is a complete rupture of the tendon with loss of function. The complete rupture of any tendon or ligament requires tissue transfer and/or an intervention that promotes healing (e.g., surgery and/or immobilization plus other interventions). The transfer of a healthy ligament or tendon involves morbidity. The use of allograft substitutes (taken from cadavers) overcomes the need for autologous tissues, avoiding donor-site morbidity. However, this approach does have limitations, including risks of disease transmission, graft rejection and inflammation [22,23]. Tissue engineering offers the opportunity to target and control various biophysiological parameters of tissue development to make it competent for implantation; for example, collagen fibers and cells can be aligned in culture under defined conditions (Fig. 59.1) [24–27]. Perhaps most importantly, bioengineered tissue substitutes are expected to respond and adapt to the unique environmental mechanical stresses that occur following implantation *in vivo* [18,27–29], as well as the biological environment (e.g., extra- vs. intra-articular environment, male vs. female, age-related factors, and co-morbidities in the elderly such as diabetes).

ACL replacement

Some tendons and ligaments are at the top of the list in terms of injury frequency and problems to repair, replace, or heal. As often reported, in contrast with the medial collateral ligament, ACL regeneration is hampered *in vivo* [30,31]. Moreover, certain anatomic factors may predispose people to ACL injury [9]. In the young and active population, reconstruction is often the best therapeutic option. In United States alone, more than 120,000 patients per year undergo tendon or ligament repairs [32]. Almost any sport that involves jumping, cutting or twisting has an inherent risk of an ACL rupture [6,21]. The completely torn or disrupted ACL does not appear to heal very well, at least to the extent that healing leads to a return to function [29,33]. The therapeutic options for ACL reconstruction include three categories of ACL substitutes:

1) Synthetic prostheses,
2) Allogenic natural substitutes (ACL excised from cadavers), and
3) Natural autografts (central portion of the patellar tendon or hamstring tendon of the patient).

Unfortunately, none of these surgical alternatives provides a long-term optimal solution. Synthetic material implantation was a very popular surgical technique in the 1980s, but it frequently led to implant degeneration and failure [34–36]. Joint laxity occurs with synthetic prostheses about 12 months post-surgery, including long-term failure due to synovitis,

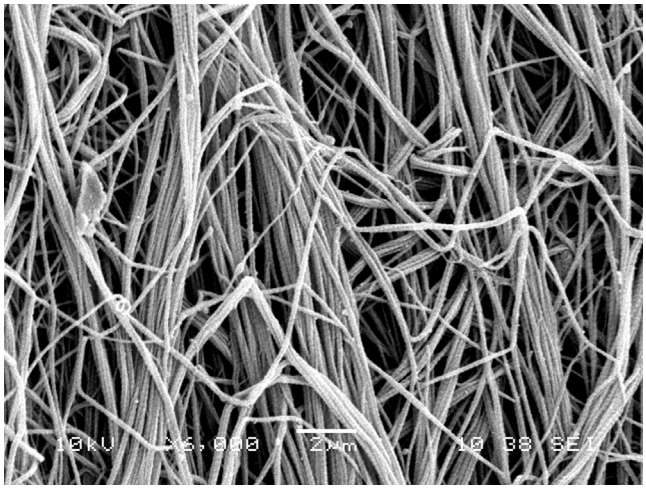

FIGURE 59.1

bACL native collagen scaffold. Photomicrograph taken during scanning electron microscope analyses of an acellular bACL bovine type I collagen scaffold that shows the alignment of the fibers in response to static tension applied for 24 h on the tissue in culture (×6000).

arthritis, and mechanical deterioration. For these reasons, synthetic prostheses are not the first option chosen [35]. However, some new biosynthetic materials are under investigation [37,38]. Assuming proper processing techniques, autografts using the patellar tendon, quadriceps tendon-patellar bone, or the hamstring tendons avoid immune rejection problems [39,40]. Permanent attachment of ACL prosthesis to bone is of critical importance. Over the last decade, the best solution has been the bone-patellar tendon-bone grafts [41]. Unfortunately, ligament translation or laxity is observed post-surgery [40], adding to the morbidity associated with the partial loss of a healthy tendon, chronic knee pain, loss of motion, knee instability, quadriceps weakness, and patella rupture [8]. Little is known about the physiological and pathological events that regulate the histological organization of ACL *in vivo*. It is well recognized that a delay of six weeks after injury is required before ACL surgery is performed. In fact, it is better to perform surgical intervention of the ACL when the nearly normal range of motion of the knee has returned and inflammation has been mostly eliminated [42]. This delay could be long enough to allow for the production of a bioengineered ACL *in vitro* with the required characteristics.

Decellularized and acellular ACL grafts

Decellularized allogeneic ACLs have been investigated as potential grafts for torn ACL replacement [43]. All decellularization treatments affect the tissue ultrastructural and mechanical properties of the remaining scaffold. The abrasive effects of detergents on the tissues to be decellularized can modify their protein contents and consequently, these ultrastructural alterations may impair cell adhesion, migration and proliferation by generating an unrecognizable environment for the cells. In fact, one of the main problems associated with this option for ACL replacement is the presence of dead cells in these tissues. They must be effectively eliminated to reduce the risks of immune rejection post-grafting. Detergents are often used to reach this goal, but they can also alter the structure of the scaffold and the quality of the collagen fibers that support the tissues. Moreover, residual traces of detergents cannot be totally eliminated from the allografts, potentially hampering repopulation of the implants by host cells *in situ* post-grafting. Acellular scaffolds produced *in vitro* overcome these issues but in the end both acellular and decellularized implants will have to be entirely colonized by host cells post-implantation to regenerate and maintain its functional features *in situ* [44]. Such processes slow down the integration of the grafts and their functional recovery [18].

Recent studies have shown that depending on the cells and available growth factor concentrations, repopulation of decellularized scaffolds remains difficult to achieve [45]. Animal experimentation could be initiated to evaluate the histological and biomechanical properties of ACL substitutes seeded with fibroblasts as compared to grafts populated with other sources of cells [18,28]. The phenotypic stability of stem cell-derived fibroblasts would have to be monitored during several passages in culture before considering cellularization of bACLs with these cells. Stem cell-derived fibroblasts, expected to show faster doubling times and a longer lifespan than fibroblasts *in vitro*, may differentiate into various cellular phenotypes in response to the physiological environment and the biomechanical stimuli that characterized functional knee joints. Therefore, until long-term animal experimentation is performed and the results validated, it may be premature to use stem cells to populate tendon and ligament grafts. However, some experiments performed in a sheep model were recently reported as successful at six months post-grafting [19]. Indeed, the success of tissue-engineering procedures also depends on the choice of an appropriate biomaterial [46]. However, further work is still needed to validate the best clinical application of each option.

Tissue-engineered ACL grafts

To overcome the drawbacks associated with ACL repair and healing, several efforts have been made to produce a bioengineered ACL model over the last decade [47–49]. Selecting the right scaffold for the starting point to build a bACL is going to impact the outcome. The use of native collagen for the production of tissue-engineered ligaments leads to a better understanding of the mechanisms responsible for its secretion by the cells, its assembly, and its remodeling. Thus, collagen alone or with cells isolated from the tissue of origin was used to produce different three-dimensional human tissue models *in vitro* [25,50–53]. The main goal behind such achievements was to exploit the tissue-engineering approach to create body parts using natural scaffolds and implant them permanently *in vivo*, where their histological and functional properties should be improved by local and systemic stimuli. The first bACL entirely reconstructed *in vitro* and grafted *in vivo* was developed by seeding autologous ligament or skin fibroblasts in a hydrated collagen matrix [18,27,28,54]. This bACL is anchored with two bones, since bone-to-bone insertion is reported as the most secure method for ligament fixation [7,10]. Such an ACL substitute is a good tool to study connective tissue repair and the environmental and cellular factors that may affect collagen alignment, crosslinking, and remodeling *in vitro* [44]. The main differences between such bACL and other ligament models are the following advantages:

1) It contains living ACL fibroblasts that contract, synthesize, and initiate remodeling and organization of the extracellular matrix in which they are initially seeded in culture;
2) It could be a very useful tool to study connective tissue repair *in vitro*, since the cells can maintain their activities for at least two months in culture;
3) It is produced without chemical crosslinking agents and synthetic material; and
4) It avoids healthy ligament or tendon donor-site morbidity.

Such an ACL substitute would highly reduce the risk of immune reactions and infections, and promote permanent graft integration post-transplantation. This approach also eliminates the risks for chemical cytotoxicity and the production of foreign molecules due to mechanical friction, which could occur *in vivo*, in the situation of its eventual use as an ACL substitute [34]. It may also become a therapeutic alternative for the torn ACL replacement, considering the promising outcomes observed *in vivo* post-implantation in the caprine model [18,28].

Matrix remodeling and gain of strength in response to mechanical stimuli

Various research groups have shown that fibroblasts seeded in collagen gels can degrade and reorganize the surrounding extracellular matrix and adopt a specific orientation in a

contracted collagen lattice as a function of culture conditions and time [55–63]. Several studies have also shown that application of mechanical stimuli to biological tissues can produce ultrastructural and histological content variations in response to the stimuli. For instance, it is well known that ligament remodeling depends on the mechanical stresses to which it is subjected *in vivo* [10]. In addition, bACL produced thus far have collagen and cells aligned in a parallel direction to the stresses exerted [27,28,54]. Finally, Huang et al. [24] have previously reported that the mechanical properties, along with the ultrastructure of ligament equivalents, change in response to mechanical strengthening *in vitro*. Similar observations were made following *in vitro* elongation studies on rabbit ACL [64]. Thus, it is possible and structurally relevant to induce the parallel alignment of the matrix fibers that compose the scaffold of a tissue-engineered ACL substitute in culture. This alignment can be achieved by subjecting the bACL to static or dynamic longitudinal stretching *in vitro* [27]. We previously reported that fibroblasts isolated from human ACL biopsies and seeded in a collagen matrix can secrete and reorganize matricial fibers in response to static tension [54]. Collagen lattices seeded with fibroblasts gain strength *in vitro* after being subjected to cyclic traction over a few days in culture, with stretching amplitudes varying from 0–30 mm at a frequency of up to 1 Hz [54,65]. Rupture and stiffness assays allow the measurement of the strength of the tissue in culture, before and after stretching stimulation [24,65–69].

Native collagen scaffolds for tissue-engineering applications

Collagen is the main constituent of tendon and ligament scaffolds. Thus, it is the protein of choice to use for the development of tissue-engineered grafts. Collagen can be extracted from animal skin or other connective tissue and purified, or it can be synthesized from human or animal cells grown in monolayers for 3–4 weeks, to obtain sheets of collagen seeded with cells according to the self-assembly approach [70]. Native collagen type I polymerizes *in vitro* and the resulting network can be lyophilized to gather fibers together and strengthen its structure (Fig. 59.2a). When rehydrated, despite fibers being arranged in bundles, the strength of the resulting collagen scaffold is sub-optimal and does not compare with the strength of the native ligament or tendon. Therefore, to produce a graftable tendon or ligament substitute made of native collagen, a resorbable threading core must be added to reinforce it in order to enhance the survival of the grafted tissue. For example, we have used polyglyconate MAXON surgical thread (size 3–0), resorbable within six weeks post-grafting, to reinforce tissue-engineered ACL grafts [28]. Following rehydration of the lyophilized central core in fresh DMEM, a second coating of collagen seeded with live cells is applied as described earlier (Fig. 59.2b). A bilayered bACL is thus obtained with a rehydrated central core and a living, cell-populated outer layer. The bACLs are viable prior to implantation, since the fibroblasts progressively contract the outer collagen layer *in vitro*. The resultant bACLs are kept in culture for about a week until grafted into their respective hosts. After a year *in situ* post-grafting in a goat knee joint, the bACL shows many macroscopic features comparable to those of the contralateral native ACL (Fig. 59.2c). Interestingly, results obtained at six months post-implantation showed that the collagen scaffold of the bACL contained nerves endings, sign of proprioceptive restoration (Fig. 59.3). Similarly, highly organized fibrocartilage colonized by chondrocytes was observed at the bone-ligament junction at six months post-grafting (Fig. 59.4). Such observations strongly suggest that chondrocytes or their precursors migrated, proliferated, and became differentiated in the collagen scaffold of the bACL grafts *in vivo* [28]. Collagen I will likely remain the best matrix component to initiate development of biocompatible scaffolds for tendon and ligament bioengineering. The use of *de novo* synthesized collagen sheets has also led to the successful integration of a stem cell seeded ACL graft [19]. Collagen remodeling starts in culture and continues post-grafting, leading to major gains in ultimate strength and rigidity. Such properties can be measured using proper mechanical devices (Fig. 59.4).

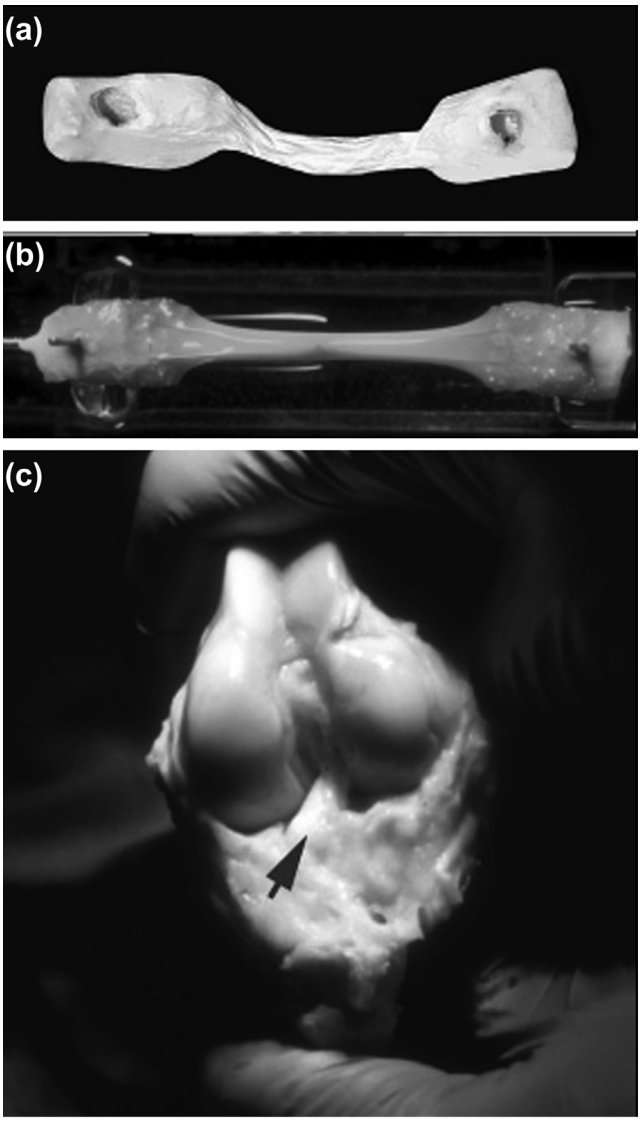

FIGURE 59.2
Construction of bACL and postgrafting view *in situ*. (a) Macroscopic appearance of the initial collagen layer contracted onto two bone plugs and lyophilized. (b) A bACL after the second collagen coating and ready for grafting. (c) Macroscopic view of a bACL *in situ* (arrow) grafted for one year into a goat knee joint. *Reprinted from Applied Bionics and Biomechanics 1:115-121, Copyright (2004), with permission from IOS Press.*

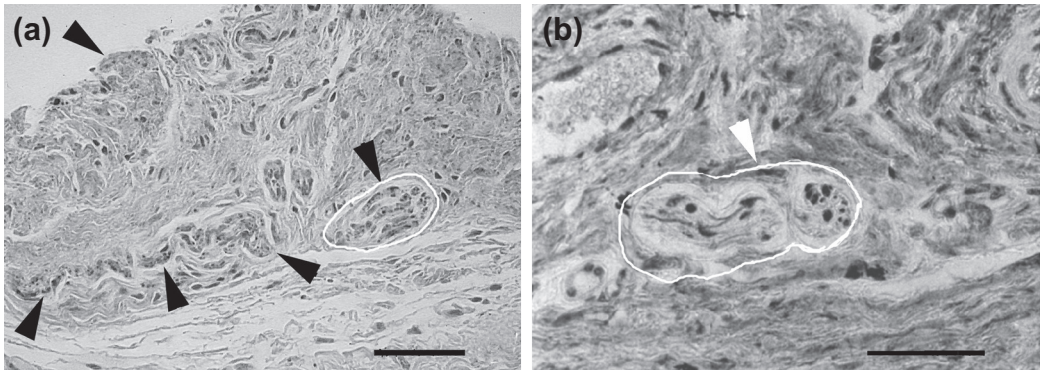

FIGURE 59.3
Light microscope histology of paraffin embedded sections of nerve endings immunolabeled with an anti-neurofilament-H+M phosphorylated form antibody revealed by peroxidase (arrow heads) (a,b), in the regenerated synovial membranes attached to a bACL initially seeded with autologous DFs at 6 months post-grafting (a), like in a native goat ACL (b). (Scale bars= 60 mm). *Reprinted from Cell Transplant 2011; 20:535-42. Epub 2010 Nov 5. (DOI 10.3727/096368910X536482) with permission from Cognizant Communication Corporation.*

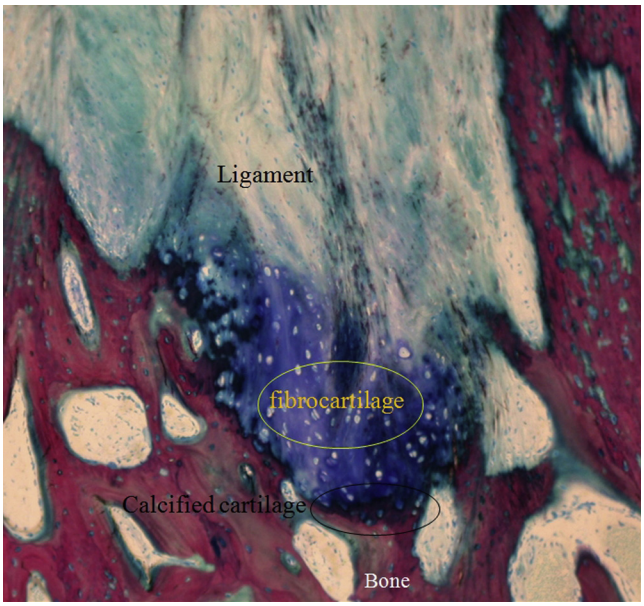

FIGURE 59.4
Direct graft-bone insertion at the tibial insertion site. Note the four distinct zones; Ligament, Fibrocartilage, Calcified fibrocartilage, and Bone. (Scale bar = 100 mm). *Reprinted from Cell Transplant 2011; 20:535-42. Epub 2010 Nov 5. (DOI 10.3727/096368910X536482) with permission from Cognizant Communication Corporation.*

Tissue-engineered Achilles tendon

Chronic tears of the Achilles tendon with a resultant gap between the torn ends exceeding 6 cm are a surgical challenge [71]. Treatment of tears to the Achilles tendon with various tendon graft transfer strategies have been reported in the current literature, including free gracilis [71] and autologous peroneus brevis tendon [72] grafts. At 8 to 15 years post-engraftment, many patients retained good functional results, despite a permanently impaired ankle plantar flexion strength and decreased calf circumference [71,72]. The use of free semitendinosus tendon has also led to good clinical and functional outcomes, and is deemed to be safe [73]. However, the possibility to permanently graft a strong tissue-engineered Achilles tendon would be very useful in order to spare autologous healthy tendons in these patients. Various strategies to produce Achilles tendon substitutes using a collagen matrix [74], silk-collagen scaffolds [75], and connective tissue stem cells [75,76] are in progress. The combination of the tissue-engineering approach with conventional treatments might lead to the development of new options to promote effective long-term Achilles tendon repair [77].

Tissue engineering and rotator cuff repair

The rotator cuff is a sensitive tendon complex of the shoulder which is involved when sustaining high levels of force during weight lifting. Soft connective tissues weaken with age and rotator cuff tears may occur as a result of acute trauma, repetitive overhead work, or sports activity, with increased risk during aging. In fact, tears affect 40% or more of people aged older than 60 years, and repair failure rates of 20% to 70% remain a significant clinical challenge [78]. Dermal grafts are often used for rotator cuff repair and augmentation. Compared to surgery without augmentation, acellular human dermal matrix augmentation of large (>3 cm) rotator cuff tears involving two tendons showed better repair with more frequent intact cuffs detected as determined by gadolinium-enhanced MRI. Intact repairs were found in 85% of the augmented group and 40% of the non-augmented group ($P < .01$) [79]. Tissue-engineering strategies to improve rotator cuff healing include the use of scaffolds, growth factors, and cell seeding, or a combination of these approaches. Scaffolds have been the most common strategy investigated to date [78].

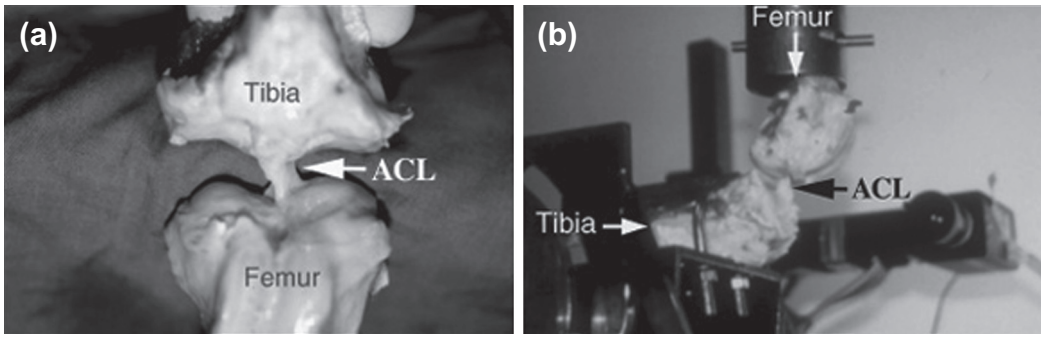

FIGURE 59.5
Rupture assay of a bACL *ex vivo*. Macroscopic view of an 11-month-old graft *ex vivo* as prepared for biomechanical testing before (a) and during (b) application of tension prior to rupture. *Reprinted from Applied Bionics and Biomechanics 1:115-121, Copyright (2004), with permission from IOS Press.*

Animal models

Many of the features of a tissue-engineered tendon or ligament depend on the type of biomaterials that are used to compose its scaffold. The potential and the functionality of any reconstructed tissue must be assessed in animal models [33]. This is the critical step to evaluate the feasibility, the viability, and the potential factors contributing to graft failure associated with all new ligament and tendon replacement strategies under development [29].

Small animals such as rat models are often used to study tendons of the shoulder's rotator cuff [80]. Rabbit and goat models have been used to assess torn ACL replacement strategies [18,27,81,82]. A major advantage of the rabbit remains the wide choice of antibodies and biomarkers commercially available to analyze the various constituents of ACL substitutes post-grafting and *ex vivo*. The main limitation of this model is its size, and the fact that it does not compare well with the anatomy and functioning of the human knee joint. The goat is larger and the size of its knee joint is close to the human structures. This facilitates development of a preclinical protocol for the implantation of a new type of ACL replacement. However, significant differences are observed between the magnitude of force experienced by the goat ACL and its anteromedial and posterolateral bundles when compared with the corresponding human ACL. Nonetheless, the caprine model is widely used for ACL characterization (Fig 59.5) [83]. More recently, the sheep is another large animal that can be used to assess the level of integration of tissue-engineered ACL substitutes in knee joint [19]. The most challenging preclinical model to study ACL repair is certainly the dog knee joint. As in humans, spontaneous ACL injuries are frequently observed in large dog knee joints. The medial meniscus is commonly damaged along with the ACL. The canine model also involves several other issues, including early osteoarthritis development after a torn ACL injury [84]. For this reason, an ACL substitute tested with success in the canine knee joint would certainly indicate serious potential for use in the human knee. As well, success in the canine model could also have veterinary implications for prevention of osteoarthritis development in valuable companion, working and show animals.

Thus, there are a number of preclinical models available to assess and validate tissue-engineered ACL constructs. A similar range of models are also available for investigating tendon constructs. However, some tendons such as the Achilles tendon may be structurally different in some species, so consideration must be given to the relationship of a construct to the analogous human tissue since repair of injuries in humans is a priority.

CONCLUSION

Recent advances in bioengineering and cell/molecular biology have led to revolutionary new therapeutic applications in many medical fields. Tissue-engineered tendons and ligaments

are under development to provide valuable alternatives and support to conventional reconstructive surgery.

The main advantages of bioengineered tendon and ligament substitutes stem from their capacity to be integrated *in situ* post-grafting, being progressively modulated by the living cells that migrate from the tissues of the host into the scaffold of the bioengineered graft. In the case of bioengineered grafts seeded with living cells prior to implantation, extracellular matrix constituents are secreted and organized in culture [28]. Such remodeling of the graft's ultrastructural features contributes to accelerate its integration post-grafting [18].

This chapter was meant to give the reader an overview of the recent achievements and approaches that combine cell biology and tissue-engineering concepts to produce tendons and ligaments in culture. These bioengineered tissues will indeed have to be optimized prior to human transplantation. However, investigators involved in this domain have not yet started to exploit all the possibilities offered by such a new biotechnological concept. For instance, gene therapy might find some interesting applications by introducing living transfected cells within various types of bioengineered tissues.

Despite the fact that challenges lie ahead for those aiming at reproducing tissues and organs *in vitro*, we can all be hopeful, looking at the ongoing advances reached in various medical fields through tissue bioengineering [32]. It is quite probable that in the near future, people will benefit from bioengineered tendon and ligament substitutes. Their production would start in a laboratory and reach its final completion in a human body. Most importantly, engineering of tendons and ligaments would avoid the need for detrimental harvesting of the patient's tissues used for transfer. This approach may allow the surgical interventions to be performed by arthroscopy, instead of more invasive approaches. In addition, the risk of bioengineered tissues being rejected post-grafting is minimized when produced with autologous cells. That is the joint ultimate goal shared by researchers and clinicians in the various research teams around the world. Such a realization would certainly revolutionize the present therapeutic approaches for tendon and ligament implantation.

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CHAPTER 60

Skeletal Tissue Engineering

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INTRODUCTION

With an aging US population, skeletal tissue injury and loss from trauma or disease represents a burgeoning socioeconomic burden to the healthcare system. Skeletal related injuries and diseases represent a significant portion of this cost; according to data from the 2007 US Health Cost and Utilization Project, there were approximately 1 million hospital admissions for appendicular skeletal fractures in the USA, with an aggregate cost of over $26 billion [1]. Furthermore, skull and facial fractures contributed an additional $1.3 billion. Of all fractures, approximately 10% are complicated with impaired healing [2]. With the culmination of developments in materials science, bioengineering, stem cell biology, and molecular biology, the potential of tissue engineering to accelerate bone regeneration offers an exciting new option for addressing the reconstruction of osseous defects.

The clinician is confronted with an array of large bone defects resulting from trauma, resection of malignancies, nonunion of fractures, and congenital malformations. Autografts remain the best option for reconstructing bony defects because they provide osteogenic cells, osteoinductive factors, and a lattice needed for bone regeneration. They can be obtained from a patient's calvarium, ribs, iliac crest, distal femur, greater trochanter, or proximal tibia. Autografts, however, subject the patient to inherent risks of surgery during harvest. In a retrospective study of autografts harvested from the iliac crest, a complication rate nearing 10% was found, including infection, fracture, large hematomas, pain, paresthesia, and nerve injury [3]. Furthermore, autografts are a relatively scarce resource and are limited by the amount of bone that can be removed without causing undue morbidity to the donor site. In situations where autografts are not sufficient or possible, allogeneic bone from donors or cadavers may be used. Allografts, however, may be accompanied by the risk for disease transmission, immunologic rejection, and graft-versus-host disease.

As an alternative, ceramics and metals are bone substitutes available to repair skeletal defects. Each, however, have their own associated problems. Metals provide immediate mechanical support, but fail to integrate with surrounding tissue [4]. Metals are also at higher risk for infection and fatigue loading. Ceramics, mimicking the inorganic composition of bone, tend to integrate well, but possess low tensile strength and are very brittle. Numerous other synthetic materials have been developed to aid in bone reconstruction, including the use of plaster of Paris, polymer-based substitutes, and bioactive glass.

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00060-4

The wide range of options available for osseous reconstruction is indicative of the many advances made in biomaterials, but also reflects the inadequacies of any single method. As such, tissue engineering holds great promise for being able to more adequately address the issue of repairing bone defects. The delivery of osteogenic cytokines is already employed clinically, specifically BMPs, for the induction of bone regeneration. The ultimate translational goal, however, is to deliver both osteogenic cells and cytokines via a biologically active scaffold, in order to heal a bony defect in an accelerated fashion.

This chapter will begin with an overview of distraction osteogenesis, a modality that translates directed mechanical force into endogenous bone formation, to emphasize the important role of the mechanical environment in bone formation and to highlight the growth factors which play a role in inducing bone formation. This chapter will then discuss the necessary components of bone tissue engineering, namely candidate cell types, osteogenic cytokines, and scaffolds, with an emphasis on recent developments.

DISTRACTION OSTEOGENESIS

Distraction osteogenesis provides a model for the study of endogenous bone formation in large skeletal defects. First described by Alexander Codivilla in 1905 for limb lengthening and later codified by Gavril Ilizarov in the 1950s, distraction osteogenesis represents an endogenous form of bone tissue engineering [5,6]. New bone is formed during the process of separating osteogenic fronts with gradual but constant mechanical force. As a first step, an osteotomy is usually performed on the bone of interest, followed by application of rigid fixation. This is followed by a variable latency period of several days, during which a soft callus forms between the two osteogenic fronts. Gradual distraction is then applied, followed by stable fixation/consolidation, until a robust osseous regenerate is formed. It is thought that the mechanical forces applied by distraction can contribute to bone formation by induction of cytokines that guide mesenchymal cells in the bony gap to differentiate along an osteogenic lineage. Distraction osteogenesis not only triggers bone formation, but also stimulates local angiogenesis as part of the process [7].

Much research has been devoted towards characterizing the association between stress and strain patterns with bone formation. Correlating tensile force measurements with histology, Loboa et al., demonstrated that the greatest amount of bone formation occurs during active distraction, the period of greatest strain [8]. Loboa went on to further characterize the forces of distraction, using finite element analysis models created from three-dimensional computed tomography image data of rat mandibles at different phases of distraction osteogenesis [9]. The models described patterns of moderate hydrostatic stress within the gap, predictive of intramembranous ossification, and patterns of mild compressive stress in the periphery, consistent with endochondral ossification. These data derived from finite element analysis were consistent with previous histological findings.

Great interest surrounds research characterizing how mechanical forces may be translated into molecular signals that promote bone regeneration. Tong et al. described the role of focal adhesion kinase (FAK), a regulator of the integrin-mediated signal transduction cascade, in distraction osteogenesis [10]. In a rat model of mandibular distraction osteogenesis, immunolocalization of FAK in regions of new bone formation secondary to distraction was observed, but was absent in the control groups where new bone formation occurred without distraction [10]. Similarly, recent work has also co-localized c-SRC, a kinase involved with activation of the mechanical transduction complex (p130), in regions of bone regeneration secondary to distraction osteogenesis [11]. While signaling molecules involved with transduction of mechanical forces are being identified, further work elucidating the mechanisms of these messenger molecules is required to clarify the influence of the mechanical environment on skeletal tissue engineering.

Distraction osteogenesis has also emphasized the multitude of growth factors which participate in bone regeneration. Elucidation of their roles and mechanisms will be a key element in successful tissue engineering. Transforming growth factor β-1 (TGF-β1), a potent growth factor known to stimulate osteoblast proliferation, has been demonstrated to have increased expression after osteotomy [12]. Immunohistochemistry revealed that TGF-β1 is localized to osteoblasts, primitive mesenchymal cells, and the extracellular matrix during the active distraction phase.

Insulin-like growth factor-1 (IGF-1) has also been implicated to play a role in the early stage of distraction osteogenesis. IGF-1 has been shown both *in vitro* and *in vivo* to stimulate osteoprogenitor cell mitosis and differentiation. In a canine tibia lengthening model, serum levels of IGF-1 increased initially during the early distraction period, followed by elevations in local IGF-1 at the region of distraction [13]. Schumacher et al., also presented evidence supporting the role of IGF-1 in early distraction, where periosteal IGF-1 levels in the rat tibia were increased only during active lengthening [14]. Exogenous addition of IGF-1 in a rabbit model of mandible distraction osteogenesis resulted in increased bone formation compared to control groups [15].

Like TFG-β1 and IGF-1, studies have also shown FGF to mediate bone formation in distraction osteogenesis. Immunohistochemical staining of sheep mandibles revealed FGF-2 staining in the region of distraction, with greater staining in animals found during high-rate distraction [16]. Exogenous administration of recombinant human FGF-2 to a rabbit model of callotasis bone lengthening, during the end of the distraction period, increased the mineral content of the callus [17].

Lastly, BMPs, known mediators in bone formation, have been localized to regions of successful distraction osteogenesis. In examining femoral lengthening in rats, gene expression levels of BMP-2 and BMP-4 were found to be elevated during the period of distraction [18]. Likewise, in distraction osteogenesis of rabbit tibia, immunohistochemical staining revealed intense BMP-2, -4, and -7 staining in fibroblast-like cells and chondrocytes during the distraction phase [19]. This was confirmed in membranous bone, where analysis of the bone regenerate from mandibular lengthening in rabbits revealed BMP-2 and BMP-4 to be highly expressed in osteoblasts during distraction and in chondrocytes during consolidation [20]. Repeated local application of BMP-2 during distraction osteogenesis in a sheep model has been shown to significantly enhance the rigidity and architecture of the callus, indicating that enhancement of the endogenous mechanism may improve the clinical outcome of the procedure [21].

Distraction osteogenesis is thus a useful modality for translating discrete applications of mechanical force into molecular signals that induce skeletal regeneration. This process is not without its own morbidities, however, such as soft-tissue infection, osteomyelitis, pintract infection or loosening, and patient discomfort [22]. Distraction osteogenesis is also limited by the large hardware necessary to accomplish distraction, and by the length of time required for large defects. Given these considerations, work continues on the development of alternative cell-based bone tissue engineering strategies to address large osseous defects.

CRITICALLY SIZED DEFECTS

In the search for optimal elements of successful skeletal tissue engineering, the critically-sized defect has proven to be an indispensable tool for evaluating the performance of various constructs in the *in vivo* context. Critically-sized defects are bone defects that fail to heal without intervention. They provide an easily accessible and quantifiable platform to evaluate the performance, dosages, and combinations of various cellular sources, osteogenic cytokines, and scaffolds.

Well described bone defect assays include calvarial intramembranous bone, endochondral long bone, and mandible segmental defects [4]. The calvarial model is particularly useful in evaluating constructs for craniofacial defects, given the relative ease in handling of the calvarial plate and support provided by the surrounding intact bone, absolving the need for fixation. And, as it bears minimal mechanical load, the influence of exogenous forces on the investigation of specific cellular elements is reduced. Long bone segmental defects can also be created in the radius, where the ulna provides endogenous fixation of that limb. Alternatively, in situations where load bearing of the construct is desired, femur defects can be used.

All of these models are accessible to serial radiographic examination of the amount and density of mineralization through the use of modalities such as plain films and micro-computed tomography. Histomorphometric analysis, however, allows cellular level resolution and evaluation of bone resorption, changes in endogenous structure, and quality of the bone regenerate [4]. Serving as functional *in vivo* bone regeneration assays, critically-sized defects allow researchers to assess the rate and degree of bone healing for individual components of a bone construct and the combined effects of multi-modality treatments.

CELLULAR THERAPY

The two broad categories of populations available as sources of osteoprogenitor cells include osteoblasts and multipotent cells. Osteoblasts are cells committed along a bone lineage and readily form mineralized matrix. They are not, however, an ideal cellular source for skeletal tissue engineering given the limited number of cells available, low expansion rate, and donor site morbidity.

Stem cells, on the other hand, have the ability for prolonged division while maintaining the capacity to differentiate along multiple lineages with proper biological cues [23]. Resident stem cells can be found in many adult tissues, and are active in endogenous mechanisms of repair and regeneration. Isolation, characterization, and utilization of these cells have provided many new promising treatment options. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) maintain differentiation capability across all cell types and lack dedicated differentiation pathways. Outside of strict culture conditions, these cells spontaneously differentiate, resulting in tumor formation *in vivo*. A growing body of work, however, has shown progress toward harnessing their differentiation potential for regenerative therapies [23,24].

Postnatal stem cells have been identified in bone marrow, periosteum, muscle, fat, brain, skin and umbilical cord blood [4]. Though the limits of their differentiation potential have not been rigorously defined, research in this area has demonstrated the capacity of these postnatal progenitor cells to differentiate along multiple lineages. The most commonly studied source of postnatal multiprogenitor cells is from the bone marrow. The major components of bone marrow consist of hematopoietic and mesenchymal stromal cells. The hematopoietic component provides progenitors which will differentiate into all the mature blood cell phenotypes. Bone marrow mesenchymal stromal cells (BMSCs) are easily isolated from hematopoietic components because of their unique adherent quality to polystyrene culture dishes. Substantial work has demonstrated the ability of BMSCs to be guided along multiple mesenchymal lineages, including bone, cartilage, muscle, ligament, tendon, and stroma [25]. BMSCs have been shown, *in vitro*, to differentiate along an osteogenic pathway when cultured in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate [25].

BMSCs have been successfully applied across multiple animal models for skeletal tissue engineering. Oghushi and colleagues implanted bone marrow cells loaded on calcium phosphate ceramics into critically-sized defects of rat femurs and found higher rates of complete bone union and accelerated healing in the bone marrow cell group compared to controls [26]. Similar results were shown by Bruder et al., where human BMSCs, seeded on a ceramic carrier,

were implanted into femur defects of athymic rats. Defects with BMSC implants healed and were significantly stronger under mechanical loading conditions than defects implanted with cell-free ceramics [27]. In a rabbit calvarial model, BMSC seeded on a fibrin scaffold demonstrated osseous regeneration similar to control implants seeded with osteoblasts [28].

Although the use of BMSCs is attractive for tissue engineering, it is limited by the low frequency of mesenchymal stem cells within bone marrow stroma. Analysis of human bone marrow aspirates have found that the incidence of BMSCs can be as low as 1 in 27,000 cells [29]. Furthermore, the procedure of acquiring bone marrow is a painful one. Other limitations on the use of BMSCs include the need for sera and growth factor supplements for *in vitro* expansion prior to use. Although subject to debate, BMSCs from older animals have also been observed *in vitro* to form less bone than that from young animals, however, this may be a reflection of differences in proliferative ability.

An alternative mesenchymal cell population composed of adipose-derived stromal cells (ASCs) has been isolated from the stromal component of adipose tissue and has been similarly demonstrated to be multipotent [30]. This work describes the isolation of an adherent cell population from human adipose tissue and induction of these cells from multipotent into adipogenic, chondrogenic, osteogenic and myogenic lineages. Comparison of surface marker antigens have demonstrated a similar expression profile between ASCs and BMSCs [30]. Osteogenic differentiation conditions were also found to be identical to that for BMSCs. Confirmation of osteogenic differentiation was obtained with alkaline phosphatase and von Kossa staining, as well as expression of bone-related genes, including Cbfa-1, alkaline phosphatase, osteopontin, and osteocalcin [30]. As an abundant multipotent cell population, ASCs are attractive candidates for reconstruction because they can be procured in large numbers from individuals with minimal morbidity, and used in an autologous fashion for tissue regeneration or reconstruction [31]. Additionally, because adult ASCs have been shown to retain osteogenic differentiation capabilities, despite some attenuation of proliferation with age, ASCs remain a viable cell therapy candidate in older patients [32].

Lee et al. demonstrated the *in vivo* ability of ASCs to differentiate into bone by implanting polylactic glycolic acid (PLGA) scaffolds seeded with predifferentiated rat ASCs subcutaneously [33]. Bone formation was demonstrated 8 weeks later by tissue morphology on hematoxylin-eosin staining and immunohistochemical staining for osteocalcin. The efficacy of ASCs in healing osseous defects was first demonstrated in a model of critically-sized calvarial defects in mice [81]. Significant bone formation was demonstrated by X-ray, histology, and molecular imaging following implantation of ASC seeded on hydroxyapatite-coated PLGA scaffolds into calvarial defects. Significant bone formation was seen in the defect at two weeks, with complete bony bridging by 12 weeks. Comparison of defects implanted with ASC seeded scaffolds versus BMSC seeded scaffolds demonstrated similar rates of bone formation. This experiment established an important proof of principle that ASCs could contribute to bone formation in a critically-sized, skeletal defect in mice. Human ASCs were shown to stimulate bone regeneration in critically-sized defects in both rat and mouse models [35,36].

When isolated from the vascular fraction, ASCs exist as a mixed cell population with subgroups possessing varying differentiation capabilities. For the purposes of skeletal reconstruction, subpopulations with increased capabilities and responsiveness toward osteogenic differentiation are desirable. Though *in vitro* culture of ASCs can cause drift in the surface expression profile, there has been some level of success using fluorescence-activated cell sorting to enrich for an osteogenic subpopulation. A CD105 enriched osteogenic fraction has been shown to have enhanced responsiveness to osteogenic differentiation *in vitro*, demonstrated by alizarin red and alkaline phosphatase staining, and faster healing of a mouse critical calvarial defect than the unsorted population [37]. In addition to cell surface drift, recent attempts to further enrich osteogenic cells with additional surface markers has resulted in prohibitively low yields.

Isolating subpopulations on factors such as mechanical properties, however, have been shown to provide an additional or alternative method for enrichment [38].

Current research in skeletal regeneration is exploring the *in vitro* manipulation of ASCs in order to enhance osteogenesis prior to transplantation. Reduction of the BMP-2 signaling antagonist, noggin, was shown to increase osteogenic signaling *in vitro* and accelerate bone regeneration in mouse critically-sized calvarial defects [39]. The combination of strategic scaffold design and cytokine treatment, both discussed in the following sections, provide additional avenues for the enhancement of ASC transplants for bone regeneration.

In contrast to the lineage-restricted potential of stromal cells, work has also been done with pluripotent stem cells for skeletal tissue engineering. Despite the broad differentiation potential of ESCs, ethical and political controversies have fueled pluripotent cell research toward adult-derived induced pluripotent stem cells for tissue engineering [40]. Overexpression of key embryonic genes in differentiated cells stimulates chromatin remodeling and altered gene expression, resetting cells to an embryonic-like state of pluripotency [24]. While iPSCs have the capacity to spontaneously differentiate across all three primary germ layers, researchers are developing methods to drive differentiation of iPSCs into pure populations of a single lineage for developmental research, drug screening and disease modeling, and for cell and tissue replacement. Barriers to use of iPSC technology for clinical applications include availability of a large autologous cell source, efficiency and safety of reprogramming, defined optimal culture conditions, efficiency of directed differentiation, tumorigenicity risk, and stability and immunogenicity of transplant. Since the discovery of how to generate human iPSCs by Yamanaka and colleagues [24] significant progress has been made in addressing clinical hurdles. This section will review some of the recent breakthroughs and ongoing research relevant to skeletal reconstruction.

A number of adult tissues, from skeletal muscle stem cells to circulating T cells, can serve as a source of patient-specific cells for iPSC reprogramming [41,42]. Notable for reconstructive surgeons, fibroblasts from skin biopsies and ASCs from liposuction aspirate provide readily available, large quantity cell sources that can readily yield iPSCs [43]. Initial reprogramming experiments used lentiviruses expressing cancer-related genes c-Myc and Klf4, and transcription factors Oct4 and Sox2, introducing risk of insertional mutagenesis at the sites of viral integration and downstream tumorigenic risk from continued oncogene expression [44]. A variety of reprogramming methods have successfully improved on lentiviral technology using non-integrating minicircle plasmids [45], microRNA [46], stabilized mRNA [47] and nucleotide-free recombinant protein [48]. Though these methods result in lower efficiency of reprogramming than the lentivirus, they carry a reduced risk of mutagenesis and have better safety profiles.

Important to engineering of skeletal bone, iPSCs have been demonstrated to respond robustly to osteogenic inductive media containing β-glyerophosphate and ascorbic acid to form osteoblasts *in vitro* [49]. This study initiated differentiation of mouse iPSCs into mesodermal lineage with embryoid body suspension culture and withdrawal of cytokines before adherent plating in osteogenic media. Over a period of embryoid body culture and two passages in osteogenic media, differentiation to an osteogenic lineage was demonstrated with qPCR for *RUNX2* and *Col1a1* genes, and functionally confirmed with Von Kossa and Alizarin Red stains. Embryoid body culture methods and TGFβ-1 treatment caused spontaneous differentiation of iPSCs toward MSC lineage and significantly increased the amount of mineralization after treatment with osteogenic media [82].

For the application of iPSCs *in vivo*, cells must be differentiated sufficiently to avoid uncontrolled and undesired proliferation, or be driven by a robust differentiation program. MSCs derived from iPSCs were shown to give rise to adipogenic, chondrogenic, and osteoblastic lineages *in vitro*. When the iPSC-MSCs were transplanted into critical calvarial defects,

observed stimulation of new bone bone growth, and histomorphological analysis demonstrated participation of these cells in osteogenic healing of the defect [51]. Transgenic iPSCs expressing SATB2, a gene critical in normal bone development, transplanted directly into a mouse critically-sized calvarial defect resulted in new bone growth and no incidence of teratoma [52]. Though it may be an unexpected result that overexpression of a single gene is sufficient to both turn off self-renewal and drive osteogenesis, the calvarial niche is composed of high concentrations of cytokines and bone progenitors which might act in cooperation with transgene expression. Preliminary data from our own laboratory supports the result that delivery of undifferentiated iPSCs on a biobimetic scaffold, in conjunction with osteogenic factors, is sufficient to both strongly drive osteogenic differentiation and dramatically decrease the incidence of teratoma formation.

Research granting agencies, both private and public, have dedicated significant funds to advancing the translation of stem cell applications toward clinical trials and therapeutic development. Continued work to advance tissue-specific stem cell, BMSC, ASC, ESC, and iPSC transplantation toward the actualization of novel therapies will need to address challenges of immune privilege and rejection, the establishment of standard operating protocols for defined culture and storage conditions, tailoring cell/scaffold combinations for specialized craniofacial regions, and defined predictability of graft retention and integration into host. As these are active areas of research undergoing continuous rapid advancements, the field continues to hold promise for future skeletal reconstructive applications.

CYTOKINES

Studies from developmental biology and distraction osteogenesis have revealed a host of growth factors involved in cell proliferation, differentiation, adhesion, and migration during bone formation. An understanding of these interactions will undoubtedly allow for their use in bone tissue engineering. Successful skeletal tissue engineering will likely involve the incorporation of or stimulation by appropriate cytokines combined with the delivery or recruitment of an osteoprogenitor population.

Already in use clinically, BMPs seem to be the most promising candidate cytokine in skeletal tissue engineering. Members of the TGF-β superfamily, BMPs were originally isolated by Urist from bovine bone extracts and found to induce ectopic bone formation subcutaneously in rats [53]. This large group of proteins, comprising nearly one third of the TGF-β proteins, has also been found to be involved in mesoderm induction, skeletal patterning, and limb development. BMPs are known to control both intramembranous as well as endochondral ossification through chemotaxis and mitosis of mesenchymal cells, induction of mesenchymal commitment to osteogenic or chondrogenic differentiation, and programmed cell death [54]. BMPs transmit their signals via ligand binding to the heteromeric complex of type I and II serine/threonine kinase receptors on the cell surface. The ligand signal is then transduced intracellularly via activation of SMAD (signaling, mothers against decapentaplegic) proteins, which subsequently migrate to the nucleus to effect gene expression. BMP signaling has also been demonstrated to be transmitted via the MAP-kinase (mitogen-activated protein) pathway.

BMP-2, -4, -6, -7, and -9 are known to be the most osteoinductive. It is thought that BMPs regulate osteoblast differentiation via increased transcription of core-binding factor-1/Runt related family 2 (Cbfa1/Runx2, a molecule known to be necessary for commitment along an osteoblastic lineage [55]. These osteoinductive BMPs have been demonstrated to stimulate osteogenic differentiation in multiple cell lines, including fibroblasts, chondrocytes, BMSCs, and ASCs. The effect of BMPs has also been noted to be concentration dependent; at low concentrations, they foster chemotaxis and cellular proliferation, but at high concentrations, BMPs induce bone formation [56]. In culture and *in vivo*, BMP-2/6, BMP-2/7, and BMP-4/7 heterodimers have been shown to more effectively enhance osteogenesis than homodimer

combinations [57,58]. BMP-2 has been approved for use by the Food and Drug Administration for surgical reconstructions, and though some controversy exists over clinical outcomes, most studies show that treatment with BMP-2 has promising clinical efficacy.

Fibroblast growth factors (FGFs) consist of a highly conserved family of 24 known peptides that transmit their signals via a family of four transmembrane tyrosine kinases. FGF-2, the most abundant ligand of the family, is known to increase osteoblast proliferation and enhance bone formation *in vitro* and *in vivo* [59]. In FGF-2 haploinsufficient and null mice, decreased bone mineral density has been observed and correlated with decreased expression of FGF receptor-2 and Runx2 at a molecular level [60]. Application of exogenous FGF-2 rescued bone nodule formation in osteoblast cultures from these FGF-2 mutant mice.

Insulin-like growth factors IGF-1 and IGF-2 are 7.6 kD polypeptides that have been demonstrated to stimulate bone collagen synthesis, as well as osteogenesis and chondrogenesis. A transgenic mouse with upregulated IGF expression in osteoblasts resulted in increased bone formation of the distal femur as compared to control mice [61]. Of interest, however, histology did not reveal an increase in the number of osteoblasts, suggesting that IGF-1 increased the activity of existing bone progenitor cells. Conversely, in an IGF-1 null transgenic mouse model, the size and bone formation rates of the knockout mice were significantly reduced as compared to their wild-type littermates [62].

Platelet derived growth factor (PDGF), a 30 kD polypeptide, has also been demonstrated to be a potent stimulus for osteoblast proliferation, chemotaxis, as well as collagen activity. The utility of PDGF *in vivo* has been shown with enhanced bone formation after local application to tibial osteotomies in rabbits and canine mandibles after periodontal surgery. PDGF, commonly used in chronic wounds, is now being studied clinically in periodontic indications. A recent multi-center trial demonstrated that the application of recombinant human PDGF in a tricalcium phosphate matrix resulted in significantly increased periodontal bone formation [63].

The recruitment of blood vessels is important for the support of newly forming tissue. Application of VEGF to both intramembranous and endochondrial bone defects results in increased blood vessel formation, ossification, and new bone in mouse and rabbit models [64]. Putatively through a direct autocrine effect, VEGF was also demonstrated in this study to play a role in osteoblast differentiation. Inhibition of VEGF by application of a receptor neutralizing agent diminished blood vessel formation and osteogenesis.

A direct comparison of the ability of VEGFA, BMP-2, and FGF-2 to enhance intramembranous bone healing in a critical calvarial defect showed that application of each of these cytokines increases proliferation of osteoblasts and progenitors in the defect region [65]. While all three cytokines significantly enhanced healing of the defect, VEGFA and BMP-2 increased angiogenic support, while the effects of FGF-2 were independent of new blood vessel formation. Supporting the hypothesis that angiogenesis is critical to calvarial bone healing, VEGFA and BMP-2 application also resulted in the most bone formation, with nearly complete healing in a mouse critically-sized defect at three weeks [65].

SCAFFOLDS

The challenges of incorporating cytokines in skeletal tissue engineering involve identifying cell populations and molecules that stimulate and participate in bone formation, but the effectiveness of these treatments is largely based on the mode of delivery, effective dosages, and compatible carrier mechanisms. Advances in materials science have provided an abundance of innovations for developing an appropriate carrier for these cells and molecules. In the selection of a biomimetic scaffold for engineering bone, the material should be osteoconductive, osteoinductive, biocompatible, and biodegradeable without eliciting an

immune response. Osteoconductivity refers to the ability of the graft to support the attachment of cells and allows new cell migration and vessel formation. The osteoinductive quality of scaffolds describes their ability to induce undifferentiated stem cells or progenitor cells along an osteogenic lineage. The challenge in designing the structure of scaffolds is to maximize the porosity of the scaffold to promote cellular and neovascular ingrowth, while maintaining the structural integrity of the lattice [4]. Potential matrices can be generalized into three categories: natural, mineral-based, and synthetic polymers.

Natural scaffolds include the use of collagen, hyaluronic acids, calcium alginate, and chitosan, and are typically biodegradable. In many instances, they exhibit osteoinductive properties, exclusive of cells or cytokines. Implantation of collagen type I alone into critically-sized defects of rat mandibles resulted in partial bone healing [66]. The disadvantage of natural scaffolds is their lack of mechanical stability, hence, their limited utility for use in load-bearing regions of the skeleton. The use of natural scaffolds in humans is also limited by the biochemical changes often induced by sterilization procedures.

Mineral-based scaffolds include calcium phosphate ceramics and bioactive glass. Calcium phosphate ceramics are available as hydroxyapatite or β-tricalcium phosphate, with hydroxyapatite most closely mimicking the structural and chemical characteristics of the mineral component of bone. Tricalcium phosphate is marked by a high-dissolution rate that accelerates material resorption and elicits an immune response, while hydroxyapatite has high chemical stability. Mineral-based scaffolds provide an oseteoinductive signal to encourage differentiation of progenitor cells along an osteogenic lineage. There is, however, great variability in the quality of calcium phosphate ceramics to support osteogenesis due to the difficulty in reproducibly creating these scaffolds [67].

Polymer scaffolds, including polylactic acid, polyglycolic acid, polydoxanonone, and polycaprolactone, have been engineered to provide a greater ability to withstand mechanical forces. These lattices are marked by their incredible strength. They are also designed to be biodegradable via hydrolysis as a result of their local mileau. Polymer scaffolds allow ingrowth of bony tissue but lack osteoinductive properties. The combination of structural stability of polymer scaffolds with mineral coating results in an osteoconductive niche with enhanced architectural strength.

Hydrogels represent another class of polymer scaffolds. They are formed by polymerization and crosslinking of molecules like acrylic acid, and N-isoproplylacrylamide [68]. Hydrogels are an attractive option because of their temperature dependent physical properties. They can be designed to be gelatinous at room temperature, but take on more rigid qualities at body temperatures. This property allows for the administration of tissue-engineering constructs via injection. Hydrogels also allow for relatively easy chemical manipulation of individual peptides, making them bioavailable as free molecules in the matrix or tethered for sustained release. Incorporation of Arginine-Glycine-Aparganine (RGD) peptide motifs on these polymers has been demonstrated to enhance osteoblast adhesion and proliferation [69]. By combining collagen hydrogels with PLGA microparticles to deliver osteogenic MSCs, researchers have demonstrated a cell delivery mechanism that is elastic enough to adapt to the shape of a defect, but will mineralize and acquire structural rigidity in the presence of differentiating cells [70]. Another group has used a hybrid scaffold by combining an osteoconductive load-bearing polymer matrix with a peptide hydrogel providing controlled release of rhBMP-2 [71]. Cells seeded in this scaffold demonstrated increased expression of bone differentiation markers in the group with rhBMP2 tethered within the hydrogel compared to an untethered control group during *in vitro* culture. Hybrid gels thus provide for multiple reconstructive goals including structural integrity, sustained release of cytokines, support of cell differentiation, and are promising candidates for actualizing large defect reconstruction.

Electrospinning has emerged as a novel method to construct scaffolds that closely mimic natural extracellular matrix. Nanowoven fibers are generated by shooting a jet of polymer

solution through a high electric field [50]. Adjusting the polymer solution and the electric field allows the fiber diameter and porosity to be controlled with high precision. Osteogenic materials or cytokines can be incorporated into this scaffold, accurately reproducing surrounding bone matrix as well as osteoinductive factors. Silk fibroin fiber scaffolds incorporating BMP-2 and nanoparticles of hydroxyapatite demonstrated higher mineralization of human MSCs cultured on these scaffolds than controls *in vitro* [50]. Electrospun biodegradable fibers seeded with amniotic-derived MSCs were used to generate allogeneic fetal bone grafts with more consistent *in vivo* reconstructions of craniofacial defects than prosthetic repairs in a rabbit model [72].

TISSUE ENGINEERING IN PRACTICE

Current clinical applications for tissue engineering are limited to the delivery of BMP cytokines and cytokine-scaffold devices. A significant criticism of these applications is the high doses of cytokines necessary to obtain clinical relevance and the resulting costs associated with manufacturing recombinant human BMPs. Concern also exists about the rare, but reported incidences of high blood pressure and even myocardial infarctions in animal models, which could be explained by an immunologic response to the cytokine-scaffold constructs [34].

The Infuse® Bone Graft/LT Cage® fusion device is currently in use for tibia fractures, lumbar fusion, and oral maxillary procedures (Medtronic Sofamor Danek, Minneapolis, MN). This product involves an absorbable, bovine collagen implant soaked with recombinant human BMP-2 (rhBMP-2). Humans require supraphysiologic doses ranging from 0.4 to 1.5mg/mL of rhBMP-2 to form new bone. Because of the high doses, there have been concerns about uncontrolled bone growth around the implantation site or cancer, given links of osteosarcoma with BMP activity [73]. A clinical study of Infuse® in 74 consecutive patients revealed radiographic fusion was 100% at 12 and 24 months, in which no bone overgrowth or sarcomas were observed. The study was significantly weakened, however, by the lack of a control group [74]. Off-label use for some procedures in the cervical and lumbar spine have been reported to cause complications including tissue swelling, seroma, ectopic bone formation, paralysis and death [75,76]. This has led many surgeons to reconsider the broad use of Infuse® for off-label applications.

Recombinant human BMP-2 (rhBMP2) has also been used in patients requiring maxillary sinus floor augmentation, in preparation for enodosseous dental implants. In one study, rhBMP-2 was applied to the maxillary sinus floor on an absorbable collagen sponge at 0.75 and 1.5mg/mL [77]. Radiographic evaluation for bone mass in the maxillary sinus floor revealed that rhBMP-2 was able to induce bone formation, but it proved less effective than autogenous bone grafting in fostering osseous formation. Notably, however, rates of successful placement of dental implants were similar between the bone graft treatment arm and patients receiving rhBMP-2 [77].

Recombinant human BMP-7 (rhBMP-7) has also been approved by the FDA for clinical use in cases where previous treatment with autograft failed (Stryker Orthopedics, Mahwah, NJ). It is also delivered via a bovine collagen matrix and has been approved for the treatment of nonunion in the tibia of at least nine months, secondary to trauma, in skeletally mature patients. In a small case series report, the use of rhBMP-7 in five patients who had failed allografts did not result in any significant healing [78]. Recombinant human BMP-7 has also been in clinical use to aid intertransverse process fusion in the lumbar spine [79]. In a putty formulation consisting of rhBMP-7, bony fusion rates were comparable to groups treated with autograft. They did not report any adverse events, ectopic bone formation, or systemic toxicities associated with use of this product over a two year period. Clinical trials are currently being conducted on BMP-7 to test the therapeutic effects on matrix protein restoration, pain reduction, and biomechanical function after vertebral disk injection [80].

CONCLUSION

The intersection of advances in stem cell biology, molecular biology, biochemistry, bioengineering, and materials sciences has brought to the forefront the ability of regenerative medicine to address problems of skeletal defects. Efforts are furthered by continued research on the functions and interactions of osteogenic cytokines, as well as the identification of postnatal osteoprogenitor cells in bone marrow and adipose tissue. The influence of nanotechnology on scaffold design and the advancements of growth factor releasing scaffolds and hydrogels are promising developments to avoid the need for supraphysiologic doses.

Efforts also need to be directed toward developing methods to provide vascular support of large osseous constructs, potentially through the use of pro-angiogenic agents. Further investigation into identifying and enriching osteoprogenitor populations from adult stem cells along with continued advancements in pluripotent cell research may translate into human clinical trials and more available tools for reconstructive surgeons. Given the immense biomedical burden of skeletal defects and the significant developments in skeletal tissue engineering, osseous regeneration provides a promising and attainable goal that will be achieved through interdisciplinary collaboration.

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PART 15

Nervous System

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CHAPTER 61

The Nervous System

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INTRODUCTION

Over the last few years pluripotent stem (PS) cells including embryonic stem (ES) and induced pluripotent stem (iPS) cells have emerged as a powerful tool to study brain development and function *in vitro* and *in vivo*. The isolation of ES cells [1] and iPS cells [2–4] of human origin has further stimulated ES cell research directed towards cell therapeutic applications assessing the potential PS cells as a source of a wide-range of somatic cell types affected by disease. The central nervous system (CNS) has been proposed as one of the prime targets for PS cell-based therapies, due to early successes in directing PS cell towards neural fates, due to the experience with fetal tissue transplantation in neurodegenerative diseases and due to the devastating nature of many CNS diseases that often lack efficient alternative treatments. Some of the most striking advantages of PS cells as compared with any other cell type are: extensive self-renewal capacity and differentiation potential, access to the earliest stages of neural development, and ease of inducing stable genetic manipulations.

The mammalian brain is one of the most complex biological structures known. The study of brain function in the normal and diseased brain is therefore a daunting task. However, an understanding of the basic elements that give rise to the extraordinary cell diversity in the brain is an important step towards that end. Developmental biology has taught us that all the cells of the CNS are derived from a small set of neuroepithelial cells that sequentially give rise to the various neuronal and glial subtypes that comprise the CNS and peripheral (PNS). ES cells provide a powerful model system to study developmental neurobiology, to test hypotheses on how neural identity and regional specification is established, and how neural stem cells decide to undergo neuronal or glial differentiation programs. Protocols for the directed differentiation of ES and iPS cells into specific neuronal or glial subtypes can serve as a cell-based developmental screen to identify novel genes involved in specific aspects of CNS development, and to assess the role of known genes under conditions that can bypass embryonic lethality often observed when studying gene function *in vivo*. This interplay between ES cell and developmental biology is mutually beneficial and will hopefully result not only in novel insights for basic biology, but will also provide a comprehensive framework of differentiation strategies for cell therapeutic applications. While work with mouse ES cells has pioneered this approach, human PS cells have developed into a particularly interesting tool given the lack of alternative model systems for the study of early human neural development and the drive to develop directed differentiation protocols for applications in human disease modeling [5] and in regenerative medicine [6].

This review will provide a short introduction to neural development, followed by a section on neural stem cells, contrasting their advantages and limitations for both basic and clinical

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applications with the use of ES cells. Subsequently, we will introduce the various strategies available for inducing neural differentiation in mouse ES cells, and we will give various examples of neuronal, glial and neural crest specification *in vitro*. This section will be followed by a brief summary of published work on lineage selection via surface makers and cell types specific promoters, a strategy that can be used as an alternative or in parallel to directed differentiation protocols. A synopsis of the current studies of neural differentiation in human and non-human primate ES cells will be followed by more general remarks on the potential for ES cells to address developmental questions via cell-based screens. The final section will summarize recent work with ES cells for cell therapeutic applications in preclinical models of neural disease.

NEURAL DEVELOPMENT

A basic understanding of neural development is a prerequisite for developing rational strategies of stem cell differentiation. The neural plate is derived from the dorsal ectoderm and is induced by 'organizer' signals derived from the underlying notochord. The dominant model of neural induction is the default hypothesis. This hypothesis states that neural tissue is formed spontaneously in the absence of bone morphogenetic protein (BMP) signaling during early gastrulation, while exposure to BMP signals causes epidermal differentiation [7]. Accordingly, signals emanating from the organizer essential for neural induction are BMP inhibitors such as Chordin [8], Noggin [9], and Follistatin [10] and cerberus. However, there are also reports indicating that FGF signals emanating from precursors of the organizer prior to gastrulation are involved in a 'pre-pattern' of neural induction via activation of Sox3 and ERNI [11]. Subsequent studies have identified Churchill (ChCh), a zinc finger transcriptional activator induced by low doses of fibroblast growth factor (FGF) signaling, which inhibits the meso-endoderm-inducing effects of FGF and sensitizes cells to BMP signals, thereby acting as a switch from gastrulation to neurulation [12]. Other essential players during neural induction are IGF [13] and Wnt signaling [14,15].

After the formation of the neural plate, cells undergo a well-defined set of morphological and molecular changes, leading to the formation of neural folds and neural tube closure. This is followed by orchestrated waves of neural proliferation and differentiation. Of particular importance in determining specific neural fates are signals which provide regional identity both in the antero-posterior (AP) and dorso-ventral (DV) axis, and also define domains of distinct expression of homeodomain proteins and bHLH transcription factors. The leading hypothesis of AP axis specification states that anterior fates are established as a default during early neural induction, while FGF, Wnt and retinoid signals are essential for establishing posterior cell fates (for review see [16]). DV identity is determined by the antagonistic action of sonic hedgehog (SHH) secreted ventrally from the notochord and floor plate and BMPs from the roof plate (for review see [17]). There is ample evidence from explant studies (e.g., [18–20]) – and more recently from ES cell differentiation studies [21–25] – which confirm such a concentration-dependent role of SHH in defining specific progenitor domains within the neural tube by activating class II genes in the ventral spinal cord [26]. However, genetic studies with SHH/Gli3 [27–29], or SHH/Rab23 [30] double mutant mice have shown that dorso-ventral patterning can occur in the absence of SHH, questioning an exclusive role for SHH gradients in DV patterning. Work has demonstrated that the timing of DV patterning is controlled by the antagonistic action of FGF and retionid signals [31], with FGF signals inhibiting the establishment DV homeodomain transcription factors [31]. Other findings have suggested a more specific role for FGFs in inhibiting dorsal gene expression [32] and have identified retinoids as an activator of class I genes [32] essential for DV patterning. A SHH antagonistic concentration dependent role for BMPs in dorsal neural patterning has been proposed from work with explants *in vitro* (e.g., [33], [34]) and partly confirmed in ES cell differentiation studies [23]. While some *in vivo* data from genetic ablation studies [35] and work in transgenic mice that

overexpress the BMP receptor type 1a (BMPR1a/Alk3 under control of the regulatory elements of the nestin gene [36] are compatible with a dorsal patterning role for BMPs, loss of function studies of BMPR1b suggested a much more limited role for BMPs in selectively determining choroids plexus fate [37]. In addition to BMPs Wnt signals might also contribute to dorsal neural patterning; particularly the establishment of the neural crest [38].

Subsequent differentiation of patterned neural precursor cells occurs in a stereotypic fashion with neurons being born first followed by astroglial and oligodendroglial differentiation. Onset of neuronal differentiation is controlled via inhibition of the Notch pathway that represses proneural bHLH genes (for review see [39,40]). Astrocytic fate is established via activation of Jak/Stat signals, which exert an instructive role on multipotent neural progenitor to drive astrocytic differentiation [41,42]. However, recent insights into the neurogenic properties of radial glial [43] cells as well as the identification of adult neural stem cells as a cell expressing astrocytic markers [44] suggest a more complex and dynamic interaction between neural stem cell and astrocytic fates. Oligodendrocytes were believed to derive from bi-potent glial precursor termed O2A progenitors [45] or from other glially committed precursors [46]. However, more recently data have emerged that suggest a lineage relationship between motor neurons and oligodendrocytes in the spinal cord [47,48], as well as GABAergic neurons and oligodendrocytes in the forebrain [49,50] due to their shared requirement for expression of the bHLH gene Olig 2. A review of the developmental signals that control the various neuronal subtypes is beyond the scope of this current chapter, but some of the necessary signals will be discussed in the individual chapters in the section Derivation of ES- and IPS-cell-derived neurons. Excellent reviews on the specification of catecholaminergic and neurons [51], ventral and dorsal spinal cord neuron subtypes [52,53], and forebrain patterning [54–57] are available for a more in depth discussion of this topic.

NEURAL STEM CELLS

The isolation of neural stem cells provided a first step towards developing rational strategies for *in vitro* neuronal and glial differentiation. Neural stem cell culture systems had a significant influence on protocols of directed neural differentiation from ES cells. We therefore briefly highlight some of the basic neural stem cell techniques, with a view towards discussing commonalities and discrepancies between the two *in vitro* differentiation strategies. Neural stem cells have been isolated both from the developing and adult brain (for review see [58–61]). Over 20 years of intensive research have convincingly demonstrated the capacity of neural stem cells for self-renewal and multi-lineage differentiation into neurons, astrocytes and oligodendrocytes; the three major CNS lineages. However, while many tissue specific stem cells, such as hematopoietic stem cells, are capable of differentiation into all progeny within an organ system [62], neural stem cells typically do not give rise to all neuronal subtypes present in the adult brain but are largely limited to the production of GABA and glutamaterigic neurons after long-term expansion [63,64]. Early attempts at isolating and propagating neural stem cells *in vitro* were based purely on selective growth and proliferation condition. The most commonly used method is the neurosphere culture system [65–67]. Under these conditions neural precursors are grown at low density and allowed to proliferate as free-floating spheres in the presence of epidermal growth factor (EGF) and FGF-2. Human neurospheres cultures are typically supplemented with LIF [68] in addition to EGF and FGF-2. Neurospheres can be formed from single cells and the capacity for neurosphere formation is often used as an assay to test stem cell properties of neural cells. For example, the isolation of prospectively identified neural stem cells based on surface markers such as AC133 [69], Lex1 [70] or combinations of surface markers [71] was largely based on the ability of the cells to form neurospheres *in vitro*. These data need to be interpreted cautiously, as neurosphere formation is not necessarily a true test of stemness, and neurospheres do contain many differentiated cells in addition to the presumptive progenitor/stem cell population. In fact studies have

demonstrated that neurospheres are formed more efficiently from transient amplifying populations than from true stem cells in the adult subventricular zone (SVZ) [72] and that neurosphere formation is prone to a number of technical pitfalls that need to be carefully considered when using this assay to define neural stem cell properties.

An alternative approach to the neurosphere technology is the growth of neural precursor/stem cells as monolayer culture attached on a matrix – typically fibronectin or laminin – in the presence of FGF2 [74,75] or FGF/EGF [76]. These conditions are more amenable for studying the precise lineage relationship, and allow for precise manipulations at the single cell level. In fact complete lineage trees for single cortical stem cells have been worked out under such conditions [77]. Among the most important limitations of current neural stem cell technology are the limited *in vitro* control of neural patterning and neuronal subtype specification.

The derivation of midbrain dopamine neurons has served as a model for these difficulties. Functional midbrain dopamine neurons can be derived from short-term expanded precursor cells isolated from the early rodent and human midbrain [78–80]. However, long-term expansion causes a dramatic loss in the efficiency of midbrain dopamine neuron generation [81]. Several strategies have been developed in an attempt to overcome these problems, ranging from exposure to complex growth factor cocktails [82,83] to changing oxygen levels [209] to the transgenic expression of Nurr1 [84,85] a key transcription factor during midbrain dopamine neuron development [86–88]. However, none of these approaches has succeeded in deriving midbrain dopamine neurons that exhibit full functionality *in vitro* and *in vivo* from naïve long-term expanded neural stem cells. While the mechanisms of the restricted neuronal differentiation potential remain to be elucidated, cell types most difficult to derive from neural stem cells are neurons born at developmental stages prior to stem cell isolation. This suggests that the competence of the precursor population to generate these neuronal subtypes might be lost, or that the environmental co-factors required for appropriate neuron subtype specification are missing. Alternatively, the mitogens used for propagation of the neural precursor cells might select for progeny incapable of generating early neuronal subtypes, bias competent precursors into a non-competent state or directly deregulate the neural patterning state as recently suggested for FGF-2 expanded precursors in the spinal cord [89]. Possible future solutions might consist of isolating neural stem cells at an earlier developmental state, and defining conditions that allow these cells to retain early competency for patterning factors, the identification of growth factors that do not bias neuronal subtype or are able to reinduce competence in later precursors, or completely novel patterning strategies that can bypass current limitations in neuronal subtype potential. One early example in this direction is the growth of midbrain-derived precursors in the absence of FGF2 but in the presence of SHH and FGF8 as well as TGFβ3 [90], a combination capable of increasing midbrain dopamine neurons generated *in vitro*, but still with limited capacity for cell expansion. While these problems continue to hamper *in vitro* differentiation studies with primary neural stem cells ES cells offer a simple and efficient alternative solution to overcome such concerns. Furthermore, several recent studies have shown the derivation of either neural stem cells (for review see [91]) or differentiated dopamine neurons [92–94] directly from fibroblasts using extrinsic expression of lineages specific transcription factors.

NEURAL DIFFERENTIATION OF MOUSE ES CELLS AND IPS CELLS

ES and iPS cells are PS cells capable of virtually unlimited *in vitro* proliferation at the undifferentiated stage, overcoming many of the problems associated with instability of stem cell phenotype observed in tissue specific stem cells including neural stem cells. In addition to their proliferation potential, ES cells offer many additional important advantages for both basic and applied research, such as their ease of genetic manipulation, access to the earliest stages of neural development, and their comprehensive differentiation potential. The cell fate potential of ES cells is most vividly illustrated upon injection of ES cells into the developing blastocyst,

where PS progeny contribute to all tissues including the germline. Neural differentiation has been one of the best-studied *in vitro* differentiation pathways in PS cell research. This is in part due to the ease by which ES cells give rise to neural progeny, but also due to the potential of the neural progeny for cell therapeutic applications in the CNS. The use of PS cells in regenerative medicine got a significant boost with the discovery of human ES cells [1,95] and the induction of iPS cells in mouse [96] and human [2–4] somatic cells.

Neural induction

The initial requirement for generating defined neural subtypes from ES cells is appropriate control of neural induction. There are at least three main strategies for neural induction of ES cell *in vitro* to induced neural differentiation in mouse ES cells: embryoid-body- (EB) based systems, stromal feeder mediated neural induction and protocols based on default differentiation into neural fates.

EMBRYOID-BODY-BASED PROTOCOLS

EBs are formed upon aggregation of ES cells in suspension cultures. The interactions of cells within the EB causes cell differentiation in a framework that mimics normal development, particularly the steps of gastrulation. Accordingly, derivatives of all three germ layers can be found in EBs (Doetschman et al. 1985 [96a], for review, see Weiss and Orkin, 1996 [96b]), and EB culture is often used as a first screening tool to demonstrate pluripotency in putative ES cell lines. While the derivation of neural progeny is inefficient under basic EB conditions, a number of protocols have been developed to enhance neural induction and to select and expand EB-derived neural precursors

The first EB-mediated neural differentiation protocol was based on exposure to retinoic acid (RA) for four days after EBs had been formed for four days in the absence of RA (the so-called 4−/4+ protocol [97]). RA is a vitamin A derivative, released primarily by surrounding mesodermal cells, and it exhibits a strong neural induction and patterning effect (for review, see Maden 2002 [96c]). Many variations on the basic protocol have been developed [98–100]. However, no clear mechanistic understanding of the action of RA in EBs has emerged, and the overall cell composition under these conditions remains quite heterogeneous. One major additional concern in RA-based neural induction protocols is the concomitant effect of RA on AP patterning mediated through activating the Hox gene cascade [101].

An alternative EB-based strategy is the exposure to conditioned medium derived from hepatocarcinoma cell line (HepG2), which appears to induce neuroectodermal fate directly [102]. Accordingly, HepG2 treated aggregates do not express endodermal or mesodermal markers but directly give rise to neural progeny expressing progenitor markers such as Sox 1, 2, and nestin. The active component within HepG2 conditioned medium remains to be isolated. Some evidence indicates that at least two separable components are responsible for this activity, and one of these components is apparently a known extracellular matrix molecule [102]. Unlike the RA protocols, HepG2 conditions are not thought to bias neural subtype composition towards specific AP or DV fates. However, no detailed studies are available that have addressed this issue experimentally or developed neural subtype specific protocols.

A third strategy for achieving neural induction is a RA-free EB-based protocol that subjects EB progeny to neural-selective growth conditions [22, 103,104]. Neural selection from EB progeny is achieved under miminal growth conditions in a serum free medium supplemented with insulin, transferrrin and selenite (ITS medium). Under these conditions most EB-derived cells die and a distinct population of immature cells emerges that expresses increasing levels of the intermediate filament nestin. These nestin+ precursors can be replated and directed towards various neuronal and glial fates using a combination of patterning, survival and lineage promoting factors (see sections Derivation of ES- and IPS cell derived neurons and ES-derived glia). This technique is quite robust and highly modular for generating a wide

variety of neural subtypes (see below). Moreover, commercially available kits have become available that provide a simple entry point for setting up the system. However, ES cell line specific differences in the efficiency of neural induction can occur, particularly at stage III of differentiation (selection of nestin+ precursors from EBs in ITS medium) and can be limiting for some applications [21, 105]. Finally, the most widely used EB technology to date for inducing neural fates from PS cells is the serum free EB (SFEB) technology [106]. Under those conditions, neural fates are induced directly without evidence of inducting other germ-layers during EB formation. This is a highly efficient protocol that can be adapted to generate a broad range of neural lineages, including hypothalamic [107], pituitary [108] retinal [109] or cortical [106] lineages.

STROMAL FEEDER MEDIATED NEURAL INDUCTION

Bone marrow-derived stromal cell lines have been used for many years to support the growth of undifferentiated hematopoietic stem cells [110–113], acting in part via expression of the membrane protein mKirre, a mammalian homolog of the gene *kirre* of *Drosophila melanogaster* [114].

Interestingly, many of the stromal cell lines which support hematopoietic stem cell growth exhibit neural inducing properties in co-culture with mouse ES cells [21,115]. Stromal cell lines with the highest efficiencies of neural induction are typically at the preadipocytic stage of differentiation. The main activity resides within the cell surface of the stromal cells and does not diffuse efficiently over long distances [115]. The molecular nature of this stromal-derived inducing activity remains poorly characterized. The efficiency and robustness of neural induction using stromal feeder cells is high compared to alternative protocols [21] and differentiation appears to occur without any bias towards regional specification or neuronal versus glial fate choice [21,23].

NEURAL DIFFERENTIATION BY DEFAULT

Co-culture free direct neural differentiation protocols are based on the default hypothesis proposing that in the absence of cell-cell signaling, particularly in the absence of exposure to BMPs, ectodermal cells will adopt a neural fates [7]. Two independent studies with mouse ES cells confirmed that under minimal conditions in the absence of BMP but in the presence of a required endogenous FGF signal, neural induction does occur in non-adherent [116] or adherent [117] monocultures. The adherent monoculture system has developed into a particularly powerful system for generating neural [61] and forebrain cortical lineages that appear by default [118] or upon inhibition of endogenous SHH [119] signaling. The development of those refined culture systems has been particularly useful for defining the minimal molecular requirements that drive neural fate specification in early development.

DERIVATION OF ES AND iPS Cell-Derived Neurons

Neuronal differentiation occurs rapidly upon neural induction of mouse ES cells. The efficiency of neuronal versus glial differentiation varies widely. Neuronal subtype specification can be influenced by the mode of neuronal induction. This is particularly the case for RA induction protocols that are known to induce the Hox gene cascade in a dose-dependent fashion and promote hindbrain and spinal cord fates at the expense of forebrain differentiation. The basic strategy for achieving neuronal subtype differentiation relies on the application of signals that mimic early patterning events in the embryo to define AP and DV domains within the body axis of the developing embryo. AP patterning is thought to involve factors that actively posteriorize regional identity including RA, FGFs and Wnts. DV patterning is thought to be controlled by the antagonistic action of SHH and BMP signals. While early mouse ES cell differentiation strategies yielded neurons not well characterized and typically of GABA or glutamatergic origin, more refined protocols have been developed

recently for many neuronal subtypes. Protocols for a selection of neuronal subtypes are discussed in more detail below.

Midbrain dopaminergic neurons

Derivation of midbrain dopamine neurons from ES cells has been of particular interest, due to the clinical potential for dopamine neuron transplants in treatment of Parkinson's disease (for review see [120,208]). Protocols for the dopaminergic differentiation of mouse ES cells closely follow findings obtained in explant studies that identified FGF8 and SHH as critical factors in midbrain dopamine neurons specification [20,121–123]. The effect of SHH/FGF8 on ES-derived neural precursors was first described using an EB-based five-step differentiation protocol [22]. Under these conditions, up to 34% of all neurons expressed tyrosine hydroxylase (TH) the rate-limiting enzyme in the synthesis of dopamine. However, the effect of SHH and FGF8 was relatively modest and did not exceed that of another dopamine neuron promoting factor, ascorbic acid (AA, Vitamin C) [22,81]. A further increase in dopamine neuron yield was obtained in ES cells overexpressing Nurr1 [24]. Nearly 80% of all neurons express TH under these conditions, and the expression of midbrain dopaminergic markers remains stable even after transgenic expression of Nurr1 has been silenced. Midbrain dopaminergic differentiation was also obtained using co-culture of ES cells on the stromal feeder cell line (PA6) [115]. PA6 cells mediated neural induction yielded dopaminergic differentiation in up to 16% of all neurons without requiring any exposure to exogenous SHH and FGF8 but in the presence of AA. These results were initially interpreted as PA6 exhibiting a specific action promoting dopamine neuron fate in addition to its neural inducing properties [124]. However, later studies demonstrated that there is no irreversible bias towards the generation of midbrain dopamine neurons using PA6, but neural precursors derived under these conditions remain highly amenable to AP and DV patterning [21,23]. When combining stromal feeder mediated neural induction with exposure to SHH/FGF8 about 50% of all neurons express TH [21] without requiring any transgene such as Nurr1 to further push dopamine neuron phenotype. Numbers of TH neurons need to be interpreted carefully in all *in vitro* differentiation studies, as TH is an unreliable marker for identifying dopamine neurons. TH is also expressed in other catecholaminergic neurons including noradrenergic and adrenergic cells, and it is induced in many cell types under various conditions including cell stress, hypoxia and exposure to a variety of growth hormones. It is therefore essential that studies reporting on the derivation of midbrain dopamine neurons provide additional markers and evidence of dopamine neuron function *in vitro* and *in vivo*. We have discussed these parameters in detail in recent and past reviews on this topic [125,210]. However, *in vitro* derived dopamine neurons generated under these conditions and selected for expression of Nurr1::GFP express all the known markers of endogenous midbrain dopamine neurons and can be used as a tool for discovering novel genes associated with midbrain dopamine neuron lineages [126].

The derivation of TH neurons has also been achieved using a monolayer default neural induction protocol [117]. However, the efficiency of dopamine neuron induction under these conditions remained unclear. In contrast, more recent studies using overexpression of key transcription factors such as LMX1A [204], or more sophisticated patterning strategies [127] can yield *bona fide* mouse midbrain dopamine neurons at high efficiencies.

Serorotergic neurons

The developmental origin of serotonergic neurons is closely related to that of midbrain dopamine neurons. Both neuronal subtypes are dependent on signals emanating from the isthmic organizer [51]. Accordingly, serotonergic neurons are a major 'contaminating' neuronal subtype in protocols aimed at the derivation of midbrain dopaminergic cells. Protocols specifically designed to increase serotonergic versus dopaminergic differentiation have been based on the exposure to FGF4. Application of exogeous FGF4 preceding FGF8 and SHH application ectopically induces serotonergic neurons in explant culture [20]. While

application of FGF4 to neural precursors in the presence of FGF2 at stage IV (neural precursor cell proliferation) of the multistep EB differentiation protocol does not yield a significant increase in serotonergic differentiation [22], FGF4 added in the absence of FGF2 however, causes a dramatic shift from dopaminergic to serotonergic differentiation [24]. Efficient derivation of hindbrain serotonergic neurons using stromal feeder mediated neural induction also involves early FGF4 exposure in the absence of FGF2, followed by FGF2, FGF8 and SHH application [21]. Novel strategies to refine serotonergic differentiation protocols might make use of novel basic developmental insights that demonstrated the importance of the transcription factor Lmx1b in serotonergic differentiation [128], and studies in zebrafish where several novel genes have been isolated that affect the proportion of dopaminergic versus serotonergic neurons including the elongation factor foggy [129] and the zinc finger protein too few [130]. While cell therapy might be not a primary goal for optimizing serotonergic differentiation protocols , the derivation of ES-derived serotonin neurons may provide insights into brain development and offer an unlimited source of cells for pharmacological screens in a neurotransmitter system involved in various psychiatric disorders [131–134].

Motor neurons

Development of spinal motor neurons has been studied in great detail using a variety of mouse loss of function ad chick gain of function models as well as explant culture systems (for review see [17,52]). A more advanced understanding of the developmental signals involved in motor neuron specification and a wealth of reagents available for their phenotypic characterization make the derivation of motor neurons from ES cells an obvious target. Early studies have demonstrated that cells expressing markers of motor neurons can be generated using an EB induction protocol in combination with RA exposure (2−/7+) [99]. More systematic approaches using RA exposure in combination with exogenous SHH to promote ventral fates have yielded ES-derived motor neurons at high efficiency and provided a demonstration of how developmental pathways can be harnessed to direct ES cell fate *in vitro* [25]. By creating an ES cell line, which expresses GFP under the control of the motor neuron specific gene HB9, these ES cell-derived motor neurons could also be readily identified and purified. The *in vivo* properties of ES-derived motor neurons were demonstrated upon transplantation into the spinal cord of early chick embryos. ES-derived motor neurons were detected in the ventral spinal cord, extended axons and innervated nearby muscle targets [25].

Efficient derivation of motor neurons has also been achieved using stromal feeders such as PA6 [23] or MS5 cells [21] in combination with SHH and RA treatment. An important challenge for *in vitro* motor neuron differentiation protocols is the selective generation of motor neurons of distinct AP and columnar identity as demonstrated recently using RA-dependent and RA-independent motor neuron induction conditions [135]. Recent insights from developmental biology studies will provide a good starting point towards that end [136,137]. The potential of ES-derived motor neurons for future therapeutic applications in spinal cord injury or amyotrophic lateral sclerosis (ALS) will heavily rely on the ability to generate precise motor neuron subtypes *in vitro*. Additional requirements are controlling axonal outgrowth, target selection and specificity of muscle innervation. One particularly useful approach might be the genetic or pharmacological manipulation of ES-derived motor neurons to overcome growth inhibitors present in an adult environment, a strategy used successfully to overcome growth inhibition of primary dorsal root ganglion cells upon activation of the cAMP pathway [138,139].

GABA neurons

GABA cells are the main inhibitor neuron type within the brain and are found at high densities in basal forebrain structures, particularly in the striatum. The presence of GABAergic neurons during ES cell differentiation *in vitro* has been reported under various conditions

including the classic 4−/4+ EB-based differentiation protocols [97], which yields approximately 25% GABA neurons [140]. Interestingly, protocols with shorter periods of RA-free EB formation followed by extended RA treatment (2−/7+) select for motor neurons rather than GABA neurons [99]. This suggests that the timing of RA application might be crucial for neural fate specification. The presence of GABA neurons has also been reported under default neural induction conditions [117].

Directed differentiation to GABA neurons has been achieved using a stromal feeder-based approach [21]. Neural induction on MS5 is followed by neural precursor proliferation in FGF2 and subsequent exposure to SHH, FGF8. The delayed application of FGF8 and SHH promotes ventral forebrain identities, as determined by the expression of the forebrain-specific marker FOXG1B (BF-1) [141], and the increase in GABAergic differentiation [21]. In addition to forebrain striatal and cortical GABA neurons there are many other types of GABA neurons in various brain regions including the thalamus [142], midbrain [143] and cerebellum [144]. Recent studies have demonstrated the efficient derivation of cerebellar GABA-ergic neuron lineages from mouse ES cells such as granule cells and Purkinje neurons [145–147]. Other examples of the generation of specific subtypes of GABA neurons include the directed differentiation of cortical interneuron lineages using an SFEB-based strategy in combination with ventral patterning, and lineage selection using an Lhx6::GFP reporter line [148]. GABA neurons are highly relevant for a variety of neurological disorders including Huntington's disease, epilepsy, and stroke, and their dysfunction may also be implicated in neurodevelopmental and neuropsychiatric disorders such as autism or schizophrenia.

Glutamate neurons

Glutamate neurons can be readily obtained at high efficiencies from mouse ES cells. For example in the Bain protocol [97] approximately 70% of all neurons are glutamatergic and ES-derived neurons with N-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes have been described [149]. Very similar neuron subtype compositions have been obtained with various related protocols [100,150]. More detailed phsyiological data on glutamatergic neurons have been reported after co-culture of ES-derived neurons on hippocampal brain slices [151]. Cortical glutamatergic projection neurons are also readily obtained in monolayer or SFEB culture systems that recreate aspects of cortical neurogenesis *in vitro* [106,118,119]

Other neuronal and neural subtypes

The presence of about 5% glycinergic neurons has been reported using the classic 4−/4+ EB protocol [97,140]. However, directed differentiation into this main inhibitory neuronal subtype in the spinal cord has not been demonstrated. Other interesting neural types generated from ES cells are precursors of the otic anlage [152], pituitary lineage [108] or ES-derived cells capable of recreating optic cup morphogensis [109].

Neural crest differentiation

The neural crest is a transient structure formed from the most dorsal aspects of the neural tube of the vertebrate embryo. It contains migratory cells that form the peripheral nervous system, including sensory, sympathetic and enteric ganglia, large parts of the facial skeleton as well as various other cell types including Schwann cells, melanocytes and adrenomedullary cells. ES cells provide a powerful assay for the study of neural crest development *in vitro*. In early studies, the main strategy for deriving neural crest-like structures from ES cells was based on the exposure to BMPs (BMP2, BMP4 or BMP7) following neural induction. The feasibility of this approach was demonstrated for mouse and partly non-human primate ES cells using the PA6 stromal feeder cell system [23]. This study showed the development of both sensory as well as sympathetic neurons in a BMP dose-dependent manner. The derivation of smooth muscle cells required growth in chicken extract in combination with BMP withdrawal. No

melanocytes or Schwann cells could be obtained under these conditions [23]. However, more recent studies suggest that activation of Wnt signaling is particularly critical for the efficient induction of neural crest lineages in particular in studies using human ES cells [153,154].

ES-DERIVED GLIA

Neural progenitors derived from mouse ES cells can be readily differentiated into astrocytic and oligodendroglial progeny under conditions similar to those described from primary neural precursors. The first reports on the glial differentiation of mouse ES cells were based on the 4−/4+ EB protocols [97,98] or multistep EB differentiation protocols [103]. Most of the glial progeny under these conditions are astrocytes with only few immature oligodendrocytes present. However, subsequent studies have defined conditions for the selective generation of both astrocytes and oligodendrocytes.

Oliogodendrocytes

Highly efficient differentiation into oligodendrocytes was reported first using a modified multistep EB-based protocol [104]. ES-derived neural precursors were expanded with FGF2 followed by FGF2 + EGF and FGF2 + platelet-derived growth factor (PDGF). These conditions yielded a population of A2B5+ glial precursors that are capable of differentiation into both astrocytic (∼36% GFAP+) and oligodendrocytic (∼38% O4+) progeny upon mitogen withdrawal. The 4−/4+ EB RA induction protocol has recently been optimized for the production of oligodendrocytic progeny. This study [155] demonstrated efficient selection of neural progeny by both positive (Sox1-eGFP) and negative (Oct4-HSV-Thymidine kinase) selection, oligodendrocytic differentiation in RA-induced EBs after expansion in FGF2, followed by dissociation and replating in serum-free medium containing FGF2 and SHH. The final step involved SHH and FGF2 withdrawal and the addition of PDGF and thryoidhormone (T3). Under these conditions ∼50% of all cells express oligodendroglial markers. Optimized conditions for oligodendrocyte differentiation using HepG2 or default neural differentiation protocols have not yet been reported. However, stromal feeder mediated induction, initially thought to bias towards neuronal progeny [115], can be readily adapted to derive oligodendrocytes at very high efficiencies and without requiring any genetic selection [21].

Astrocytes

Highly efficient differentiation of ES cells into astrocytes has been recently reported using stromal feeder mediated neural induction, followed by sequential exposure to FGF2, bFGF/EGF, EGF/CNTF and ciliary neurotrophic factor (CNTF). Over 90% of all cells expressed the astrocytic marker GFAP under these conditions [21]. Significant numbers of GFAP cells were also obtained using HepG2 mediated neural differentiation [102] or multistep EB protocols [104]. Glial progenitors obtained with a multistep EB protocol were recently 'transplanted' *in vitro* into hippocampal slices and revealed that full physiological maturation of ES-derived astrocytes can be achieved upon interaction with an appropriate host environment [156]. Under these conditions ES-derived astrocytes integrated seamlessly into host astrocytic networks tightly coupled via gap-junctions.

LINEAGE SELECTION

Lineage selection based on surface markers or the cell type specific expression of promoter-driven selectable markers provides an alternative approach to directed *in vitro* differentiation protocols. The use of genetic markers in ES cells is particularly attractive due to ease of inducing stable genetic modifications and the availability of large libraries of transgenic and gene targeted mice and ES cells. Efficient purification of neural progeny from mouse ES cells *in vitro* was demonstrated via positive selection using a Sox1-EGFP knock-in cell line [206] and

combined with negative selectable marker controlled by endogenous Oct4 locus [155]. Other ES lines successfully used for the genetic identification and purification of neural precursor, neuronal and glial progeny *in vitro* include a tau-eGFP knock-in cell line, a GFAP transgenic line [157], GAD-lacZ knock-in ES cells [158], BF1-lacZ knock-in ES cells [21], and ES cell lines driven under the regulatory elements of the Nestin gene [159,160]. Promoter-driven lineage selection for motor neuron fate *in vitro* includes ES cell lines expressing eGFP in the Olig2 locus [161] or as a transgene under the HB9 promoter [25]. In the case of midbrain dopamine neurons, we have described the use of reporter lines that mark sequential stages of dopamine neuron lineages from the precursor stage (Hes5::GFP) to post-mitotic dopamine neuron precursors (Nurr1::GFP), to the specified midbrain dopamine neuron lineage (Pitx3::YFP) [125]. Those reporter lines have been particularly useful for defining optimized grafting protocols and for gene discovery studies [125,162]. Finally, cortical interneuron precursor lineages can be captured using an Lhx6::GFP reporter line that has been particularly useful for lineage selection in cell transplantation studies.

NEURAL DIFFERENTIATION OF HUMAN AND NON-HUMAN PRIMATE ES CELLS

Neural differentiation potential was readily observed when primate ES, or ES-like cells were first established in both monkey and human ES cells [95,163], as well as in human EG cells [164] and monkey parthenogenetic stem cells [165]. However, the derivation of purified populations of neural progeny from human ES cells required more systematic studies. A highly efficient protocol for the neural differentiation of human ES cells was based on a modified multistep EB approach [166]. ES cells are aggregated short-time (~4 days) and subsequently replated under serum-free conditions in the presence of FGF-2. Under these conditions, neural precursors can be readily identified based on the formation of multilayered epithelia termed rosettes. It was suggested that such rosettes might mimic neural tube like structures *in vitro*. These neural precursor cells can be enzymatically separated from the surrounding cell types and grown to purity under neurosphere-like conditions in the presence of FGF-2. Differentiated neural progeny derived from these neurosphere-like structures are neurons and astrocytes. The main neuronal subtypes are GABAergic and glutamatergic phenotypes [166] (see also [167]). A similar spectrum of differentiated progeny was obtained when human ES-derived neural precursors were obtained after spontaneous neural differentiation by overgrowth of undifferentiated cells [207]. Rosette-like structures were manually isolated under microscopic view and subsequently grown and passaged under neurosphere-like conditions. A third strategy that yielded differentiated cell populations enriched for neural precursors was based on a modified EB RA induction protocol, followed by lineage selection in attached cultures using serum free conditions and supplementation with bFGF [168].

Protocols that yield better control over neuronal subtype specification are still in development, but first examples have been provided for deriving midbrain-like dopamine neurons from non-human primate ES, [165,169,170]. Interestingly, PA6 mediated neural induction caused differentiation into retinal pigment epithelial cells [169] in addition to neural differentiation and the derivation of TH+ neurons. A bias towards eye differentiation was not observed with standard mouse ES protocols using stromal feeder cells. However, more systematic efforts have identified conditions that yield eye-like differentiation in mouse ES cells [171], suggesting that eye phenotypes can be obtained not only with human ES cells but also mouse ES cells.

One interesting difference between human and mouse ES cell differentiation is the presence of large numbers of rosette-like structures during the early stages of neural differentiation in human ES cells, while these structures are more rarely observed during mouse ES cell differentiation. Human ES cell-derived neural rosettes can be isolated and propagated *in vitro* as rosette (R-)NSCs [172]. R-NSCs express a set of specific markers such as PLZF that distinguish them from previously described NSC populations. Furthermore, early stage R-NSCs retain the

ability to adapt a broad range of region-specific fates in response to appropriate anterior-posterior and DV patterning cues [172]. However, those properties are unstable using currently described methods for R-NSC maintenance. A similar human ES-derived rosette-like cell has been described that retains developmental potential after long-term expansion [173]. Those long-term (lt-) NSCs are highly neurogenic even after extensive *in vitro* expansion and retain some limited patterning potential towards various midbrain/hindbrain lineages. However, in contrast to R-NSCs those cells appear to be unable to generate forebrain neuronal lineages.

A key breakthrough in the last few years was the identification of defined neural induction protocols in human ES and IPS cells. Based on the developmental principles, we have shown that inhibition of BMP signaling in combination with the inhibition of TGFb signaling yields neural fates at very high efficiencies and without the need for neural-inducing feeders or EB formation [174]. Those dual-SMAD inhibition conditions can be triggered using small molecules and have become a new standard in the field. We and other groups have adapted the dual-SMAD protocol to a wide variety of conditions and CNS and PNS lineages. Some of those key lineages include human ES cell-derived midbrain dopamine neurons [175], floor plate cells [176], neural crest lineages [153,174] and sensory neurons [174], motor neurons [177] or cortical forebrain neurons [178,179] to name only a few. The rapid progress in directing neural specification of human ES and iPS cell raises intriguing possibilities for their use in human disease modeling and in cell therapy.

THERAPEUTIC PERSPECTIVES

One of the driving forces behind deciphering the developmental program that controls cell fate specification is the hope that such insights could be harnessed for generating specialized cells for therapy and for human disease modeling. However, despite the excitement about the potential of ES and iPS cells for application in regenerative medicine, there are only a limited set of examples where human translation is currently being contemplated. Similarly, while there is a large body of work showing the feasibility of modeling human neural disease using iPSC technology [5], there are only few examples in the nervous system and beyond where this approach has yielded novel potential therapeutic targets. While a discussion on the current state of human neural disease modeling is beyond the scope of the current review, I will briefly summarize some progress in the area of cell therapies and specific applications in regenerative medicine

Parkinson's disease (PD)

One of the most widely discussed applications is the derivation of unlimited numbers of dopamine neurons from human ES cells for the treatment of Parkinson's disease. PD is particularly attractive for cell transplantation due to the relatively defined pathology that mainly affects midbrain dopamine neurons, and the largely unknown etiology that currently precludes causative treatment. At the onset of clinical symptoms, the majority of midbrain dopamine neurons have already died, providing further rationale for a cell replacement approach. The first ES cell-based study which showed functional improvement in 6OHDA lesioned rats, an animal model of PD, was based on the transplantation of low numbers of largely undifferentiated mouse ES cells isolated after short-term differentiation in EB cultures [205]. Spontaneous differentiation into large numbers of neurons with midbrain dopamine characteristics was observed. However, the clinical relevance of this approach is rather limited due to the high rate of tumor formation (>50% of the animals with surviving grafts developed teratomas).

Remarkable functional improvement was obtained after transplantation of dopamine neurons derived from mouse ES cells overexpressing Nurr1 [24]. This study was based on a multistep EB differentiation protocol. In addition to behavioral restoration in 6OHDA lesioned rats,

this study demonstrated *in vivo* functionality via electrophysiological recordings from grafted dopamine neurons in acute brain slices obtained from the grafted animals. However, transgenic expression of Nurr1 may complicate clinical translation and it does not necessarily yield an authentic midbrain dopamine neuron fate [180]. Functional recovery with dopamine neurons derived from naïve mouse ES cells has been reported in using mouse ES cells [21], nuclear transfer ESCs [21] and iPS [182] cells. Successful grafting of ntES-derived dopamine neurons provided a first example of therapeutic cloning in neural disease [21,172]. The differentiation of human ES cells into midbrain dopamine neurons was first reported using a stromal feeder-based approach [183]. However, transplantation studies of human ES cell-derived dopamine neurons has been hampered over the last few years by the poor *in vivo* survival/maintenance of the dopamine neuron phenotype. Only recently, we were able to develop a novel strategy for midbrain dopamine neuron induction [175] that is based on the dual-SMAD inhibition protocol [174]. Using this novel protocol, which generates midbrain dopamine neurons via a floor plate intermediate [176], we were able to report highly efficient *in vivo* survival of midbrain dopamine neurons in Parkinsonian mice, rat and rhesus monkey hosts [175] and very robust functional effects associated with behavioral recovery.

Several issues remain to be addressed before clinical trials with human ES cell-derived dopamine neurons should be initiated. Twenty years of fetal tissue research have demonstrated that fetal midbrain dopamine neurons can survive and function long-term (>10 years [184]) in the brain of Parkinson's patients. However, these studies have also shown limited efficacy in placebo-controlled clinical trials and demonstrated potential for side effects [185,186]. Stem cells will have to learn from the fetal tissue transplantation trials and better define and address the critical parameters that can take cell therapy in PD to the next level [120]. However, the ability to derive highly purified populations of authentic substantia nigra type dopamine neurons from human ES cells [175] is an important step on this road, and major efforts are geared toward clinical translation in the near future.

Huntington's disease (HD)

Similar to PD, the pathology in HD preferentially affects selective neuronal subtypes, particularly GABAergic medium spiny neurons, the main neuron subtype in the striatum. Fetal tissue transplantation trials [187–189] provide experience on how to design stem cell-based approaches for the treatment of HD. However, unlike in PD, grafted cells are required to reconnect, not to local targets within the striatum, but to project from the striatum to the targets in the globus pallidus and the substantia nigra pars reticulata. Data on whether fetal tissue grafts have been able to re-establish such long-distance connections in patients remains controversial. Recent studies have demonstrated the feasibility of generating medium-spiny type striatal GABA neurons from human ES cells [190–192] with solid evidence of *in vivo* survival and function [192].

However, transplantation into the HD brain remains daunting given the large distance in the human brain that would need to be reconnected by striatal grafts. Therefore it remains to be determined whether the main use of human ES and iPS cell-derived striatal neurons is cell therapy or in using those cells for modeling HD *in vitro*, an approach that has shown considerable promise.

Spinal cord injury and other motor neuron disorders

Traumatic or degenerative injuries to the spinal cord are often devastating and irreversible. Cell replacement using stem cells has been touted as prime application of stem cell research. However, the complexity of cell therapy in spinal cord injury is enormous and far from resolved. Motor neurons are one of the main cell types affected by spinal cord injuries and by various degenerative diseases such as ALS, Lou Gehrig's disease). The efficient

derivation of motor neurons has been reported from mouse and human ES cell sources, [25,193,194]. However, the integration of ES-derived motor neurons *in vivo* has been mostly addressed via xenografts into the developing chick spinal cord [25,135]. The application of ES-derived motor neurons in the adult CNS and in animal models of spinal cord injury or motor neuron disorders remains challenging with only a small number of cells surviving [194].

However, there is compelling evidence that neural stem cell population and oligodendrocyte precursor cells can engraft in animal models of spinal cord injury and induce functional improvement in the host animal. While the specific mechanisms of action in those studies remain to be determined [195], the field is rapidly moving towards testing those cells in early stages clinical trials given the devastating nature of the disease and preliminary evidence for the safety of those cells from *in vivo* studies.

Stroke

Cell replacement therapies for treating stroke are complicated by the fact that the cell types lost in the disease differ considerably from patient to patient depending on the exact location of the stroke within the brain. Nonetheless, several groups have pursued transplantation studies using ES-derived cells in stroke models. Early work has shown that grafted ES cells can survive in a rat stroke model induced by transient ischemia via occlusion of the middle cerebral artery. This study used non-invasive imaging to track cells by high resolution magnetic resonance imaging (MRI) after transfection with ultrasmall superparamagnetic iron-oxide particles [196]. More recent studies have focused on the use of primary or ES-derived neural stem cell populations and shown functional effects of neural stem cell grafts [197]. While the mechamism of action of those grafts are complex [198,199] and remain poorly understood, there are active ongoing efforts to translate those efforts into human patients.

Demyelination

The capacity of mouse ES-derived progeny to remyelinate *in vivo* was first demonstrated after transplantation of highly purified ES-derived glial progenitors into the spinal cord of *md* rats [104] that lack the X-linked gene encoding myelin proteolipid protein (PLP), an animal model of Pelizaeus-Merzbacher Syndrome. This study showed impressive *in vivo* differentiation results and yielded large grafts comprised of myelinating oligodendrocytes. However, the grafted cells were not able to extend the short lifespan of these animals, precluding detailed functional analyses. A second study demonstrated remyelination after grafting purified oligosphere cultures derived from 4−/4+ EBs into the spinal cord of shiverer mice or into the chemically demyelinated spinal cord [200].

In the case of human ES cell-based studies, there was a lot of excitement about the potential use of ES-derived oligodendrocyte precursors for applications in spinal cord injury with the idea to remyelinate denuded axons [201]. This study evolved into the first human ES cell-based clinical study in spinal cord injury patients. However, those studies are not being further pursued by the company involved (Geron), and the ability of those cells to remyelinate the human CNS remains to be determined. In fact those early human ES cell-derived oligdendorcyte precursors expressed very immature markers and did not remyelinate very efficiently *in vivo*. More recently, better defined protocols for generating oligodendrocyts were developed [202], but those protocols are difficult to scale and require up to 100 days of *in vitro* differentiation. Nevertheless, human ES-derived oligodendrocytes transplanted after long-term *in vitro* differentiation have shown remarkable *in vivo* remyelination efficiencies and induced functional recovery in shiverer mice [203]. Those studies indicate considerable promise for future clinical translation.

Other diseases

Several disease models that have been approached with fetal neural progenitors have not yet been tested using ES cell-based approaches. These include epilepsy and enzymatic deficiencies such as lyosomal storage diseases. Other CNS disorders such as Alzheimer's disease have been touted as future applications for ES cell therapy. However, at the current stage of research, the challenges for cell therapy in Alzheimer's disease seem to be overwhelming and it appears more likely that the role for ES cells might be in providing cellular models of disease rather than for cell replacement.

CONCLUSION

The development of protocols that allow the directed differentiation from ES and IPS cells to specific neural fates provides an essential basis for all PS cell-based approaches in neural repair. Over the last few years, directed differentiation protocols have rapidly progressed, and neural induction is now a routine procedure for studies in both mouse and human PS cells. In fact it is conceivable that over the next 5–10 years there will be strategies to generate any neural cell type of interest using a combination of directed differentiation protocols, transcription factor based programming and lineage selection. Access to all major neural lineages will greatly improve our ability to design rational cell-based strategies in regenerative medicine. However, those applications should remain limited to disorders that are amenable to cell replacement based on an understanding of the disease process. Some of the key examples include cell replacement in PD, eye disorders or demyelinating conditions. A potentially even broader range of applications for PS derived cell types is their use in human disease modeling. The ability to generate patient specific neurons from a myriad of neural disorders should offer novel insights into disease mechanisms and enable the development of cell-based pharmacological screens. Finally, one of the most important contributions is the use of PD-derived cells as a basic research tool that allows us to unravel step by step the complex signals that govern the development of single pluripotent ES cells to the amazing diversity of cell types that comprise the mammalian CNS.

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CHAPTER 62

Brain Implants

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INTRODUCTION

Even though regulatory approved cellular brain implants are not yet available, experimental data strongly support the use of brain implants and tissue-engineering concepts in the treatment of brain disorders and promise new disease modifying medical products for patients. Various cellular implants have already been applied in the clinic in proof-of-concept studies in Parkinson's disease (PD) [1], Huntington's disease (HD) [2], Alzheimer's disease (AD) [3], epilepsy [4] and stroke [5]. PD is a major target for brain implants and is highlighted in this chapter as a disease example to illustrate the tissue-engineering concepts applied to date.

Despite the longstanding success of many drugs for PD, such as L-dihydroxyphenylalanine (L-DOPA) therapy, the current treatments of PD do not stop the progressive dopamine neuron dysfunction and cell death. Over time, patients on chronic L-DOPA therapy develop both progressive symptoms and drug-induced side effects and require additional treatment options. In the early 1990s, surgical treatments initially developed during the 1950s, such as the ventrolateral pallidotomy for Parkinsonian rigidity, were rejuvenated [6]. With improvements in imaging and surgical techniques, ablative procedures have yielded excellent results in selected PD patients. Implantable neural stimulators, which inhibit neuronal transmission in local areas by high frequency stimulation, have more recently replaced much of the ablative therapies and can yield good therapeutic results without inducing permanent lesions in the brain [7]. Despite successful applications of neurosurgical procedures for the treatment of PD, these procedures are based on the inhibition or destruction of normal neurons to compensate for the disease damage. They do not address the biology of the underlying disease itself and, albeit successfully applied in many patients, the destruction or inhibition of normal tissue is not an optimal treatment for neurological disorders. There is therefore a need for new treatment strategies that can address the pathology more directly and offer disease modifying effects. Fortunately, the accumulated knowledge of the pathological processes, molecular and cell biology, biomaterials, imaging, and surgical procedures make it now possible to implement disease modifying tissue-engineering concepts to the treatment of PD and other neurological diseases.

In many untreatable neurological disorders, the progressive loss of neurons and their associated function is the primary underlying cause for the symptoms of the disease. Therefore, various cell implant strategies have been designed to either **replace** the neurons or their function or to **protect and/or regenerate** the function and health of the diseased neurons, or a combination of both (Fig. 62.1).

Clinical applications to replace the dopaminergic function in patients with PD have so far utilized primary tissues or cells. Dopaminergic neurons derived from stem cells and more sophisticated tissue-engineered implants have not yet been applied in the clinic but rapid

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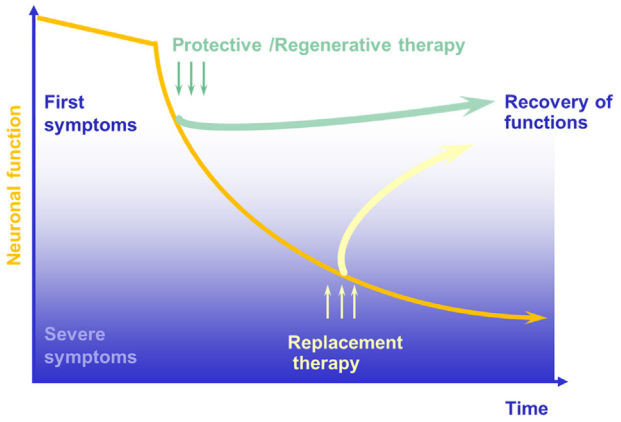

FIGURE 62.1

The concepts of cell replacement and protection/regeneration are depicted. A loss of neurons or their function (orange line) occurs with aging but in a disease, such as PD, neuronal loss is accelerated and after a while, the loss is significant enough to cause symptoms of the disease. At this stage, further deterioration could be prevented by protecting the neurons and the system could even be improved by applying regenerative factors to the diseased host cells. After significant loss of neurons, regeneration of the host system is not possible. However, the implantation of cells capable of replacing the function of host cells could be possible. Both approaches have been applied in Parkinson's disease and a combined treatment of neuroprotection/regeneration with replacement may be achieved in future brain implants.

progress in preclinical efforts is being made. In neuroprotective or restorative strategies, cellular implants secreting either endogenous or engineered growth factors or cytokines have successfully been applied in animal models and even in the clinic for amyotrophic lateral sclerosis (ALS) [8], Huntington's disease (HD) [9], and Alzheimer's disease (AD) [10–12].

This chapter reviews some of the cell replacement and regenerative brain implants applied in the clinic and touches on what may be developed in the future.

CELL REPLACEMENT IMPLANTS

Primary tissue implants

As mentioned above, oral L-DOPA therapy is the main treatment for PD. L-DOPA is a precursor to dopamine that passes the blood-brain barrier and is mainly taken up by the dopaminergic neurons that convert L-DOPA to dopamine and increase their dopamine production and storage. However, with the progressive loss of dopaminergic neurons, the L-DOPA therapy eventually becomes ineffective and severe fluctuations in the ability to initiate movements occur. Because L-DOPA can increase the production of dopamine and alleviate the symptoms of PD, a reasonable therapeutic approach may be to implant dopamine or L-DOPA secreting cells in the relevant areas of the brain (striatum).

Considering this idea, the first clinical transplantation for PD using a cellular brain implant was performed at the Karolinska Hospital in Stockholm, Sweden in the early 1980s [13]. Autologous dopamine-secreting adrenal chromaffin cells were harvested from one of the patient's adrenal glands and successfully transplanted to the striatum. The procedure was adopted very quickly by the medical community and initial reports indicated good clinical results. With time though, other studies showed poor survival of the cells and minimal positive clinical effects, resulting in the cessation of the treatment [14]. However, the concept of cellular brain implants had made its definite entry into the clinic and many lessons were learned along the way.

At about the same time as the first chromaffin cell transplants were made in Stockholm, a promising cell transplantation strategy for PD was developed by Anders Björklund and co-workers at Lund University in southern Sweden [4]. They collected discarded aborted fetal

tissue and dissected out the ventral mesencephalon to create cell suspensions containing developing dopaminergic neurons for transplantation experiments. After several years of extensive validation of the concept in animal models, cells were transplanted to the striatum of two PD patients [15]. The first results were safe but relatively unimpressive prompting modifications to various parts of the experimental procedure, and a second pair of patients transplanted about one year later fared much better [16]. These patients showed positive clinical recovery starting about four months after the procedure. Positron emission tomography (PET) data indicated that the grafts survived and took up and secreted dopamine. More than 10 years after the procedure, one of the patients showed persistent graft viability on PET scanning and required only minimal L-DOPA therapy [17,18]. To date, more than 300 patients have been transplanted with fetal ventral mesencephalic tissue at different centers around the world with some encouraging results. However, the lack of suitable donor material, the heterogeneity of the tissues and preparations, and the inability to industrialize the process, have all made it difficult to make standardized medical trials and therapy geared for a large number of patients. Two controlled trials with fetal transplantation showed only minimal efficacy and some patients developed movement side effects (dyskinesias) that appear related to the grafting procedure [19,20]. More recently, it has been observed that grafted fetal neurons may be able to survive for many years after surgery but these same grafted neurons are subject to the same pathological processes that underlie the loss of endogeneous dopaminergic neurons in PD [21]. Therefore, fetal transplantation as a therapy for PD is no longer actively pursued and cell replacement strategies for PD are awaiting alternative cells and implants that lend themselves to a reproducible industrial process applied in well controlled trials. It also should be noted that clinical trials with porcine-derived ventral mesencephalic cells that did lend themselves to a more rigorous industrial process also failed in clinical trials (Pollack, 2001). With concerns regarding the transmission of animal diseases to humans (zoonosis), few if any additional clinical applications of animal-derived cellular brain implants are being contemplated. In fact, the use of animal-derived products in the manufacturing of human brain implants should be avoided to the largest degree possible to minimize safety concerns. Despite disappointing results in the clinical application of both chromaffin and fetal derived primary tissues from both human and pig sources, the experimental work surrounding primary tissue transplantation in PD demonstrated several important points that continue to facilitate the development of tissue-engineered implants for the treatment of PD and other neurological disorders.

1) Allogeneic cells can survive over many years in the brain after a relatively brief initial immunosuppression therapy (18 months) but xenogeneic (porcine) implants do not.
2) Grafted neurons can integrate, function, and interact with the host brain in a physiological and reciprocal manner.
3) Trial designs and outcome measures have been developed that facilitate safety and efficacy measures in the clinic.
4) Surgical techniques have been developed that allow for the safe injection and implantation of cells and tissue-engineered products in the brain.
5) Imaging techniques have been developed to evaluate the implantation and function of the brain implants.
6) Animal models have been developed to translate and scale-up experimental brain implants for the clinic.

Cell line implants

As mentioned above, important drawbacks using primary tissues are its limited supply, its heterogeneity, and the difficulty and prohibitive costs to implement the necessary good manufacturing principles (GMP) for harvesting, manipulation, storage, and later use. For example, fetal transplantation experiments for PD required fresh tissue from 4–8 donors resulting in procedural difficulties and poor quality control and may help explain the poor

and variable efficacy outcomes in controlled trials. Therefore, the ability to expand and store cells in cell banks is paramount to creating allogeneic cell alternatives to primary tissue sources. The expansion of normal and genetically unmodified cells derived from tissue donations can give rise to expandable primary cell lines potentially useful for brain implantation with or without further manipulation. The primary cell lines normally retain a limited number of cell cycles but allow for the proliferation of enough cells to transplant hundreds to thousands of patients from a single donor. Cultured primary cells can be expanded while retaining normal genotypes and phenotypes with normal contact inhibition and differentiation behaviors. These cells are therefore relatively safe to use, and the formation of tumors or other abnormal behaviors are relatively unlikely.

Retinal pigmented epithelial (RPE) cells derived from the retinas of organ donors can make primary cell lines with limited expansion capacity and have been evaluated in clinical trials for PD. The RPE cells secrete L-DOPA and are thought to function by increasing the intrastriatal L-DOPA concentration and subsequent conversion to dopamine by residual dopaminergic nerve endings and glia. These cells are grown and transplanted on gelatin microcarriers to improve survival and prevent immune rejection. A report on a clinical pilot trial showed that these implants were well tolerated and safe [22]. However, a recently completed Phase II trial failed to reveal any evidence of efficacy [23] and earlier published autopsy results demonstrated poor cell survival at six months [24].

A more versatile source of expandable primary cell lines capable of making various replacement neurons or glia are mitogen responsive human neural progenitor/stem cell cultures that can be isolated from various regions of aborted and adult human central nervous system (CNS) tissues and expanded for more than one year *in vitro*. These cells are often grown as 'neurospheres' and can be expanded in defined and animal-free media containing growth factors [25]. These cells can form the three major phenotypes of the nervous system (neurons, astrocytes, and oligodendrocytes) *in vitro* and *in vivo* and show excellent survival without the formation of tumors *in vivo*. These human neural stem cell-containing cultures have been transplanted to various regions in animals and survive, integrate, migrate, differentiate, extend neurites, and arborize [26]. Even though the cells tend to retain the markers consistent with the anatomical region from which they were isolated [27], the cells can be manipulated with epigenetic and genetic factors to make specific cellular subtypes potentially useful for cell replacement implants. For a PD application, relevant nigral dopaminergic neurons need to be generated from these cells. However, so far only dopaminergic neurons with limited differentiation have been generated [28] although improvements are consistently being made [29].

In addition to neurospheres, adherent primary cell lines of neurogenic glia have been made from both mouse and human developing neural tissues that display a glial phenotype during expansion but are capable of generating neurons during differentiating conditions [30]. Similarly, so called NS cells, expressing glia and stem cell markers have been derived from ES cells and appear to differentiate into similar neural phenotypes as the adherent glia and neurosphere cultures [31]. The adherent cultures may have an advantage over neurospheres from an industrial point of view as adherent cells can be readily cloned, expanded, and have shown to retain their phenotype during expansion without differentiating into a heterogeneous mixture of progenitors. It is currently unclear if neurospheres or adherent NS cells can be made into dopaminergic neurons suitable for replacement therapy in PD. Reportedly, clinical applications in Batten's disease with unmanipulated human neurosphere cell lines are currently being explored [32].

In general, all primary cell lines derived from tissue stem cells have a large but limited expansion potential and show senescence [33,34]. This may be due to the successive loss of immortal stem cells through the asymmetric division into progenitors (as seen in neurospheres) or alternatively, the stem cells themselves have a limit to their proliferation.

Both mouse and human ES cells defy the normal senescence of primary cells and can be expanded from a single clone indefinitely without losing pluripotentiality [35]. From an industrial and tissue-engineering perspective, this feature is extremely attractive as a single donation could give rise to a cell line source with the capacity to make all organs of the body in unlimited numbers. One major drawback of ES cell-derived products, however, is that the ES cell itself cannot be implanted but needs to undergo the relevant development *in vitro* to make suitable cells for transplantation, e.g., dopaminergic neurons for PD or islet cells for diabetes mellitus. As it is difficult to make pure cultures without retaining one or more undifferentiated ES cell, both the risk of heterogeneous cell preparations and the risk of tumorigenesis need to be overcome to develop ES cell-derived brain implants [36]. For neural applications, the recently described NS cell may become a progenitor of choice for brain implant products as it is restricted to the neural lineage and can be expanded as a clonal cell line. It is currently unclear if the undifferentiated NS cell is immortal like its parental ES cell or if it displays senescence as its neurosphere counterpart.

Cell replacement strategies using cell lines need to employ differentiation or selection methods in order to make the relevant replacement cell, e.g., dopaminergic neurons for a PD application. The generation of functioning human dopaminergic neurons *in vivo* akin to those derived from primary VM tissues has been difficult to achieve. Although neurons with the dopaminergic machinery can be made from both growth factor expanded human neurospheres and genetically immortalized committed dopaminergic neuroblasts, it has been difficult to achieve survival and function *in vivo* [28]. Interestingly, even though ES cells would hypothetically need more steps to be differentiated into dopaminergic neurons for a PD application, relatively short-step protocols that use developmental signals involved in the rostrocaudal and ventrodorsal specification of the midbrain can push mouse ES cells into functional dopaminergic neurons in a rat model of PD [37,38]. Studies have also identified important transcription signals involving the Lmx1a and MSX homeobox genes that when overexpressed in ES cells under the nestin promoter can yield dopaminergic neurons with markers consistent with substantia nigra neurons [39]. Transplantation of these dopaminergic neurons in a rat model of PD yields excellent survival, neurite extension, and function consistent with results from primary VM tissues. However, similar to the human ES cells [36], these cells also form tumors *in vivo*. These findings may pave the way forward to make relevant 'nigral' dopaminergic neurons from human ES cells and neural stem cell cultures in the not too distant future but also shows the need for adult and fetal derived neural stem cell sources that do not form tumors.

CELL PROTECTION AND REGENERATION IMPLANTS

The use of primary tissues or cell lines in cell replacement approaches are aimed at making transplantable mimics of the cells lost in the disease process. In PD, the use of chromaffin cells, dissected developing ventral mesencephalon, RPE cells, or stem cells have all been aimed at replacing or augmenting the dopaminergic function. However, primary tissues, cell lines, and genetically modified cells also produce secreted factors that can influence the nearby host or transplanted cells in potentially beneficial ways. Several growth factors are endogenously made by cells including fibroblast growth factors, transforming growth factors, and interleukins that can have neuroprotective and regenerative effects on nearby nerve cells. Custom therapeutic cell lines can also be made by genetic engineering to secrete specific growth factors such as nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) that when implanted in relevant anatomical areas can affect specific neuronal populations in neuroprotective and regenerative ways (*ex vivo* gene therapy). To protect the transplanted cells from immune rejection and to allow for the retrieval of the therapeutic cell implants an encapsulated device can be used.

Cell implants secreting endogenous factors

A carcinoma cell line derived from a human testicular teratocarcinoma isolated from a metastasis in a patient has been used in clinical applications to treat the neurological sequelae of stroke [40]. This immortal cell line is pluripotent and can be induced to stop dividing and to differentiate into a neuronal phenotype using retinoic acid. A preparation of this cell line was investigated in the clinic for the treatment of ischemic stroke based on animal data suggesting that the post-injury transplantation of this cell line into an infarcted area can improve recovery. The mechanisms surrounding this effect are unclear but are likely related to beneficial factors released from the cells. In a study in patients with lacunar stroke in a randomized controlled Phase II trials at the University of Pittsburgh, USA the therapy with this cell line failed to meet the efficacy endpoints. The transplantation of a cell line derived from a human cancer has obvious risks associated with it. Importantly, the approval of this trial demonstrates that cell transplantation for severe neurological disorders is seen as a reasonable strategy by the regulatory agency, as long as safety and some efficacy can be demonstrated in animal models.

Other groups are investigating the transplantation of immortal cell lines but are using genetic engineering to immortalize cells. Advances in genetic engineering have made it possible to extend the number of doublings a primary cell line can go through by inserting various oncogenes and cell cycle regulators. This allows for the selection, clonal expansion, and banking of a large number of cells. Besides the genetic modification, these cells retain otherwise normal genotypic characteristics. ReNeuron, a British biotechnology company, has made immortal human neural stem cells that have shown regenerative effects in stroke models [41] and have initiated clinical trials in stroke patients [42]. Similarly, StemCells Inc., is using growth factor expanded human neurosphere cell lines in a strategy to treat Batten's disease, a rare neurometabolic disorder, with the idea that the endogenous enzymes and factors made by the stem cells will have a therapeutic effect [32,42]. Lastly, implanted autologous mesenchymal stem cells are being studied in clinical trials for stroke [43] and potentially in neurodegenerative disorders [44].

The use of non-specific neuroprotective and regenerative strategies based on the implantation of cells with unclear mechanisms may pose regulatory problems as the risk benefit analyses become difficult to make. For example, even though positive results were inferred from the published trial with the human teratocarcinoma derived cell line in stroke, the clinical data were not convincing enough to continue clinical development [45]. In this trial, no significant adverse events or tumors were reported but, if it they had occurred, a major set-back for tissue-engineered brain implants could have been the result. The risk benefit analysis is often difficult and the predictive value and scale-up issues by the use of animal models are not straight forward. The regulatory agencies have therefore a real dilemma and, similar to the setbacks experienced in the field of gene therapy, a push to do clinical trials with poorly characterized cell preparations and mechanisms in patients desperate for a treatment may cause significant adverse events that can create set-backs for the whole field of tissue engineering. On the other hand, a too restrictive regulatory body may make the hurdle of bringing potentially beneficial but complex tissue-engineered products into the clinic too costly and difficult. These regulatory issues are hard to resolve but as experience with cell-containing implants build, it is likely that the decision making and risk benefit analyses will improve. Some of the clinical trials using primary autologous cells such as hematopoietic or mesenchymal stem cells derived from the bone marrow can also by-pass regulatory scrutiny and only need approval by a local ethics committee. Unfortunately, this has led to the initiation of clinical trials based on very little evidence of preclinical beneficial effects causing potentially false hopes, personal expenses, and potentially harmful side effects to patients desperate for therapy.

Cell implants secreting engineered factors (*ex vivo* gene therapy)

As PD involves a slow and progressive degeneration of dopaminergic neurons a protective and/or regenerative strategy could be applied. Many protein factors have been shown to

protect fetal dopaminergic neurons both *in vitro* and *in vivo* and one of the most powerful factors is glial cell line-derived neurotrophic factor (GDNF) [46]. This factor promotes the survival (neuroprotective effect) and neurite extension (regenerative effect) of dopaminergic neurons both *in vitro* and *in vivo*. Based on positive animal data, GDNF has been tried in humans in two separate randomized clinical trials [47,48]. The first trial used monthly intracerebroventricular bolus injections of GDNF and failed to meet both safety and efficacy endpoints. The second trial employed intrastriatal infusion of GDNF and albeit safe, did not meet the efficacy endpoints. This is an unfortunate outcome for patients suffering from PD and critics of the trial have indicated that inconsistencies in catheter design and other parameters may explain the poor results [49]. From both the clinical and animal data, it appears necessary to deliver the GDNF in low but chronic doses to the dopaminergic nerve endings within the striatum in order to have a regenerative effect. It is currently the view of many that implantation of genetically modified cells, encapsulated cells, or the use of viral vectors may be better at delivering GDNF than utilizing available pumps and catheters. Safety concerns with unregulated and unstoppable gene therapeutic approaches may favor the use of a tissue-engineered product based on an implantable and retrievable encapsulated cell biodelivery of GDNF. Of note, however, Ceregene, inc. has completed a Phase II clinical trial demonstrating safety of AAV-mediated delivery of Neurturin (a GDNF analog) in PD patients [50] and has recently initiated a follow on trial. It is beyond the scope of this chapter to expand on the topic of *ex vivo* and *in vivo* gene therapy but it is important to mention that the delivery of growth factors to the brain via implants is an important cornerstone of tissue-engineering strategies for brain repair.

Encapsulated cell implants

The implantation of naked cells has the advantage of allowing for migration, integration and the formation of neurites and synapses in replacement strategies. The migration and homing mechanisms that neural stem cells display in models of stroke and glioma may also be utilized to deliver regenerative or tumoricidal agents respectively in genetically modified cells. However, naked cells cannot readily be removed and if a potent protein factor is being delivered to the brain, the inability to stop the treatment may pose a problem if untoward effects are noted or if the regenerative treatment is only needed for a limited amount of time. A device containing encapsulated cells that secrete the factor combines the advantages of cell and gene therapy with that of the safety of a retrievable device. One of the types of encapsulated cell devices is depicted in Fig. 62.2 and consists of a hollow fiber membrane that surrounds a core of cells seeded on a polymer matrix that in turn is attached to a tether.

These encapsulated cell implants are true tissue-engineered devices that combine genetically modified cell lines with artificial scaffolding enclosed behind an immunoprotective membrane. The polymeric membrane excludes larger molecules and cells but allows for the bi-directional passage of nutrients and transgene products. The encapsulated cells can thus be protected from immune rejection, making allogeneic or even xenogenic transplantation possible and immunosuppressive therapy unnecessary. The host is also protected from the implanted genetically modified cells and the risks of gene transfer or tumor formation are greatly diminished. The tether allows for handling, implantation, and removal. These devices can be implanted intraparenchymally, intracerebroventricularly, or intrathecally depending on the application. Cellular survival and continuous production of factors have been demonstrated for at least 12 months in the brain allowing for long-term delivery of therapeutic factors [11,51]. Encapsulated devices secreting GDNF have been studied in rodent models for PD and have shown both neuroprotective and neuroregenerative effects on dopaminergic cells [52]. A recent Phase I trial in Alzheimer's patients was completed demonstrating safe and targeted delivery of NGF to the brain using encapsulated, genetically modified ARPE-19 cells. The cells survived for up to12 months with no evidence of inflammation or device displacement [10,11].

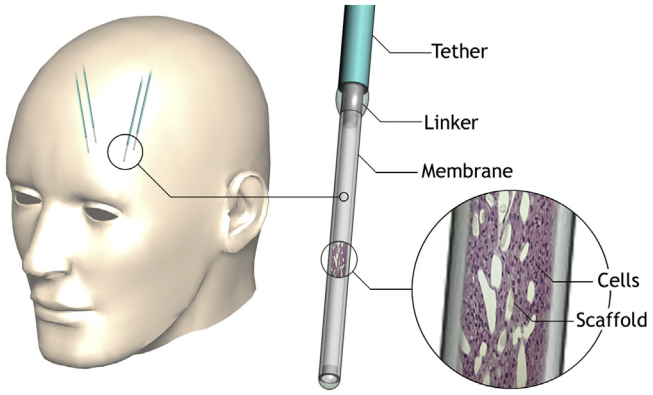

FIGURE 62.2

A schematic of the implantation of four encapsulated cell implants in the human basal cholinergic forebrain is depicted. The tip of the implant houses a human immortal cell line engineered to secrete the therapeutic protein (in this particular application NGF). The cell line is grown on a three dimensional synthetic scaffolding and the cellular core receives nutrients and oxygen from the surrounding brain interstitial fluid via the semipermeable membrane. In turn, the therapeutic protein can diffuse from the implant tip in a few millimeter wide radius into the surrounding brain at the implant target and the associated diseased neurons. The membrane protects the engineered cells from immune rejection and the entire device can be removed or replaced through the use of the tether anchored beneath the skin at the skull bur hole level. The sections are stained with hematoxylin and eosin. The implant measures approximately 1 mm in diameter.

Other investigators have published data on using encapsulated choroid plexus cells in the treatment of Huntington's disease [53] and PD [54]. These cells are reportedly therapeutic by secreting various endogenous factors that have neuroprotective and regenerative effects. The application of the choroid plexus cells in this study was made with an injectable micro-encapsulated cell configuration. In this setting, the encapsulation provided immunoprotection for the porcine-derived primary choroids plexus cells. Unlike the macro-encapsulation described above, these devices would not be retrievable and may be less suited for applications in which the treatment may need to be stopped.

Controlled release implants

Acellular synthetic polymeric brain implants that are able to deliver protein factors or other drugs to the CNS have also been developed [55,56]. These systems normally release drugs by degradation- or diffusion-based mechanisms over an extended time (weeks) but cannot sustain release over a long time (months), which is possible with cellular-based systems.

Appropriately designed, polymeric controlled release devices have several possible applications and could for example support the survival and integration of transplanted cells. Furthermore, a polymeric system can support the sequential release of growth factors that may be necessary to fully support the stepwise differentiation of immature cells. This concept could become applicable to transplanted neural stem cells that may lack important embryonic developmental signals in the adult brain.

COMBINED REPLACEMENT AND REGENERATION IMPLANTS

From a tissue engineering point of view, the future goal is to make replacement organs for the body that can take over the function of a failed organ or structure in an anatomically and physiologically correct manner. And even though it would be difficult if not impossible to make entirely new brains, it should become possible to not only replace cells but also to make new axonal pathways and restore the correct connections.

The transplantation of fetal dopaminergic cells to the striatum is called heterotopic transplantation. This means that the dopaminergic cells are transplanted into an anatomical region different from their normal location, which is the substantia nigra. The heterotopic implantation of dopaminergic neurons may result in the loss of important normal innervation and feedback loops. Many transplants for PD may thus only work as simple cellular pumps that increase the striatal dopamine levels. Although simplicity is desired, an ultimate strategy to treat PD could be to transplant the dopaminergic neurons to their anatomically correct position (homotopic), regenerate the nigrostriatal axonal pathway, and induce terminal sprouting and innervation of the striatal target neurons. This would regenerate the appropriate connections and represent a more physiologic strategy.

An initial approach may be to provide survival factors to the implanted cells. Even in the most optimal VM grafts applied heterotopically in PD, the total fraction of surviving dopaminergic neurons was only about 10–20% [57]. This required a large number of donors (4–8) to assure enough surviving cells for a clinical effect. The combination of VM grafts with a neuroprotective and regenerative effect of GDNF is therefore a logical idea. Experimental data indeed show that the application of GDNF delivered by encapsulated cells in combination with either rat or human VM grafts increase both the survival, neurite extension, and innervation of the striatum in a rat model of PD [58,59]. Similarly, it would be expected that GDNF would have survival and regenerative effects on dopaminergic cells derived from ES or other stem cells when placed *in vivo*. A combined approach with dopaminergic grafts and encapsulated cell implants secreting GDNF may therefore be contemplated in future transplantation studies in PD.

A large challenge for tissue-engineering approaches in the treatment of neurological disorders is the regeneration of axonal pathways. Axons between the cell bodies and their targets often extend for several centimeters in the brain and close to one meter between the brain and the lumbar spinal cord neurons in an adult. Compared to the relatively short distances that the axons had to grow during development, the regeneration in the adult may pose a particular challenge. Fortunately, science has made progress in this area and what was though impossible only a few years ago now seems more feasible. Several molecules are now known to both promote and guide axonal outgrowth. As mentioned, GDNF is a strong promoter of axonal outgrowth of dopaminergic neurons. In addition, certain extracellular matrix (ECM) proteins, such as laminin, can guide axonal outgrowth [60] and extensive nigrostriatal reconstruction has been accomplished using bridges of striatal tissues in combination with fetal mesencephalic grafts placed in the substantia nigra [61]. The finding that the central myelin and glial scars are inhibitory to axonal outgrowth, has led to the identification of various inhibitory molecules that can be manipulated in various ways with inhibitors and enzymes [62]. From a tissue engineering point of view, the combination of replacement cells with regeneration channels or scaffoldings capable of releasing survival and neurite promoting factors and coated with molecules that facilitate axonal outgrowth, may thus become a future reality. In combination with nanotechnology, synthetic bridges can be made that promote extensive fiber regeneration and functional restoration [63].

Even though there mounting data show that axonal bridges can improve axonal growth in animal models, these methods have not been applied to humans. As the cellular building blocks become better refined, it is likely that more 'true' tissue-engineered brain implants will enter the clinic. These types of implants could have great potential use for regeneration in many areas of the CNS, particularly the spinal cord.

DISEASE TARGETS FOR BRAIN IMPLANTS

As mentioned, PD has been a major target for cellular brain implants. However, many other neurological disorders should become amenable to tissue-engineered implants.

In Huntington's disease (HD), several neuronal populations slowly degenerate and cause the clinical signs of choreiform movements and progressive dementia. HD is inherited as an autosomal dominant disease and the responsible mutation has been located to chromosome 4. Carriers of the disease can therefore be screened for and identified before the onset of symptoms. This makes a neuroprotective strategy for HD an attractive possibility, where the delivery of neurotrophic factors could prolong the symptom-free interval. One such factor is CNTF that protects striatal neurons in both rodent and non-human primate models of HD [64]. Considering these results, a small clinical proof-of-concept trial using an intracerebroventricularly placed encapsulated cell device secreting CNTF was completed [9]. The study indicated that the placement was safe and would warrant additional trials. However, data also suggested that improved devices and an intrastriatal implantation approach may improve the efficacy. This approach may be taken in future trials.

Cell replacement strategies have also been tried in HD [2]. Primary fetal striatal tissue transplantation for HD has been performed at a handful of centers in the world. Long-term follow up has described mild improvements in some of the implanted patients [65]. Because the number of implanted patients is few, it is currently difficult to draw any major conclusions regarding the clinical efficacy of transplantation for HD. One possible advantage over PD is that the transplantation for HD involves homotopic implantation, which at least theoretically should allow for the differentiation of the fetal tissue using normal environmental cues. However, in HD, multiple sets of neuronal populations degenerate including both cortical and striatal neurons. The homotopic transplantation for HD may thus require more extensive regeneration of axonal pathways than in PD.

Other diseases that could be amenable to the implantation of cells within the brain are the myelin disorders. Animal experiments have shown the ability of neural tissue, purified oligodendrocytes, oligodendrocyte precursors, immortalized glial cells, and neural stem cells to remyelinate areas of demyelination [66].

One substitute for oligodendrocytes may be to transplant growth factor expanded Schwann cell (the myelinating cell of the PNS). This is based on the fact that patients with CNS myelin disorders do not display demyelination of the peripheral nervous system. In fact, remyelination of central axons may spontaneously occur by ingrowth of Schwann cells from the periphery. An intriguing therapeutic possibility is therefore to grow Schwann cells from a nerve biopsy and expand these cells in culture. In turn, these cells could be transplanted into demyelinated areas in the same patient. Physiologically, this may not be the best strategy as Schwann cells only myelinate single axons, whereas an oligodendrocytes myelinate multiple axons. However, animal data indicate that, for a limited volume, Schwann cells can remyelinate and restore function to central demyelinated areas. Based on the animal data, a small clinical study was performed. Reportedly, the Schwann cell transplants did not survive but the procedure appears to be safe [67]. It is currently unclear if new trials with Schwann cells are to be expected.

One of the most common neurological disorders is epilepsy that affects about 1−2% of the population. Epilepsy is characterized by recurrent abnormal electrical discharges in the brain affecting subparts of the brain or generalizing to deeper parts in the brain resulting in unconsciousness. A subgroup of these patients has temporal lobe epilepsy that is generated by a loss of neurons and an imbalance of inhibitory and excitatory neurotransmitters in the hippocampal formation. In medically intractable cases, this disease can sometimes be treated surgically with the removal of the medial hippocampus and the abnormal area. This procedure eliminates or reduces the frequency of seizures in selected patients but involves a major surgical procedure and the ablation of normal tissue. A less invasive procedure may be to implant inhibitory cells in the seizure focus that would raise the seizure threshold [4]. This idea is supported by animal experimentation data that indicate that locus coeruleus grafts and the local delivery of inhibitory substances such as GABA can increase the seizure threshold.

However, with the lack of suitable cells, such as GABA producing cells, transplantation for focal epilepsy has not been as extensively studied as for some of the aforementioned disease targets. Recent preclinical studies have suggested that interneuron precursor cells derived from the medial ganglionic eminence may be a source of inhibitory GABAergic neurons perhaps providing a useful source of transplantable cells for epilepsy [68].

Other disease indications that may benefit from brain implant strategies include stroke, brain injury from trauma, Alzheimer's disease, and rare disorders such as cerebellar degeneration and inherited metabolic disorders. Besides the brain, the spinal cord and retina are potential targets for similar approaches.

SURGICAL CONSIDERATIONS

The surgical implantation of most brain implants involves the use of stereotactic techniques. The stereotactic method (stereotaxis) in brain surgery was established in the beginning of this century and is now well established in neurosurgical practice [6]. It involves attaching a rigid frame (stereotactic frame) to the skull followed by imaging such as MRI. Attached markers (fiducials) create a three-dimensional coordinate system in which any point in the brain can be defined and related to the frame with high precision. In the operating room, the markers used during imaging are replaced with holders that guide the instruments. It is a relatively simple neurosurgical procedure often done under local anesthesia and mild sedation. The procedure is therefore safe and relatively painless. The patients are usually discharged from the hospital after an overnight observation.

CONCLUSIONS

In this chapter, various brain implants have been described that may have potential to treat PD and other neurological disorders using tissue-engineering strategies. Most of the current literature describes the transplantation of various primary cells such as fetal tissue. Tissue-engineering principles and cell lines have only more recently been introduced. Applications using growth factor support, genetic engineering, scaffolds, extracellular matrices, and encapsulation have all been able to improve the survival and function of the brain implant. The ultimate implants are yet to be developed and may combine stem cells, genetically modified cells, controlled delivery devices, axonal bridges, scaffolds, and encapsulated cells.

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CHAPTER 63

Brain-Machine Interfaces

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INTRODUCTION

The use of brain signals for interaction as well as for controlling robots and prosthetic devices has gained increasing attention over recent years. This is a rapidly emerging field of multi-disciplinary research called *brain-machine interfaces* (BMI), or *brain-computer interfaces* (BCI), which has seen impressive achievements over the past few years —prototypes for writing messages with a virtual keyboard [1—5], playing brain games [3,6—8] and even controlling robots or wheelchairs [9—15]. A BMI monitors the user's brain activity, extracts specific features from the brain signals that reflect the intent of the subject, and translates their intentions into actions — such as closing the prosthetic hand, or selecting a letter from a virtual keyboard — without using the activity of any muscle or peripheral nerve. The central tenet of a BMI is the capability to distinguish between different patterns of brain activity, each being associated to a particular intention or mental task. Hence, adaptation is a key component of a BMI, because, on the one hand, users must learn to modulate their brainwaves so as to generate distinct brain patterns, while, on the other hand, machine learning techniques need to discover the individual brain patterns characterizing the mental tasks executed by the user. This chapter provides an introduction to the field of BMI, with a particular focus on principles for reliable and long-term operation of neuroprostheses. For a more detailed coverage of BMI, the interested reader can refer to references [16—18].

BMI technology offers a natural way to augment human capabilities by providing a new interaction link with the outside world. In this respect, it is particularly relevant as an aid for patients with severe neuromuscular disabilities, although it also opens up new possibilities in human-machine interaction for able-bodied people. Fig. 63.1 shows the general architecture of a brain-actuated device such as neuroprostheses for motor restoration and recovery. Brain activity, electroencephalogram (EEG) signals in this example, is recorded with a portable device. These raw signals are first processed in order to extract some relevant features, which are then passed on to a mathematical model (e.g., statistical classifiers/regression or neural networks). This model computes, after some training process where it finds the prototypical patterns of brain activity associated to each mental command, the user's intention, and this is transformed into an appropriate action to control the device. Finally, visual feedback, and maybe other types of feedback such as haptic stimulation [19] or intracortical microstimulation [20,21], informs the subject about the performance of the neuroprosthesis so that they can learn appropriate mental control strategies and make rapid changes to achieve the task.

This chapter is organized as follows. First, we will review the different kind of brain signals that can be recorded as input for a BMI. Then, we will discuss a series of principles to build efficient BMIs that are independent of the particular signal of choice. These principles concern the

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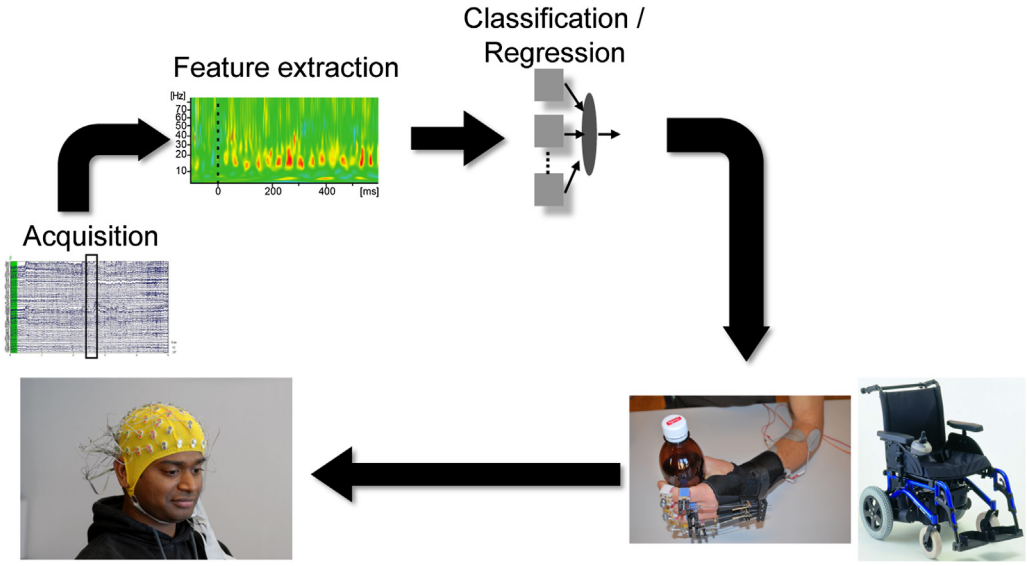

FIGURE 63.1

General architecture of a brain-computer interface (BMI) for controlling devices such as a hand orthosis, or a motorized wheelchair. In this case the BMI measures electroencephalogram (EEG) signals recorded from electrodes placed on the subject's scalp.

nature of electrical brain correlates more suitable to control neuroprosthetic devices and to promote motor rehabilitation, the use of machine learning techniques, and the design of context-aware BMIs. We will conclude discussing some future research directions in the field of BMI.

BMI SIGNALS

A BMI may monitor a variety of brain signals, such as electrical, magnetic or metabolic. Magnetic fields can be recorded with magnetoencephalography (MEG), while brain metabolic activity — reflected in changes in blood flow — can be observed with positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and near-infrared spectroscopy (NIRS). Unfortunately, MEG, PET and fMRI require sophisticated devices that can be operated only in special facilities. Moreover, metabolic signals have long latencies and thus are less appropriate for interaction. Brain electrical activity, on the other hand, can be recorded with portable devices and has an excellent time resolution — we can detect changes in brain activity at the millisecond range — what makes it the natural candidate for a BMI. We can record electrical brain activity invasively or non-invasively. The former technique employs microelectrode arrays implanted in the brain which record the activity of single neurons (single unit activity, SUA) or from small neuronal populations (local field potentials, LFP). The overall concerted activity of neuronal populations can also be recorded (semi-) invasively with electrodes placed on the surface of the brain (electrocorticography, ECoG). Non-invasive electroencephalographic (EEG) activity is recorded from electrodes placed on the scalp. It measures the synchronous activity of thousands of cortical neurons. LFP, ECoG and EEG signals are similar in nature, although at different levels — microscopic, mesoscopic and macroscopic, respectively.

Invasive approaches (LFP and SUA) produce very detailed information, what may allow decoding of complex movement intentions [12,20,22–26]. However, we need to record from many electrodes and different areas to capture the global set of motor parameters, which are encoded in a rather distributed brain network. Also, implanted microelectrode arrays damage brain tissue, and so signals do not last long. Consequently, one area of active research is

the design of safe biophysical interfaces that, in addition, should be ultralow power and wireless. On the other hand, since they are recorded on the scalp, non-invasive EEG signals suffer from a reduced spatial resolution and increased noise. In particular, EEG can be contaminated with muscular — such as subtle facial movements — and ocular artifacts that, ideally, must be filtered out. Alternatively, we must ensure that the selected control features are proper brain signals and not generated partially, or completely, by non-brain sources.

The spatial resolution of EEG can be greatly improved by means of spatial filters that estimate the electrical activity originated in radial sources immediately below each recording electrode. Such filters are known as surface Laplacian, and require a large number of electrodes and a spherical spline model. Other examples of spatial filters are common average reference (CAR) and Laplacian, which compute an approximation of the surface Laplacian. The former removes, for each electrode, the average activity over all electrodes. The latter subtracts, for each electrode, the mean activity of its four nearest neighboring electrodes. The use of spatial filters should be an essential step in the design of an EEG-based BMI, as they have largely been shown to yield significant increases in performance. Another way to increase the spatial resolution of raw EEG is to estimate the cortical activity from the scalp EEG, thus unravelling the contributions of different small cortical areas that are picked up and mixed by a single scalp electrode [27,28].

Analysis of human ECoG is attracting significant interest as, being semi-invasive, it blends the advantages of both approaches: high spatial resolution and simple surgery with minimal health risks. Although not largely used because of limited access to patients — who undergo neurosurgery for epilepsy or brain tumor — during short periods of time, initial results show high potential [29–32].

VOLUNTARY ACTIVITY VS. EVOKED POTENTIALS

A BMI can exploit signals associated with external sensory stimulation — such as visual flashes or auditory tones — or with endogenous voluntary decision processes — such as the onset of a movement. In the former case, the brain reacts with so-called evoked potentials. Two different evoked potentials have been widely explored in the field of EEG-based BMI; namely P300 [2,13,33–35] and steady-state visual evoked potential (SSVEP) [36,37]. P300 is a potential evoked by an awaited infrequent event that appears at centro-parietal locations along the midline of the scalp, independently of the sensory stimulation modality. As its name indicates, it is a positive wave peaking at around 300 ms after task-relevant stimuli. The amplitude of the P300 depends on the frequency of stimulus occurrence — less frequent stimuli produce larger responses — and task relevance. Visual evoked potentials (VEP) reflect electrophysiological mechanisms underlying the processing of visual information in the brain and vary in response to changes in visual stimuli. SSVEP are VEP induced by a stimulus repeated at a rate higher than 6 Hz. An SSVEP is composed of a series of components over the visual cortex whose frequencies are exact integer multiples of the stimulus frequency.

Evoked potentials are, in principle, easy to pick up. The necessity of external stimulation does, however, restrict the applicability of evoked potentials to a limited range of tasks. This is particularly the case when controlling robotics devices and for motor rehabilitation. In both cases, BMI has to rely upon brain correlates of voluntary mental activity, which users can modulate at different frequency ranges — or rhythms. Populations of neurons can form complex networks with feedback loops, which give rise to oscillatory activity. In general, the frequency of such oscillations becomes slower the larger the size of the synchronized neuronal assemblies. A particularly relevant rhythm can be recorded from the central region of the scalp overlying the sensorimotor cortex during the imagination of body movements. Correlates of imaginary movements can be recorded at any scale —microspopic, SUA and LFP, mesoscopic, ECoG, and macroscopic, EEG. Apart from their different degrees of spatial

resolution, microscopic and mesoscopic signals also have a broader bandwith (up to 300−500 Hz) than macroscopic signals (normally, less than 100 Hz).

In the case of frequencies below 2 or 4 Hz, we can observe slow cortical potentials (SCP). The analysis of SCP is usually done in the temporal domain. Notably, SCP measured with scalp electrodes was the basis of the first demonstration of a BMI for lock-in patients [1], who learned to modulate its amplitude. The negative amplitude of SCP is related to the overall preparatory excitation level of a given cortical network − the more negative the more active. Another example of SCP that can be observed in the EEG is the so-called Bereitschaftspotential (BP), or readiness potential − a slow negative shift over the controlateral motor cortical area starting around 400−500 ms before the onset of a movement. This makes the readiness potential particularly relevant for motor rehabilitation. However, being close to DC, its presence in single trials seems to be elusive. Nevertheless, it has been recently shown that it allows detection of self-paced reaching movement intention in single trials with humans, including stroke patients [38]. Readiness potentials are also present in intracranial LFP [39] as well as in ECoG [40]. In the former case, intracranial LFP recorded from the supplementary motor area (SMA) in an epileptic patient yielded high recognition rates in the detection of self-paced reaching movements well before the onset. The intention to execute a self-paced movement has also been decoded from SUA in humans [41], where it observed progressive neuronal recruitment in the SMA over 1500 ms before subjects made the decision to move. As a last observation, recent experiments have shown the possibility to decode 3D arm trajectories from scalp SCP [42].

Most spontaneous BMI rely on variations of brain rhythms at higher frequencies than SCP. In the case of EEG and ECoG, imagination of limb movements gives rise to amplitude suppression − event-related desynchronization, ERD − of Rolandic μ (8−12 Hz) and β (13−28 Hz) rhythms over the controlateral limb motor cortical area [43]. This imagination-related ERD shows different time courses in the two bands. In the μ band the ERD recovers to baseline level within a few seconds. On the other hand, the central β activity displays a short-lasting ERD followed by an amplitude increase − event-related synchronization, ERS. In particular, ERD has been largely exploited to build BMIs where subjects imagine movements of their left vs. right hand or arm, or upper limbs vs. feet. References are too numerous to cite them all, but see references [4,5,9,10,15,19,27−30,44,45]. Apart from these 'classical' imagined movements of hands, arms and feet, recently researchers have also shown the feasibility of recognizing different wrist movements [46,47]. Also, as mentioned before, recent results show how it is possible to decode 3D arm trajectories from EEG [42,45] as well as basic grasping patterns such as hand closing/opening [15,48] and finger extension [49,50]. Although more work remains to be done in this area, its combination with functional electrical stimulation and light exoskeletons (see Fig. 63.2) is quite relevant for motor restoration and even rehabilitation in spinal cord injury and stroke patients.

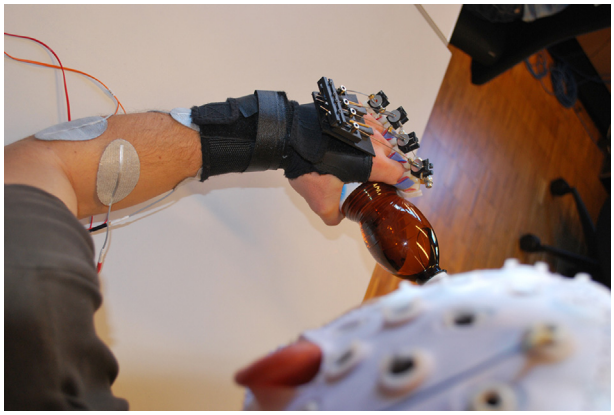

FIGURE 63.2
Example of a BMI for the restoration of grasping. The BMI is combined with functional electrical stimulation that activates the subject's peripheral nerves under the electrodes and contracts the corresponding muscles for hand closing and opening. This operation is further facilitated by the use of a light passive hand orthosis that synchronizes finger movements so as to generate natural grasping patterns.

Finally, because of its higher spatial resolution and broader frequency range, ECoG also seems to carry information about different motor parameters, such as movement kinematics [31,40,51,52], that could provide finer control of neuroprostheses and could greatly help in motor rehabilitation. As mentioned before, the decoding of complex movement intentions has been largely demonstrated with the use of intracranial recordings (LFP and SUA) [12,20,22–26].

MUTUAL LEARNING

A critical issue for the development of a BMI is training – i.e., how users learn to operate the BMI. Some groups have demonstrated that some subjects can learn to control their brain activity through appropriate, but lengthy, training in order to generate fixed EEG patterns that the BMI transforms into external actions [1,45]. In this case the subject is trained over several months to modify the amplitude of a pre-specified parameter of their brain signals, from EEG to fMRI. An alternative approach is to relieve the user of any training by using machine learning techniques to rapidly find individual patterns of brain activity associated with the mental commands the user wants to convey [53]. The drawback of this approach is that the BMI must be recalibrated at the beginning of every session. Most BMI systems lie in between these two extreme approaches, since they adopt a mutual learning principle, where the user and the BMI are coupled together and adapt to each other. Different groups have shown that, in this case, humans can learn to operate the brain-actuated device very rapidly, in a few hours which are normally split over a few days [8,9,30,42].

Mutual learning facilitates and accelerates the users' training process thanks to the use of statistical machine learning techniques that both select relevant, stable features and build optimal models to decode the user's intention. Feature selection yields user-specific brain components – normally spatio-frequency features for rhythmic activity or spatio-temporal for evoked potentials – that maximize the separability between mental commands [54,55]. In addition, because of the non-stationary nature of brain signals, selected features must also be stable over time. These initial features represent those brain components that the user can naturally modulate and, via feedback received during online BMI training, learn to control quickly and voluntarily. Selection of stable features can be enhanced by removing non-stationary brain sources [56].

Although there are reports of users who keep a stable level of performance over months and even years, BMIs suffer from the natural variability of brain signals due to changes in background activity and learning. This calls for the use of online adaptation techniques to keep the BMI tuned to drifts in the signals [57–62].

CONTEXT-AWARE BMI

Independently of the kind of BMI signal (SUA, LFP, ECoG or EEG), paradigm (voluntary activity or evoked potential) and learning approach, users cannot sustain high levels of performance over long periods of time. This is mainly due to the natural variability of brain signals. Furthermore, EEG-based approaches yield low throughput. As a consequence, it seems that current state-of-the-art BMI technology is insufficient for full dexterous control of complex applications like neuroprostheses. Nevertheless, we can cope with those limitations and achieve reliable mental control by designing context-aware BMIs [63]. Such a BMI collects information about the state of the device (e.g., position and velocity of the cursor or neuroprosthesis), as well as its environment (e.g., icons in the screen or potential targets and obstacles perceived by the prosthesis' sensors), and combines this with the user's mental commands. In this approach, the smart brain-controlled device interprets and executes the mental commands with respect to the context, thus enabling the performance of complex tasks even with a reduced, scattered set of commands. Furthermore, any eventual decrease in mental

FIGURE 63.3
Brain-controlled wheelchair. Users can drive it reliably and safely over long periods of time thanks to the incorporation of shared control (or context awareness) techniques. This wheelchair illustrates the future of intelligent neuroprostheses that, as our spinal cord and musculoskeletal system, works in tandem with motor commands decoded from the user's brain cortex. This relieves users from the need to deliver continuously all the necessary low-level control parameters and, so, reduces their cognitive workload.

control is compensated for, up to a certain degree, by the smart device. Examples of context-aware BMIs are neuroprostheses such as robots and wheelchairs [9,11,14,64,65], as well as smart virtual keyboards [66,67] and other assistive technologies (AT) software with predictive capabilities. Remarkably, in the case of the control of complex devices such as a telepresence robot, this approach yields similar performance to manual control despite the fact a BMI is not a perfect control channel. This was the case not only for expert BMI users, but also for novel BMI subjects [65] and users with physical disabilities [68]. Fig. 63.3 shows our brain-controlled wheelchair.

A common form of context awareness is shared control, a technique widely used in robotics, where the user and the smart device share responsibilities in choosing the final action to be executed that best matches the user's intent. For example, it requires quite precise control to drive a wheelchair in a home environment (scattered with chairs, tables, doors, etc.). Also, it can take a long time to navigate between rooms. In a shared control BMI, the user delivers high-level mental commands such as left, right and forward, which the wheelchair interprets based on the contextual information from its sensors to compute the actual motor commands (speed of the motors) to travel smooth trajectories and avoid obstacles. In such a way, shared control can facilitate the operation of the device by inferring goals, reducing the cognitive workload (the user does not need to care about all the low-level details), inhibiting pointless mental commands (e.g., driving zig-zag), preventing critical situations (e.g., collisions), and determining meaningful motion sequences (e.g., for an arm neuroprosthesis).

FUTURE DIRECTIONS

Current BMI technology, in particular EEG-based approaches, enables the operation of simple brain-actuated devices over short periods of time. Increasingly complex prototypes are being developed and demonstrated. No doubt this will represent an important achievement for motor-disabled people. Yet, robust and natural brain interaction with more complex devices, such as neuroprostheses, over long periods of time is a major challenge.

A related issue is the development of practical BMI technologies that can be brought out of the lab and into real-world applications in order to improve the lives of countless disabled individuals. For this we need to confirm the benefit of BMI for disabled people beyond laboratory conditions. Only a few studies have been conducted up to now. In addition, we have to design novel hardware and sensors as well as principled approaches.

One of the main barriers to the wide use of BMI technology is the limitation of today's electrodes, both invasive and non-invasive. In the former case, they damage brain tissue and do not last long before they lose signal. Consequently, one area of active research is the design of safe biophysical interfaces that, in addition, should be ultralow power and wireless. In the latter case, EEG signals suffer from a reduced spatial resolution and increased noise when measurements are taken on the scalp. Significant worldwide research is taking place on algorithms to improve EEG analysis and decoding. Another of the shortcomings of today's EEG technology, which is essentially the same as that of 30 to 50 years ago, is the need for conductive gel to reduce impedance. Success of BMI, although still modest, is creating a market for new sensors that will make the use of EEG-based systems much easier and robust over long periods of time. Examples of this new technology are dry electrodes that do not require any gel and can be integrated into aesthetic helmets [69], as well as skin sensors that can remain operational for months, if not years [70]. An associated challenge, however, is ways to develop new techniques to analyze the signals they will measure, which will probably differ from those from conventional electrodes.

Apart from addressing new sensors, and its associated signal processing techniques, we should also develop new principles to make BMI work effectively for disabled people. In this respect, it seems natural to combine BMI with existing AT [71]. This is called a hybrid BMI — a combination of different signals including at least one BMI channel [71,72]. Thus, it could be a combination of two BMI channels but, more importantly, also a combination of BMI and other residual biosignals (such as electromyographic, EMG) or special AT input devices (e.g., joysticks, switches, etc.). Recently, we have introduced a general architecture and a common software framework for hybrid BMIs [73]. Importantly, shared control (context-aware techniques) is a key component of the hybrid BMI, as it will shape the closed-loop dynamics between the user and the brain-actuated device so that tasks can be performed as easily and effectively as possible.

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CHAPTER 64

Spinal Cord

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INTRODUCTION

Spinal cord injury is a major medical problem because there currently is no way to repair the central nervous system and restore function. In this chapter, we focus on embryonic stem cells as an important research tool and potential therapy. We quickly review the epidemiology, functional anatomy, and pathophysiology of spinal cord injury and then discuss spontaneous regeneration and current limitations on repair. We also summarize features of spinal cord development that might guide restoration strategies. We then review studies that have utilized embryonic stem cells in spinal cord repair. We conclude that progress has been good, that knowledge is still too limited, and that harnessing the potential of stem cells will be important for solving the problem of spinal cord injury.

THE PROBLEM

Nearly 12,000 people in the United States suffer a traumatic spinal cord injury (SCI) every year, and about a quarter of a million Americans are living with this devastating condition (National Spinal Cord Injury Statistical Center, Birmingham, Alabama, 2012). There are also four to five times as many spinal cord injuries caused by medical conditions, such as multiple sclerosis, a common disorder that destroys the myelin insulation on nerves in the cervical spinal cord. Other medical causes include amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), polio and post-polio syndrome, human T-lymphotropic virus type 1 (HTLV-1), human immunodeficiency virus type 1 (HIV-1), metabolic deficits such as of vitamin B-12, as well as causes of myelopathy, such as stenosis and disk herniation [1]. The worldwide incidence of traumatic and non-traumatic SCI is greater per capita than in the United States.

The consequences of SCI depend on the level at which the cord is damaged. Generally, injuries in the neck produce tetraplegia, with loss of bowel and bladder function, whereas lesions in the thoracic or lumbar area may cause paraplegia, also with bowel and bladder dysfunction. In its severest form, SCI causes complete paralysis and loss of sensation throughout the body, inability to control bowel and bladder function, trouble controlling autonomic functions such as blood pressure, and inability to breathe or cough. The long-term disability from SCI results not only from the initial loss of function but also from the complications that accumulate. Up to 30% of individuals with SCI are hospitalized every year for complications [2], such as severe spasticity, infections (lung, skin, bowel, bone, urinary tract), osteoporosis and pathologic bone fractures, autonomic dysreflexia, and heterotopic ossification [1,3]. Another major long-term complication is muscle wasting. This results from disuse and the absence of nerve impulses, which are critical for maintaining junctions between nerves and

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muscles [4,5]. Therefore, patients who maintain their body in the best condition for nervous system repair will benefit most from future therapeutic strategies.

SPINAL CORD ORGANIZATION

Unlike the brain, the spinal cord has its white matter (nerve axons) on the outside and gray matter (cell bodies) on the inside (Fig. 64.1). The gray matter houses neurons that project to their level of the periphery to control movement and receive sensory signals. The white matter carries axonal connections to and from the brain, and half of all those axons are myelinated. In general, the spinal cord has much less potential for regeneration than the peripheral nervous system. Moreover, most traumatic SCIs also injure the incoming and outgoing (afferent and efferent) peripheral nerves at the injury level. In most cases, however, the caudal cord remains intact beginning several segments below the injury level. Circuitry in those segments can produce reflexes by activating a ventral motor neuron to produce muscle contraction when it receives a sensory stimulus from the periphery of the body (Fig. 64.1).

The distal spinal cord also contains groups of nerve cells that generate the patterns of activity needed for walking and running. Finally, there are central pattern generators, which govern particular motor functions in the periphery that are quite complex in nature and well controlled. Interested readers are referred to recent monographs on this subject [6–9]. Because the upper spinal cord plays only a limited role in controlling these pattern generators, people with injury to the cervical spine can walk or ride a stationary bicycle if their muscles are stimulated appropriately. The existence of additional pattern generators has also been predicted. For example, the phrenic nucleus, housed at C3 through C5 in the upper neck, appears to generate the pattern of movements needed for respiration. Unfortunately, this part of the spine is a common site of traumatic injury.

INJURY

Traumatic injury occurs when broken fragments of bone and ligament impinge on the soft spinal cord, which is no wider than the thumb. The cord responds by swelling until it

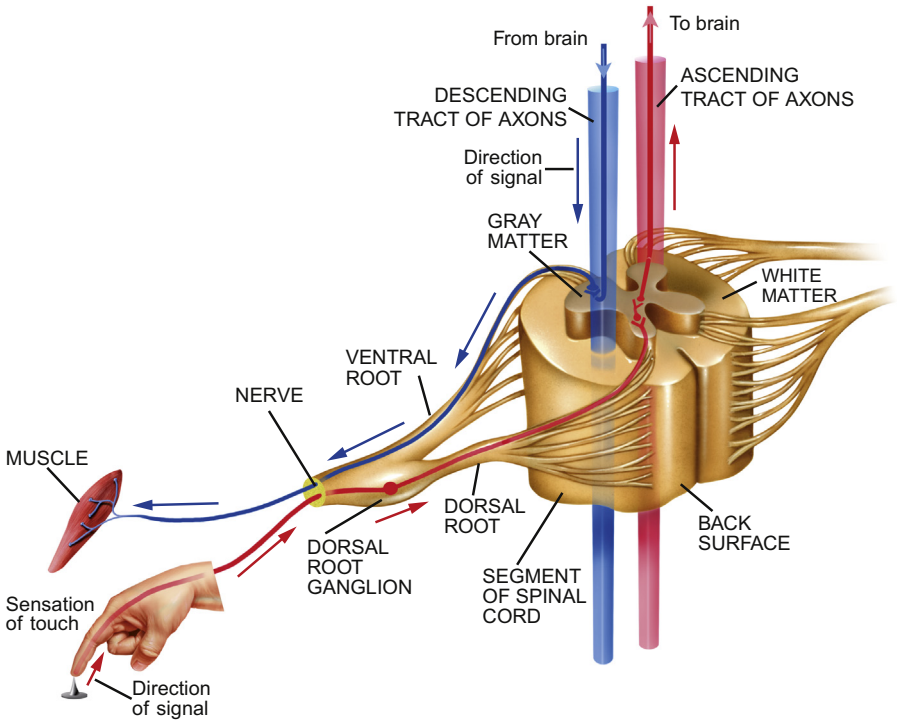

FIGURE 64.1
Organization of the spinal cord. A segment of cord reveals the butterfly-shaped gray matter at the core and an outer ring of white matter. The main components of the gray matter are neuronal cell bodies and glial cells and blood vessels. The white matter also contains astrocytes and blood vessels, but it consists mostly of axons and oligodendrocytes (glial cells that wrap axons in white, insulated myelin). Axonal tracts that ascend in the cord, such as the one shown in red, convey sensory messages received from the body; the descending tracts (blue) carry motor commands to muscles. *(Reproduced with permission of Alexander and Turner Studio, FL. © 2002 Edmond Alexander.)*

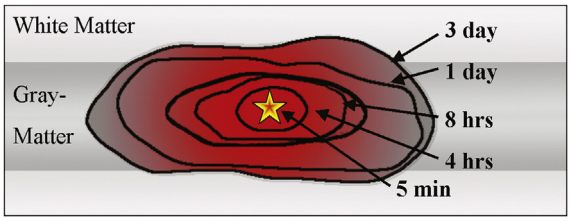

FIGURE 64.2
Evolution of primary to secondary spinal cord injury. Following the initial trauma, the injury site enlarges rapidly as a consequence of secondary injury, particularly over the first 24 hours.

encounters resistance from the bony canal of the spinal column. The swelling that compounds the initial injury reduces venous blood flow, causing a secondary venous infarct in the central part of the cord. This initiates a cascade of events that injures neighboring tissue. During this secondary phase, which occurs in the first 24 hours after the primary phase, cells die by excitotoxic necrosis (caused by release of harmful concentrations of chemicals from other cells) as well as by apoptosis (cell suicide), causing the initial injury site to enlarge rapidly into a hole in the middle of the spinal cord (Fig. 64.2). Because a donut-like rim of viable tissue usually remains at the level of injury, SCI preferentially affects gray matter.

A second wave of much delayed cell death occurs during the weeks after an SCI [10]. It removes mostly oligodendrocytes (the cells that wrap nerve axons in a fatty layer of myelin) from adjacent white matter tracts. Since each oligodendrocyte myelinates 10 to 40 different axons, loss of one oligodendrocyte leads to exponential loss of myelin. As a result, nerve axons cease to function because they cannot conduct electrical impulses when this insulating sheath is lost. This progressive phase of secondary injury is a good target for potential therapies, such as neuroprotective stem cell transplantation.

The problem does not stop with the secondary wave of cell death, however. An injured, underactive nervous system may be unable to adequately replace cells, particularly housekeeping cells called glial cells, which normally turn over. Therefore, individuals with SCI may experience a slow, progressive loss of neurological function over very long periods in addition to complications from their initial injury. It is important to consider this potential for further loss of function when designing therapeutic regimens.

SPONTANEOUS REGENERATION

Not long ago, the adult nervous system was believed to have no capacity for repair or regeneration. Now a growing body of evidence indicates that the capacity for spontaneous regeneration may be much greater than previously perceived. Data obtained since the 1960s have demonstrated that new cells are added to the nervous system continually; these cells include neurons in at least two brain regions, the hippocampus and the olfactory bulb [11,12]. Moreover, glial cells are frequently born and are capable of regeneration [13,14]. Recent work has shown that severed or intact axons can sprout even long after injury [15,16].

Nonetheless, our ability to maximize spontaneous regeneration is currently limited. The recent detection of endogenous stem cells within the spinal cord raises hope that such cells can be harnessed to repair the damaged spinal system. Although the birth of new neurons from these progenitors has never been demonstrated within the spinal cord [17], new glial cells, including oligodendrocytes and astrocytes, are continually being added. In fact, injury stimulates cell birth. Our laboratory recently showed that nearly 2 million cells are born in the spinal cord each day, though most eventually die [18]. Collectively, these data suggest that cell turnover does occur in the nervous system, albeit much more slowly than in other organs of the body. Moreover, we recently showed that patterned neural activity, generated by repetitive tasks such as cycling, can stimulate cell birth, suggesting that behavioral modification could be important for maximizing cord regeneration and functional recovery [18].

CURRENT LIMITATIONS AND APPROACHES TO REPAIR AND REDEFINING GOALS

Although some limited spontaneous regeneration is known to occur [19,20], dramatic self-repair of the nervous system does not take place. A growing body of evidence suggests that factors in the nervous system actively inhibit regeneration. Such factors include inhibitory proteins in the cord, which guide re-growing connections, and scar tissue, which contains chondroitin sulfate and other proteoglycans [21]. Reduced production of growth factors that stimulate regrowth also limits regeneration.

Figs. 64.3 and 64.15 outline the general strategies for repairing the damaged spinal cord. Note that a complete cure of nervous system injury is not practical or required. Partial repair translates into proportionally greater recovery of function. For example, only about 10% of the functional connections are required to support locomotion in cats [22], and humans missing more than half the gray matter in the cervical spinal cord can still walk and run fairly normally.

One repair strategy is to transplant stem cells or other biomaterials to fill the cyst that forms at the eye of the injury. This cyst acts as a physical barrier to the growth of anatomically intact axons in the surrounding donut of white matter. The lost cells include segmental motor neurons, sensory neurons, oligodendrocytes, and astrocytes. Although it is possible for endogenous cells to give birth to new oligodendrocytes and astrocytes, this response is limited; moreover, it has not so far been possible to obtain new neurons in the spinal cord from endogenous cells [17].

As well as filling-in the cyst, it may be necessary to repair axons that no longer function properly because they have lost their myelin or have been inappropriately myelinated. Several

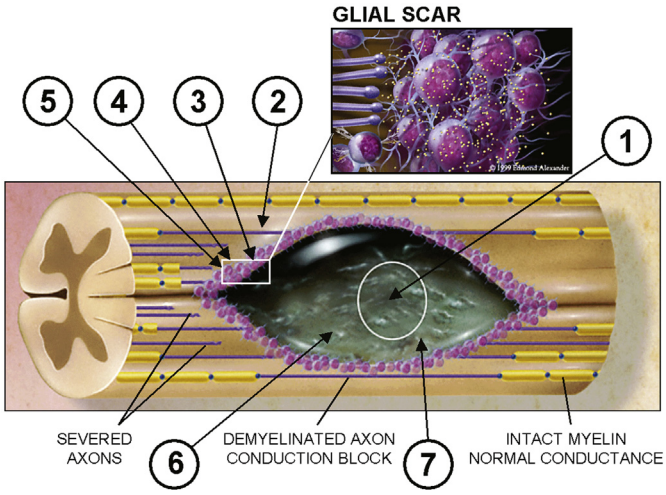

FIGURE 64.3

Common strategies toward regeneration of the damaged spinal cord. (1) Prevention of progression of secondary injury: Necrotic and apoptotic cell death would be prevented by anti-excitotoxic drugs and anti-apoptotic treatments. (2) Compensation for demyelination: Chemicals that prevent conduction block in demyelinated areas and agents that encourage surviving oligodendrocytes to remyelinate axons would be provided. Lost oligodendrocytes would be replenished. (3) Removal of inhibition: Agents that block the actions of natural inhibitors of regeneration or drugs that downregulate expression of inhibitory proteins would be provided. (4) Promotion of axonal regeneration: Growth factors that promote regeneration (sprouting) of new axons would be provided. (5) Direction of axons to proper targets: Guidance molecules would be provided or their expression would be increased in host cells. (6) Creation of bridges: Bridges would be implanted into the cyst to provide directional scaffolding that encouraged axonal growth. (7) Replacement of lost cells: Cells capable of generating all cell types (progenitor cells or embryonic stem cells) would be implanted. Substances that induce undifferentiated cells to replace dead cells would be provided. Also, cells that had been genetically engineered to deliver regenerative molecules would be transplanted. *(Reproduced with permission of Alexander and Turner Studio, FL. © 2002 Edmond Alexander.)*

approaches have been used to overcome this problem, which can manifest itself as a segmental conduction block. For example, potassium channels on dysfunctional axons can be blocked pharmacologically, and preexisting or transplanted oligodendrocytes and their progenitors can be encouraged to make new myelin [23,24].

Another strategy is to remove or mask the effects of proteins in the glial scar around the cyst that actively inhibit the regrowth of new connections. Antibodies that block the inhibitory effects of these proteins promote sprouting [25]. Alternatively, cells might be delivered to the cyst to digest inhibitory proteins in the scar; or the initial expression of such proteins might be inhibited. Moreover, certain growth factors promote the self-repair of physically broken connections. These factors can be provided exogenously or by transplanting genetically altered cells that release them [19,26,27].

It is important to understand the feasibility of various repair strategies and to redefine appropriate goals of repair. If one ranks the foregoing strategies in terms of likely success [1], it becomes clear that it will be very difficult to persuade axons to regrow across a lesion, extend all the way down the spinal cord, and make connections with the appropriate target cells. Remyelination seems much more feasible because it occurs continually at a steady rate in the damaged spinal cord. It is important to investigate all possible strategies, however, because some will materialize sooner and some later. Most importantly, we must understand that multiple strategies delivered over time will be more important than the elusive magic bullet.

When defining appropriate goals for therapy, improving patients' quality of life must surely rank first. To this end, strategies that limit complications are important. Moreover, individuals with SCI often value small gains in function more highly than larger gains, such as walking. Thus, the top goal for most patients is recovery of bowel, bladder, or sexual function. Distant seconds include recovery of respiratory function for those whose respiration is impaired and use of a single hand for those without hand function [1]. Paradoxically, most animal models focus on recovering the ability to walk. This is both the least appropriate goal and the most difficult.

SPINAL CORD DEVELOPMENT

To understand spinal cord regeneration, it is necessary to understand spinal cord development. In humans, oligodendrocytes are found in cultures of fetal spinal cord at 7 and 12 weeks of gestation [28,29]. Myelination begins at 10 to 11 weeks of gestation [30,31] and continues throughout the second year of life [32,33]. Signals derived from axons regulate the growth of progenitor cells and the survival of oligodendrocytes [34]. For example, sonic hedgehog (SHH), a protein synthesized by notocord and floor plate cells, induces the differentiation of motor neurons in the ventral cord [35], and SHH signaling induces oligodendrocyte precursors (OP) to emerge in the embryonic spinal cord [36]. Platelet-derived growth factor (PDGF) is a potent regulator of OP migration and proliferation, while insulin-like growth factor (IGF-1) acts on both neurons and oligodendrocytes. Other locally synthesized growth factors appear to control the balance between OP proliferation and differentiation [37].

Many different neuronal phenotypes arise from various progenitor pools during central nervous system (CNS) development, but most of the pathways are poorly understood. One of the best described is the generation of spinal motor neurons [38], which involves several steps. Through signaling pathways that enable cells to respond to external cues (pathways involving bone morphogenetic protein [BMP], fibroblast growth factor [FGF], and Wnt), ectodermal cells obtain a rostral neural character [39]. In response to caudalizing signals, such as retinoic acid (RA), these progenitors acquire a spinal positional identity [40]. Through the ventralizing action of SHH, spinal progenitor cells gain their motor neuron phenotype [41].

These findings raise the question of whether embryonic stem (ES) cells can be directed along specific pathways to produce specific neural cell populations for CNS repair. In fact, very

encouraging steps have already been taken. Early reports revealed the possibility of generating cells with motor neuron characteristics from ES cells [42]. Later, Wichterle and colleagues demonstrated that signaling factors operating along the rostrocaudal and dorsoventral axes of the neural tube to specify motor neuron fate *in vivo* could be harnessed *in vitro* to direct the differentiation of mouse ES cells into functional motor neurons [43].

EMBRYONIC STEM CELLS

ES cells have unique features that are important for spinal cord repair. They represent every cell type in the body and are the earliest stem cells capable of replicating indefinitely without aging, and their DNA can be modified easily, even in single cells. They also fulfill two criteria that are essential for nervous system repair through transplantation:

1) They normally belong in the spinal cord; and
2) They contain the recipient's own DNA, obviating the need for immunosuppression, which cannot be used in spinal cord patients because of the increased incidence of infections.

Several methods are available for obtaining ES cells. The most common is *in vitro* fertilization, which requires a fertilized egg and therefore produces cells that differ genetically from the host. A second strategy involves somatic nuclear transfer, which takes a nucleus from a somatic cell, such as a skin cell, and transfers it to a fertilized egg whose nucleus has been removed (Fig. 64.4a) [44]. The subsequent ES cells therefore contain only the genetic material of the recipient. Another possibility for women is parthenogenesis [45], which tricks an egg into thinking it is fertilized, allowing it to begin duplicating its DNA (Fig. 64.4b). Although this process is unable to create a viable embryo, (because factors derived from sperm are necessary for pre-implantation), it can produce normal cells.

INDUCED PLURIPOTENT STEM CELLS

The newest and most promising method to date is based on induced pluripotent stem cells (iPSC). iPSCs were first generated by inducing fibroblasts into an embryonic-like state with respect to their morphology and differentiation potential [46,47]. This is achieved by ectopic expression of transcriptions factors through different methods and use of small molecules that aid the reprogramming process [48].

The applicability of patient specific iPSCs ranges from:

1) generation of *in vitro* models for neurological disorders,
2) The generation of drug screening assays, and
3) Autologous cell replacement in neurological disorders of various etiologies.

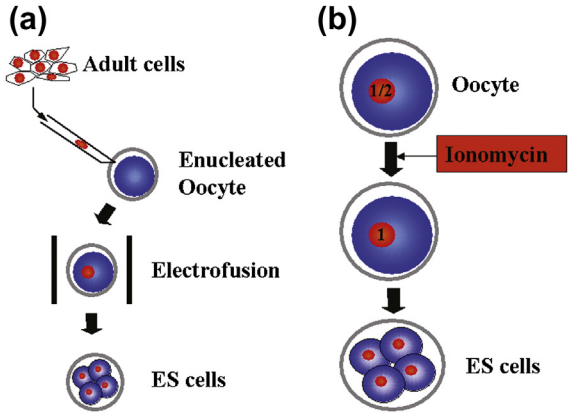

FIGURE 64.4
ES cells can be made by *in vitro* fertilization and two additional methods: (a) Somatic nuclear transfer: Donor cells are placed under the zona pelucida into the perivitelline space of enucleated oocytes. The cell nucleus is introduced into the cytoplast by electrofusion, which also activates the oocyte. It can then be grown to the blastocyst stage to harvest the ES cells. (b) Parthenogenic activation: For activation, oocytes are briefly exposed to a calcium ionophore such as ionomycin. The resulting cell can mature for ES cell harvest.

The first challenge in using patient specific iPSCs, generating differentiation protocols, has been overcome. To date human iPSCs have been differentiated into components of each germ layer *in vitro* and to form teratomas in immune deficient mice [49]. Additionally, IPSCs have been directly differentiated into neural progenitors [50], neurons [51,52], pancreatic insulin-producing cells [53], and hepatocytes [54].

The first human patient specific iPSC line to be derived from a patient with a neurological disorder came from an ALS patient in an advanced age of 83. These iPSCs were differentiated into motor neurons effectively creating an *in vitro* human model of ALS [55]. Another group generated ALS patient specific iPSC lines from three patients that carried a mutation in Tar DNA binding protein-43 (TDP-43), a protein that when mutated is found in cytosolic aggregates in the motor neurons of ALS patients that have the sporadic form of the disease. iPSC-derived motor neurons formed cytosolic aggregates containing TDP-43 and were used to screen compounds that rescue the ALS-associated phenotype through assays that included measurements of insoluble TDP-43, neurite length quantification, and TDP-43 mRNA. The screen identified anacardic acid, a histone acetyltransferase inhibitor, as a substance that rescued motor neurons from the abnormal ALS phenotype. Interestingly, while there were clonal differences within iPSCs derived from different patients as well as iPSCS from the same patient, the ability of anacardic acid to rescue the abnormal motor neuron phenotype was consistent in the two iPSC lines used in this work [56].

The generation of iPSCs has applicability in studying the differences in the pathogenesis of neurological diseases where the etiology of the disease is different as is the case with Parkinson's disease (PD) where only 10% of patients have the inheritable form of the disease while the rest manifest the sporadic form. Differentiation of iPSCs into dopaminergic neurons from patients with idiopathic PD and patients with familial PD carrying a G2019S mutation in the Leucine-Rich Repeat Kinase 2 (LRRK2) gene showed that when cultured for a short amount of time (indicating a young age) only the iPSCs with LRRK2 mutations manifested α-synuclein-containing intraneuronal inclusions known as Lewy bodies. However, when cultured for longer periods of time (indicating aging) iPSC-derived dopaminergic neurons generated from patients with idiopathic and familial PD showed signs of neurodegeneration caused by deficient autophagic machinery. Importantly, this work reports on the generation of iPSC-based model of PD that recapitulates pathological features of idiopathic and familial PD, and has the potential to be used for identification of pathogenic mechanisms of PD, and can be used to screen drugs that rescue the abnormal phenotype of dopaminergic neuron phenotype [57].

The therapeutic potential of iPSCs for autologous neural cell replacement has shown to be promising. When transplanted in utero, iPSC-derived neural precursors extensively migrate and differentiate into neurons and glial cells of the developing brain. The transplanted iPSC-derived motorneurons can be found primarily in the septum, striatum, hypothalamus and midbrain as well as olfactory bulb, cortex and thalamus but not cerebellum and brainstem. The engrafted neurons generate various neuronal subtypes such as glutamatergic neurons, GABAergic neurons, and catecholaminergic neurons that functionally integrate into the brain; the glial cells display glial phenotype and are glial fibrillary acidic protein (GFAP) positive. Preclinical studies show that transpantated iPSC-derived neural precursors in a rodent model of PD differentiate into dopaminergic neurons in the substantia nigra and induce functional recovery. However, this study highlighted the issue of teratoma formation as this was observed in the animals that did not recover in function [58].

Teratoma formation from transplanted iPSC-derived neural precursors was an issue in another preclinical study. Transplantation of iPSC-derived neural precursors in a rodent model of SCI resulted in teratoma formation. However, this problem was resolved by choosing an iPSC-derived neural precursor cell line that had no teratoma-forming activity. Transplantation of 'safe' iPSC-derived neural precursors in a rodent model of SCI promoted functional recovery. In the injured spinal cord, the engrafted cells differentiated into neurons,

oligodendrocytes and astrocytes. The engrafted cells that had differentiated into mature oligodendrocytes were integrated into myelin sheath whereas cells that differentiated into astrocytes promoted serotonergic innervation of the dorsal cord. Therefore, this preclinical study suggested the possibility that remyelination of demyelinated axons by the grafted 'safe' iPSC-derived oligodendrocytes contributed to the functional recovery of the grafted animals. The other contributing mechanism for functional recovery suggested here is axonal regrowth supported by the grafted 'safe' iPSC-derived astrocytes. Importantly, this study demonstrated the potential of iPSC for use in the repair of the injured spinal cord as well highlight the need for the evaluation of the iPSCs prior to transplantation [59].

The generation of iPSCs is delivering on the promise of creation of patient specific cells for therapeutic transplantations. The general consensus in the scientific community was that unlike hESCs the use of iPSCs in autologous transplantation-based therapeutic approaches would not face any immune related issues and that their tumorogenic potential will be reduced by the use of non-integrating episomal vectors [60], viral-free systems to generate these cells [61] or pre-transplantation characterization [59]. However, an examination of immune rejection in inbred C57BL/6 showed that iPSC lines derived from C57BL/6 fibroblasts through retroviral approach or episomal approach are immunogenic in these mice. C57BL/6 iPSC-derived teratomas in syngeneic recipients were regressed, had T cell infiltration and tissue damage. Global gene expression analysis revealed noticeable abnormal gene expression in C57BL/6 iPSC-derived teratomas that could induce T cell-dependent immune response in syngeneic recipients [63]. While this data is concerning for clinical applications of iPSCs, more preclinical studies are needed to establish the immunogenicity of autologous iPSCs particularly in an immune-privileged environment of the CNS.

ALTERNATIVE TRANSPLANTATION OPTIONS

Many studies of spinal cord repair have involved differentiated cells. The earliest studies used peripheral nerve grafts to demonstrate that the nervous system has the capacity to regenerate but that the environment in the CNS is not permissive [62]. Substantial progress has been made with transplantation of peripheral nervous system cells and non-nervous system cells, including genetically engineered fibroblasts, bone marrow cells, glial cells, neurons, and mixtures of glia and neurons [64–67]. In general, fetal sources of cells have been the most successful because derivatives of postnatal and adult cells are less able to withstand neural transplantation. One recent exception has been neural stem cells derived from the adult CNS [68].

Another very active area of investigation is transdifferentiation [69]: isolating cells from organs other than the nervous system and transforming them into neural progenitor cells. Although initial progress suggested that this strategy might work with many types of tissue, the problem of cell-to-cell fusion may have dampened enthusiasm for this approach [70]. Suffice it to say that transdifferentiation has not yet produced cells suitable for CNS transplantation, although clearly the potential exists.

EMBRYONIC STEMS CELLS AND THE NEURAL LINEAGE

Several protocols are available for converting ES cells into neural lineage cells. However, protocols for mouse and human cells differ because the constraints of the human ES cell system have not yet been clearly defined (Fig. 64.5). Differentiating murine ES cells traditionally begins with floating spheres called embryoid bodies. These bodies are akin to the neural spheres of stem cells obtained from the adult CNS. Retinoic acid is a key induction agent for producing neural progenitors from mouse ES cells [71]. Neural cells resembling anatomically normal neurons, astrocytes, and oligodendrocytes from the CNS can easily be derived from mouse ES cells using these protocols (Fig. 64.6).

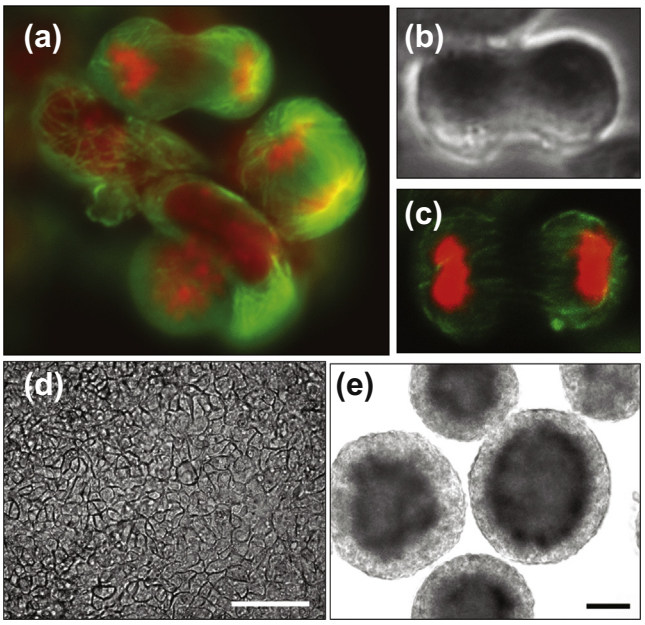

FIGURE 64.5
(a—c) Undifferentiated mouse ES cells dividing in a culture dish. (a, c) Immunofluorescence images demonstrate anti-B-tubulin (green) and anti-DNA (Hoechst; red). The phase image (b) of the identical field (c) is shown in Panel b. (d) Undifferentiated human ES cells. (e) Embryoid bodies derived from human ES cells. Immunofluorescence (green), Hoechst 33342 (blue). Scale bar d—e = 100 μm.

EMBRYONIC STEM CELL TRANSPLANTATION

Use of ES cells for neural transplantation is in its infancy, and only a very limited amount of work has been completed with the spinal cord. These early studies have relied on transplantation during the embryonic [43], postnatal [73,74], and adult [74—76] periods in the normal or injured spinal cord. Overall, they demonstrate that ES cells have a remarkable ability to integrate into the injured region of the cord and differentiate appropriately.

A study by Thomas Jessell and colleagues provides exceptional evidence that ES cells can participate in the normal development of spinal cord cells, including motor neurons [43]. It demonstrated that developmentally relevant signaling factors can induce mouse ES cells to differentiate into spinal progenitor cells and then motor neurons through the normal developmental pathway (Fig. 64.7). Thus, the signals that promote the differentiation of neural stem cells *in vivo* are also effective when applied to ES cells. This group further demonstrated

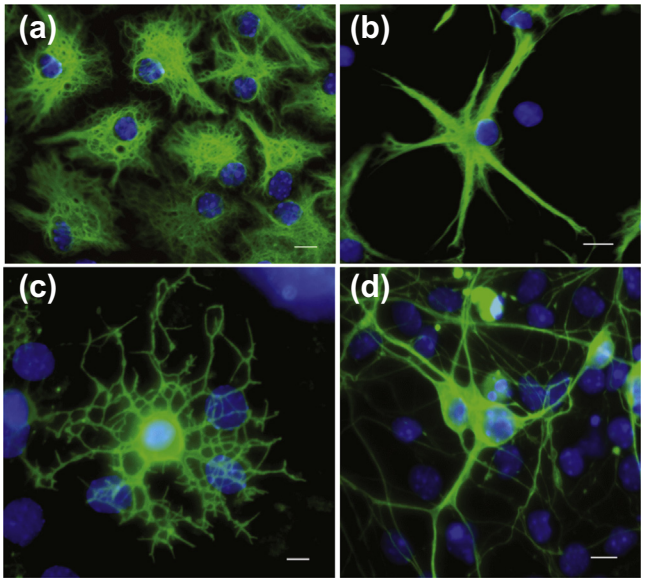

FIGURE 64.6
ES cells differentiate into the principal types of neural cells. (a) Type I and (b) type II astrocytes (anti-GFAP). (c) Oligodendrocytes (anti-O1). (d) Neurons (anti—β tubulin). Scale bar = 10 μm. From Becker et al. (2003). *(Reproduced with permission.)*

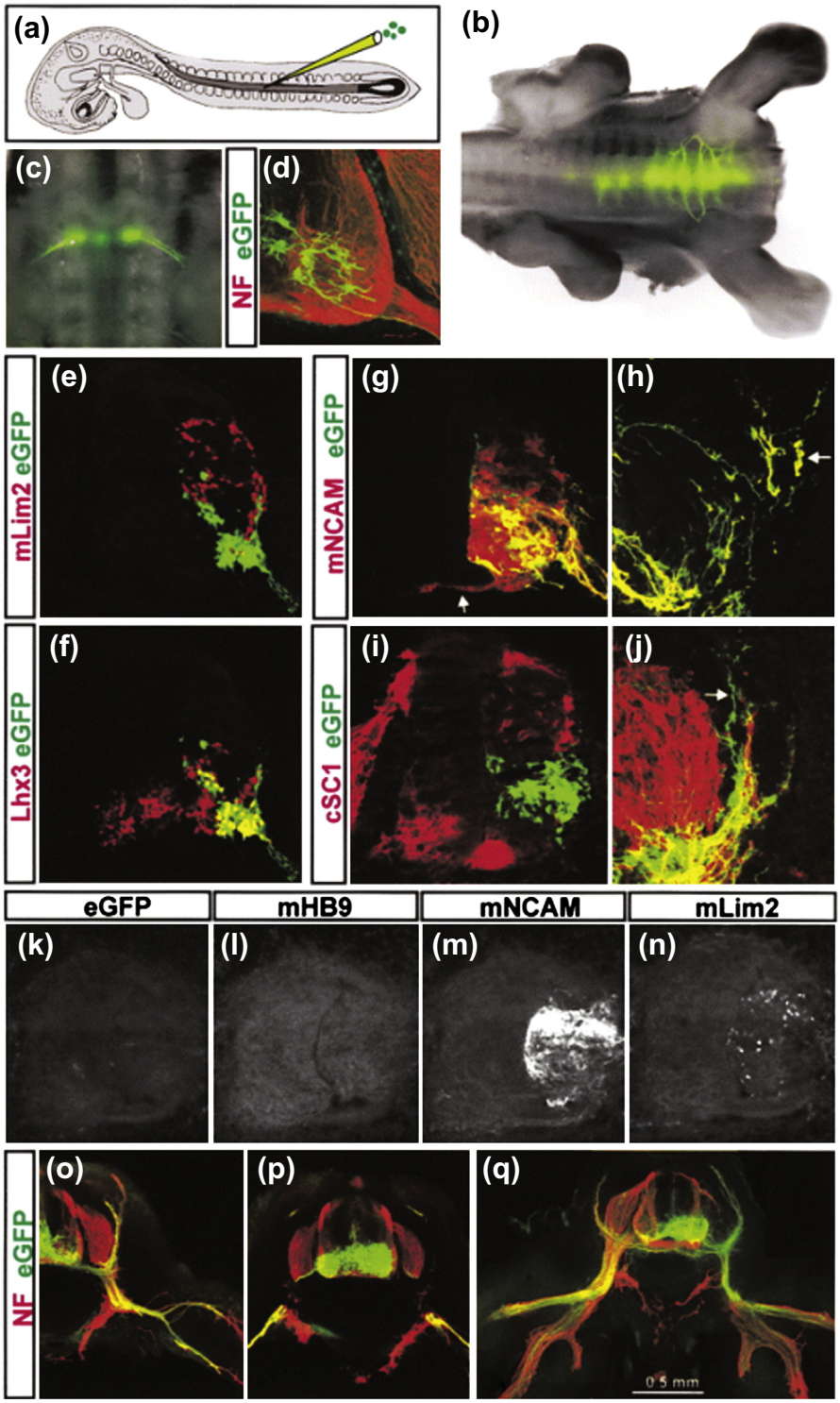

FIGURE 64.7

Embryonic transplantation of motor neurons derived from mouse ES cells. Integration of transplanted ES cell-derived motor neurons into the spinal cord *in vivo*. (a) Implantation of HBG3 ES cell-derived motor neuron-enriched embryoid bodies into stage 15—17 chick spinal cord. (b) Brightfield/fluorescence image showing eGFP+ motor neurons in thoracic and lumbar spinal cord, assayed at stage 27 (ventral view). (c, d) Location of FACS-sorted ES cell-derived eGFP+ motor neurons in thoracic spinal cord, assayed at stage 27. eGFP+ motor neurons are clustered in the ventral spinal cord (d). (e—j) Transverse sections through stage 27 chick spinal cord at rostral cervical levels after transplantation of motor neuron-enriched embryoid bodies. Motor neurons are concentrated in the ventral spinal cord and are segregated from transplanted interneurons, labeled by a mouse- specific Lim2 antibody (e). Many ES cell-derived motor neurons coexpress eGFP and Lhx3 (f). ES cell-derived motor neurons (g) and axons (arrow, h) are labeled by rodent-specific anti-NCAM anti- body but do not express the

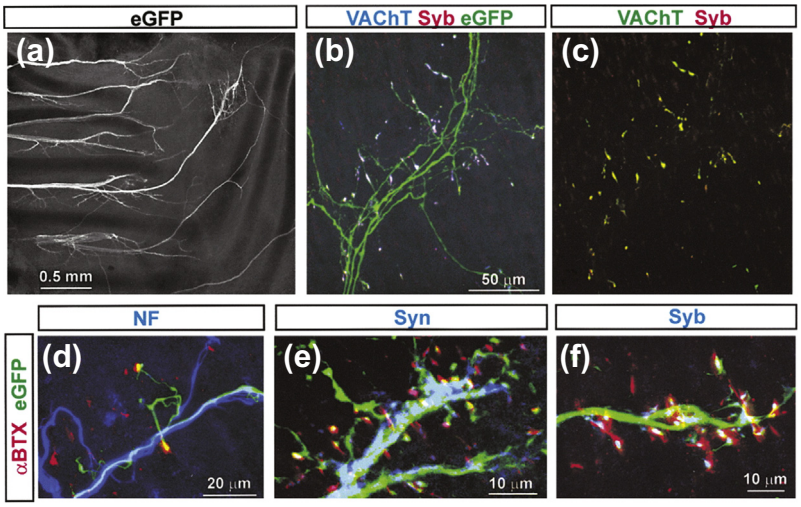

FIGURE 64.8

Anatomic integration of motor neurons derived from transplanted mouse ES cells. Synaptic differentiation of ES cell-derived motor neurons *in vivo*. (a) Whole-mount preparation of stage-35 chick embryonic rib cage. ES cell-derived eGFP+ motor axons contact intercostal muscles. (b, c) Coexpression of synaptobrevin (Syb) and vesicular acetylcholine transporter (VAChT) in the terminals of eGFP+ motor axons at sites of nerve contact with muscle. The anti-Syb and -VAChT antibodies recognize mouse but not chick proteins. (d) Neurofilament (NF) and eGFP expression in motor axons that supply intercostal muscles. eGFP+ axons lack NF expression. The terminals of eGFP+ axons coincide with clusters of acetylcholine receptors, defined by α-bungarotoxin (αBTX) labeling. (e) Coincidence of synaptotagmin (Syn) expression in eGFP+ motor axon terminals and αBTX labeling. (f) Coincidence of synaptobrevin (Syb) expression in eGFP+ motor axon terminals and αBTX labeling. *(From Wichterle et al. (2002). Reproduced with permission.)*

that motor neurons derived from ES cells can populate the embryonic spinal cord, extend axons, and form synapses with target muscles (Fig. 64.8). Therefore, they not only participate in normal development but also grow appropriately when transplanted into the embryonic spinal cord, targeting muscle. Thus, inductive signals in normal pathways of neurogenesis can direct ES cells to form specific classes of CNS neurons.

Oliver Brustle, Ian Duncan, and Ron McKay were the first to transplant progenitors derived from ES cells into the adult and embryonic spinal cord [74]. They demonstrated that ES cells that are transplanted into brain and spinal cord of normal adult animals can differentiate into oligodendrocytes that can myelinate axons. They generated OP efficiently by first supplementing cultures of ES cells with fibroblast growth factor (BFGF) and epidermal growth factor and later including PDGF. About 38% of the cells in the resulting cultures were oligodendrocytes. To investigate whether these oligodendrocytes could myelinate *in vivo*, cells grown in the presence of BFGF and PDGF were injected into the spinal cord of one-week-old myelin deficient rats, a model for a human myelin disorder. Two weeks after transplantation, numerous myelin sheaths were detected in six of the nine affected rats. The original 100,000 cells had migrated widely, and they made myelin with appropriate ultrastructure. Therefore,

chick motor neuron marker protein SC1 (i, j). eGFP−, NCAM+ axons cross the floor plate but do not project out of the spinal cord (arrows, g, h). (k—n) Transverse sections of thoracic spinal cord at stage 27, after grafting of embryoid bodies grown with RA (2 μM) and anti-Hh antibody (5E1, 30 μg/mL). No mouse-derived motor neurons were detected by either eGFP (K) or a mouse-specific anti-HB9 antibody (l). In contrast, many mouse-derived NCAM+ (m) and Lim2+ (n) interneurons are present. (o—q) Transverse sections through stage 27 spinal cord at thoracic (o, p) and lumbar (q) levels after grafting embryoid bodies enriched with motor neurons. eGFP+ motor neurons are concentrated in the ventral spinal cord. Ectopic eGFP+ motor neurons are located within the lumen of the spinal cord. eGFP+ axons exit the spinal cord, primarily via the ventral root, and project along nerve branches that supply axial (o—q), body wall (o, p), and dorsal and ventral limb (q) muscles. The pathway of axons is detected by neurofilament (NF) expression. eGFP+ axons are not detected in motor nerves that project to sympathetic neuronal targets. *(From Wichterle et al. (2002). Reproduced with permission.)*

glial precursors derived from ES cells and transplanted into the neonatal rat spinal cord migrated over several millimeters and differentiated into myelinating oligodendrocytes and astrocytes.

This group also transplanted precursors derived from ES cells into the cerebral ventricles of developing rodents (embryonic day 17) [74]. Three weeks later, proteolipid protein (PLP)-positive myelin sheaths were evident in a variety of brain regions. The cells' exogenous origin was confirmed by *in situ* hybridization with a probe to mouse satellite DNA. Importantly, there was no observable evidence of abnormal cellular differentiation or tumor formation.

In the same year, John McDonald, David Gottlieb, and Dennis Choi demonstrated for the first time that ES cells that had been induced to become neural cell precursors could be successfully transplanted into the injured spinal cord [75]. Examination of the spinal cord nine days after 1 million precursor cells were transplanted into a cyst caused by contusion injury showed that the cells had survived, grafted, migrated long distances, and differentiated into the three principle neural cell types: neurons, astrocytes, and oligodendrocytes (Figs. 64.9 and 64.10). This group also demonstrated that transplantation was associated with a significant and sizable improvement in function. Their study was the first demonstration that transplanted embryonic precursors can successfully repair the damaged adult nervous system, an important finding, given that conditions in the damaged adult nervous system are much less favorable for regeneration than those in the neonatal spinal cord. Subsequently, Liu and colleagues [76] demonstrated that precursors derived from ES cells and transplanted into the injured adult nervous system can remyelinate neurons in an anatomically appropriate manner (Fig. 64.11). Furthermore, they showed that oligodendrocytes derived from ES cells functioned normally, myelinating multiple axons in culture, just as they do in the normal nervous system (Fig. 64.12). Using patch clamp analysis, Jim Huettner and John McDonald later demonstrated that the physiologic characteristics of such oligodendrocytes are similar to those of oligodendrocytes taken from the adult spinal cord (Fig. 64.12c and Fig. 64.13). Oligodendrocytes derived from ES cells represent the entire oligodendrocyte lineage, from early OP to mature, myelinating oligodendrocytes.

In culture, neurons derived from ES cells differentiate rapidly and spontaneously create neural circuits with anatomical (Fig. 64.14) and physiological evidence of excitatory and inhibitory synapses [72,77,78]. Substantial neural differentiation is also evident *in vivo* in the model of contusion injury [75], where neurons show extensive axonal outgrowth with presumptive

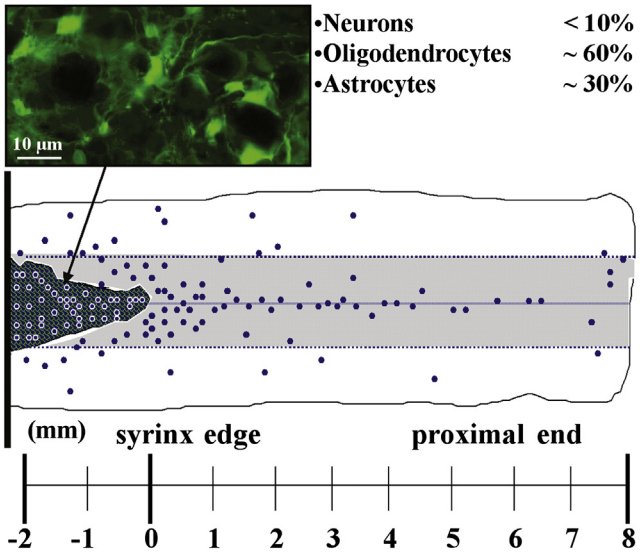

FIGURE 64.9
In the contusion-injured spinal cord of the rat, ES cell-derived neural precursors survive, migrate, and differentiate following transplantation. The schematic demonstrates the relative distribution of ES-derived cells two weeks after transplantation into the central cavity nine days after spinal contusion injury. The cavity is partially filled, and cells have migrated long distances. Most of the distant cells are identified as oligodendrocytes, while astrocytes and neurons remain restricted to the transplantation site. Left top inset shows GFP-expressing ES cell-derived neural cells.

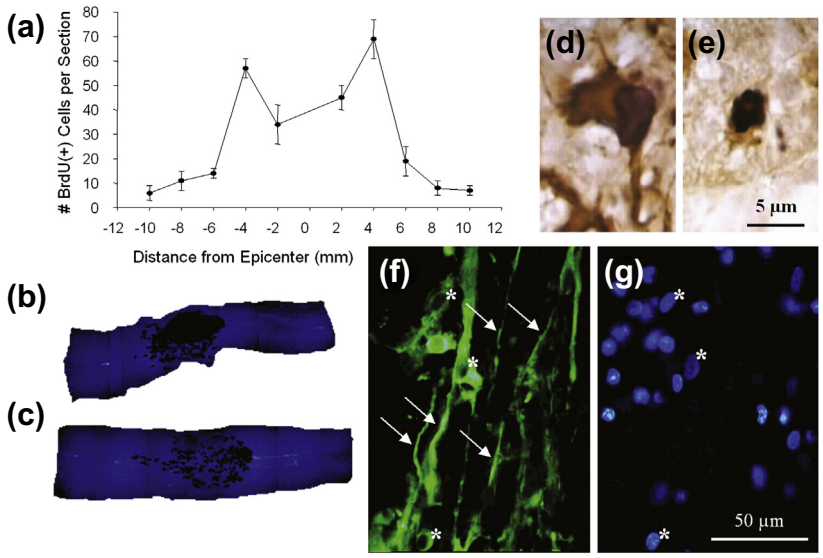

FIGURE 64.10

BrdU-labeled ES cell-derived cells two weeks after transplantation. Mean ± SEM BrdU-labeled nuclei per 1 mm segment in longitudinal sections. (a). Hoechst 33342-labeled sections 42 days after injury, transplanted with vehicle (b) or ES cells (c) nine days after injury. BrdU-positive cell colabeled with GFAP (d). BrdU-labeled cell colabeled with APC CC-1 (e). The mouse-specific marker epithelial membrane antigen (EMA) indicates processes (arrows) emanating from embryonic stem (ES) cells (f). Corresponding nuclei are marked by asterisks (g). *(Modified from McDonald et al. (1999) with permission.)*

morphological evidence of synapse formation. Moreover, immunological evidence of cholinergic, serontonergic, GABAergic, glycinergic, and glutamatergic neurons has been obtained *in vitro* and *in vivo* (data not shown).

Implantation, survival, and migration of transplanted ES cell-derived precursors in the damaged spinal cord have been verified with real-time polymerase chain reaction (PCR) and also by using magnetic resonance imaging (MRI) to track oligodendrocytes prelabeled with paramagnetic agents. In both cases, migration up to 1 cm from the transplantation site was evident [79].

After other groups demonstrated that multiple rounds of transplantation can deliver different types of stem cells to the CNS, our group systematically evaluated intravenous, intraventricular, intrathecal, and interparanchemal transplantation of embryo bodies derived from ES cells using our 4−/4+ protocol (four days in culture without retinoic acid followed by four days with retinoic acid). Although some of the cells that were transplanted intravenously entered the CNS, they remained in blood vessels and showed only limited neural differentiation. Intracerebral ventricular administration enabled some transplanted ES cells to be incorporated into the CNS, but this approach had to be discontinued because the cells obstructed the third and fourth ventricles, causing hydrocephalus. However, we made a remarkable observation when we transplanted a large number of ES cell-derived neural precursors intrathecally into the lumbar spine [80]. Three to four months later, ES cells were observed in well-formed tissues surrounding the lumbar sacral nerve roots and extending down the sacral canal. Further evaluation clearly showed that the cells had differentiated into tissue that bore a remarkable resemblance to some components of the normal spinal cord. For example, extensive myelination and cells resembling cholinergic motor neurons were observed. Zones of the peripheral nervous system were also clearly demarcated from the CNS. These results were quite surprising because intraparenchymal transplantation into the spinal cord characteristically produces abnormal macro-organization of cells. Thus, this was the first demonstration that transplanted ES cells can organize themselves into CNS-like tissues. It will now be important to understand the constraints required to produce this degree of self-organization.

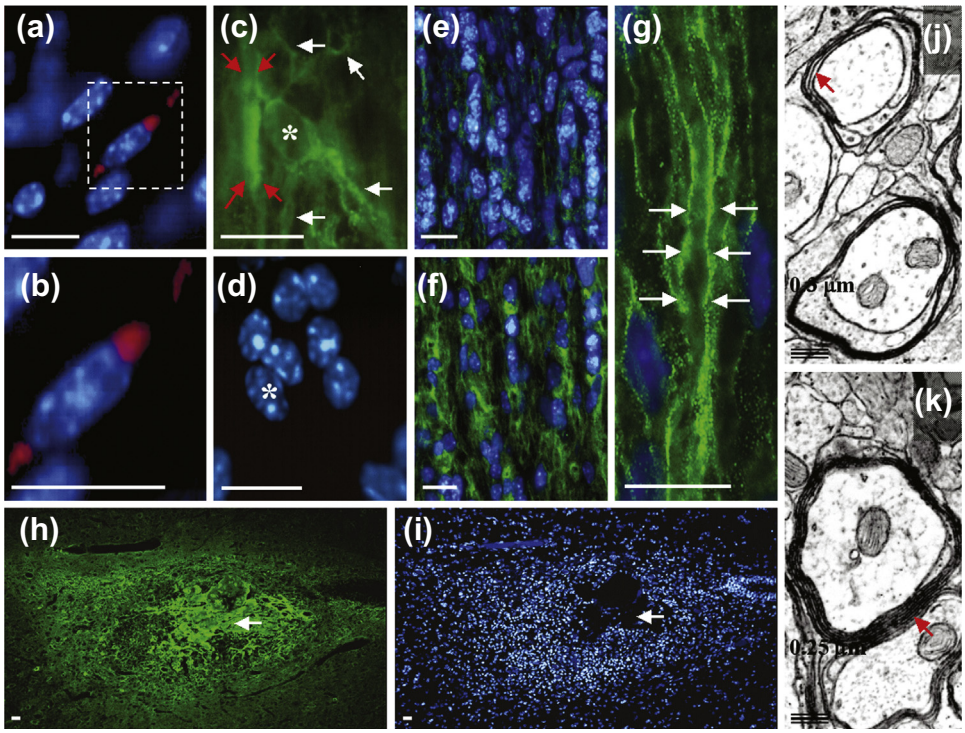

FIGURE 64.11
Cells derived from ES oligospheres can migrate and myelinate axons when transplanted into dysmyelinated spinal cords of adult Shiverer mice, which lack the gene for myelin basic protein (MBP). Transplanted cells were identified by cell tracker orange (CTO) epifluorescence (red) or immuno-reactivity for MBP (green). Hoechst 33342 (blue). CTO-labeled cells aligned with native intrafascicular oligodendrocytes in white matter (a, b). An ES cell-derived (MBP+) oligodendrocyte (asterisk) with longitudinally oriented processes (white arrows) is shown in panels c and d. Red arrows mark probable myelination around an adjacent axon (c). Little MBP immunoreactivity is seen in white matter in a longitudinal section of spinal cord from a mouse that received a sham transplantation (e). A gradient of MBP immunoreactivity centers on the site of ES cell transplantation (f). Panel g (high magnification) shows intrafascicular oligodendrocyte nuclei (blue) and MBP immunoreactivity (green), two indications of axonal myelination (white arrows), in white matter from a mouse transplanted with ES cells. The spatial distribution of MBP immunoreactivity one month after ES cell transplantation is shown at low magnification (h), with corresponding Hoechst 33342 counterstaining (i). White arrows indicate the center of the transplanted site. Transmission electron microscopy shows four loose wraps of myelin, the maximal number of layers typically seen around axons in control animals (red arrow, j), and nine or more compact wraps around axons from the transplanted area (red arrow, k). Shiverer-mutant mice lack a functional MBP gene that is required to form mature compact myelin; therefore, the presence of mature compact myelin is a standard for transplant oligodendrocyte-associated myelin. Scale bars: a–i = 10 μm; j, k = 0.3 μm. *(Reproduced with permission from Liu et al. (2000).)*

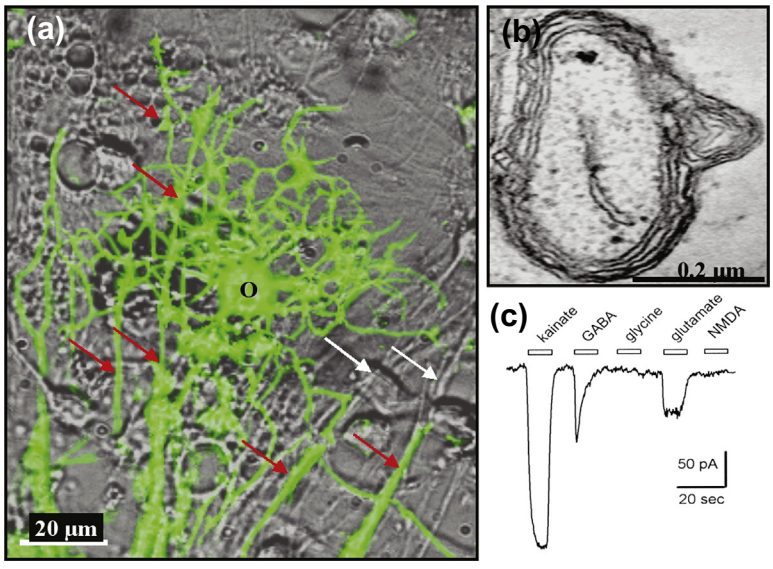

FIGURE 64.12
ES cells produced mature oligodendrocytes with normal anatomical features (panel a and b) of myelination and physiological response to neurotransmitters (panel c). *(Reproduced in adapted form with permission from Liu et al. (2000).)*

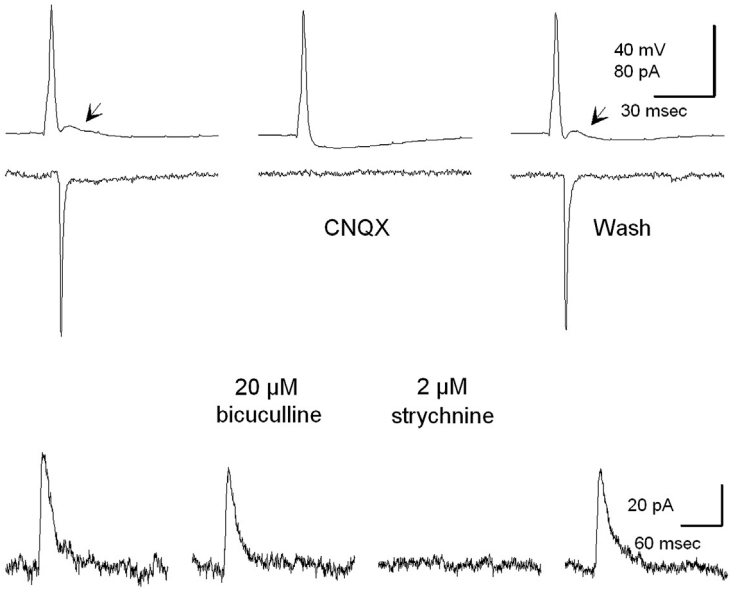

FIGURE 64.13

Excitatory (top) and inhibitory (bottom) synaptic transmission between neurons derived *in vitro* from mouse ES cells. Action potentials were evoked in the presynaptic neuron by current injection. Excitatory post-synaptic currents, blocked by superfusion with the selective glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 micromolar), were recorded under voltage clamp. Arrows point to an autaptic excitatory synaptic potential that is also blocked by CNQX. Inhibitory synaptic currents were evoked in a different ES cell pair and tested sequentially with the antagonists bicuculline and strychnine, which are selective for gamma-aminobutyric acid (GABA) and glycine receptors, respectively. For this cell pair, only the post-synaptic currents are shown. Transmission was blocked by strychnine, indicating that glycine was the transmitter. Other presynaptic cells evoked bicuculline-sensitive synaptic currents, indicating that GABA mediated transmission (not shown).

NOVEL APPROACHES TO CNS REPAIR

Most neural transplantation studies, including those using ES cell-derived neural progenitors, have used cells to replace cells lost after injury. Given that we do not know which types of differentiated cells are required for repair, neural precursors may be ideal for this purpose because environmental clues can decide their developmental fate [68,76,81]. Moreover, such precursors can serve as bridges to support axonal regrowth [64].

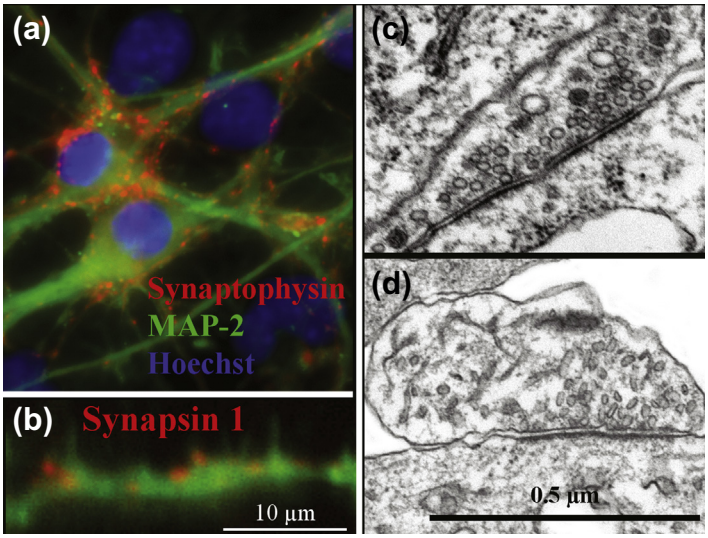

FIGURE 64.14

ES cells differentiate into neurons that spontaneously create neural circuits. Presumptive presynaptic sites (red) oppose dendrites (green) in (a) and (b). (c) and (d) demonstrate ultrastructural characteristics of synaptic profiles *in vitro*.

To try to overcome the physical and chemical constraints imposed by the injured nervous system, ES cells are often genetically altered so they will deliver growth molecules, such as neurotrophin-3, after transplantation [19,24,81], and they are particularly well suited to this task. McDonald and Silver recently adopted a novel approach to overcoming restraints by showing that early progenitors of ES cells can phagocytize key inhibitory molecules in glial scar tissue [82]. These cells therefore created an inhibitor-free bridge over which axons rapidly sprouted from the transplant into normal cord. By nine days after transplantation, axons from the graft had grown up to 1 cm — a rate of more than 1 mm per day! This rate is similar to that seen in the normal embryo [83].

Another novel approach is to use stem cell transplantation to limit the secondary injury that occurs after nervous system injury [84]. For example, we demonstrated that transplanting ES cells can limit the delayed death of neurons and oligodendrocytes. Since most transplantations are performed at the time of injury, it is possible that many of their results may be attributable to this neuroprotective role. It seems clear that even genetically unmodified ES cells release large quantities of growth factors.

Although replacing lost neurons is difficult, it might be possible to use neuronal replacements to create bridging circuits across the injury site. Descending axons could synapse onto ES cell-derived neurons that subsequently synapsed onto key pattern generators in the lower spinal cord. More global delivery of neurotransmitters via synaptic or non-synaptic mechanisms might enhance functions such as locomotion. Previous work by others has demonstrated that release of noradrenergic and serotonergic neurotransmitters can stimulate and enhance the central pattern generator for locomotion [85]. More recent work indicates that release of brain-derived neurotrophic factor and neurotrophin-3 can perform this function [86].

Because ES cells are embryonic in nature, neural progenitors derived from them may be able to reprogram the adult CNS so that damage can be repaired [87]. ES cells are also unique in that they have the potential to replace cells derived from multiple embryonic germ layers. In the CNS, it will be important to replace lost non-neural cells as well as neural cells to regain normal CNS function, such as neovascularization. Growing recognition of the link between neovascularization and neurogenesis is strengthening this approach. We found that ES cells in transplanted 4−/4+ embryoid bodies differentiate into both neural cells and vascular endothelial cells (unpublished observations).

In the embryo, stem cells differentiate into motor neurons and grow toward muscle, where they form neuromuscular junctions. Using ES-derived motor neurons to replace lost motor neurons at injured segments will not enhance function because chronically denervated muscle is unable to reassemble functional neuromuscular junctions. However, transplantation might avoid the long-term muscle wasting that results from denervation. To maintain denervated muscle, for example, it might be possible to transplant ES cells into the distal nerve stump or directly into the muscle. Once in place, the transplanted cells could differentiate into motor neurons. If they were genetically altered, the motor neurons could be selectively removed once the repair was properly achieved.

More recent studies are beginning to combine stem cells, including ES cells, with biomaterials. The advent of nanotechnology should further enhance this approach [88,89].

Finally, remyelination is one of the most pragmatic approaches to restoring function to the damaged spinal cord (Fig. 64.15). Because many potentially functional connections remain in the outer donut of surviving tissue, appropriate remyelination could substantially improve function. One must consider, however, that dysmyelination rather than demyelination is the biggest problem in the damaged nervous system. Often, inappropriate myelination is more harmful than no myelination at all.

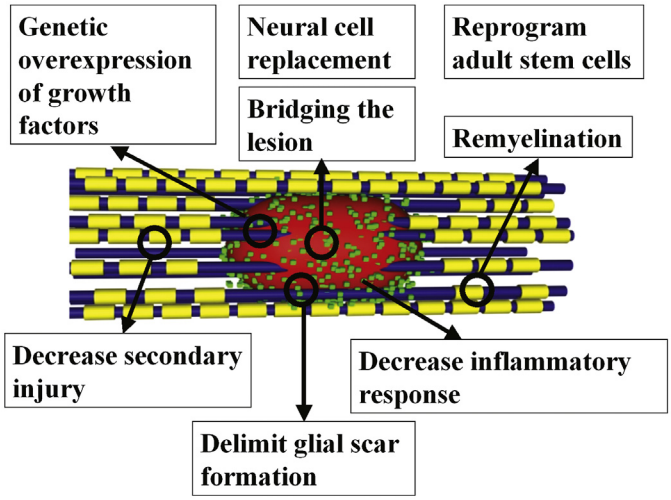

FIGURE 64.15

Novel approaches to spinal cord repair using ES cells. Remyelination is one of the most pragmatic approaches to restoring function to the damaged spinal cord. ES cell-derived precursors can serve as bridges to support axonal regrowth. They can phagocytize key inhibitory molecules in glial scar tissue. These cells therefore created an inhibitor-free bridge over which axons sprouted rapidly from the transplant into normal cord. Newly generated neurons can be used to create bridging circuits across the injury site. ES cell transplantation can limit the secondary injury. To try to overcome the constraints of the injured nervous system, ES cells are often genetically altered so that they will deliver growth molecules, such as NT3, after transplantation. Because ES cells are embryonic in nature, their progenitors may also be able to reprogram the adult CNS to optimize spontaneous host regeneration. Replacement of non-neural cells as well as neural cells will be important for regaining normal CNS function, such as neovascularization. ES cells can have similar applications in the peripheral nervous system.

TOWARD HUMAN TRIALS

Some of the animal studies described previously have prompted early human safety trials of ES cell transplantation. Most have focused on PD, where unforeseen exaggeration of dystonia has limited advances in transplantation therapy [90]. Phase I/II human trials for repairing the spinal cord are also under way. They include transplantation of porcine-derived stem cells for the purpose of remyelination (Diacrin) as well as allogeneic transplantation of olfactory ensheathing glia (Carlos Lima; Lisbon, Portugal). Although safety data are not yet available, it is important to note that such trials are paving the way for further uses of ES cells. With the recent advent of human ES cells, human transplantation appears more feasible. The California based research company Geron performed the first human ES cell research trial approved in the United States for individuals with acute traumatic SCI. A total of four patients were treated in this study. Unfortunately the company dropped its study in November 2011 amongst financial concerns. To date there have been no safety problems reported.

Considering the recent advancements in generating induced pluripotent stem cells raises the possibility that ethically acceptable procedures for human stem cell generation is on the horizon that will hopefully lead to quick translation into clinical care.

CONCLUSIONS

Studies of neurotransplantation for repairing the damaged nervous system are making good progress. Early animal studies showed that mouse ES cells can replace neurons, astrocytes, and oligodendrocytes, instigate appropriate remyelination, and even improve locomotion. As is the case for all transplantation studies, however, the mechanisms underlying functional improvement remain unclear. Nevertheless, ES cells offer a novel approach to deciphering these mechanisms. For the first time, ES cell genetics is allowing investigators to track

transplanted cells that have been engineered to express green fluorescent protein. This and other types of genetic modification permit one to track the integration and differentiation of ES cells, assess behavioral recovery, selectively remove the cells to see whether recovery wanes, and insert more cells to see if functional recovery occurs anew. This proof of principle will be required to identify mechanisms in neural repair.

Although it is impossible to predict the future of the field, it is clear that IPS and human ES cells are tools that will revolutionize neurobiology and neural transplantation by providing the unprecedented ability to selectively deliver key growth and regulatory factors. Also, ES cell transplantation promises to be one of the greatest potential therapies for chronic nervous system disorders. Only the future will reveal its full potential for treating human disease and disability, but it is possible to say with confidence that ES cells will have a major impact on repairing the human CNS within our lifetimes.

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CHAPTER 65

Protection and Repair of Hearing

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INTRODUCTION

Deafness is the USA's number-one disability, with hearing loss affecting more than 35 million individuals. Hearing loss can have a genetic cause, be acquired from causes such as ototoxic drugs, noise overstimulation or disease/trauma, or can arise from a combination of genetic and environmental influences such as occurs with age-related hearing loss. Close to one half of all hearing losses are thought to be of genetic etiology. Acquired deafness on average affects one in four to five individuals, but the incidence increases significantly with age when presbycusis (age-related hearing loss) becomes a major problem in the elderly population, affecting one in three over the age of 65 and 50% of individuals over the age of 75.

An improved understanding of the mechanisms underlying genetic, acquired and 'mixed' deafness has driven development of new interventions and treatments as well as refinement of previous approaches to prevent hearing loss and restore hearing after deafness. Hearing loss induced by cochlear damage from overstimulation, ototoxic drugs, trauma, infections and/or aging can be reduced by relieving oxidative stress to the cochlea with antioxidants and enhancing endogenous protective systems with agents such as neurotrophic factors and heat shock proteins. Genetic modification to induce regeneration of hair cells following their loss has now been shown in animal models and may become clinically feasible, with development of safe and effective vectors. Use of endogenous stem cells and placement of exogenous stem cells is being developed as a strategy to replace lost hair cells or auditory nerve and to supplement remaining function. Survival factors can be applied to the cochlea to improve auditory nerve survival following deafness and to induce regeneration of its peripheral processes. Major advances have also been made in region specific delivery of therapeutics to the cochlea to allow these interventions, including gene transfer, microcannulation and biomaterials placed into the middle ear or directly into the cochlea. Auditory prostheses are being refined, with significant improvements occurring in cochlear prostheses as well as in development of central auditory prostheses. Prostheses can also provide a mechanism for application of therapeutics through microchannels. Biomaterials on and associated with prostheses not only can improve their function and histocompatability but also can provide a means for delivering therapeutics. The strategies for tissue-engineering interventions based on recent scientific discoveries are the focus of this chapter.

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INTERVENTIONS TO PREVENT HEARING LOSS/COCHLEAR DAMAGE

Oxidative stress

Oxidative stress, the overproduction of reactive oxygen species (ROS; free radicals) in the cell, has now emerged as a common mechanism by which many traumatic insults, including noise and ototoxic drugs cause damage to the inner ear. The formation of ROS, such as super-oxide, hydroxyl radical, and nitric oxide, may occur by different mechanisms depending upon the stress, but in each it appears to occur early and throughout the traumatizing events. ROS can be formed by non-enzymatic reactions via redox-active iron complexes of drugs [1,205] and by enzymatic reactions in the cell. On the cellular level, oxidative stress may originate in the mitochondria [2] or may be due to activation of NADPH oxidase via Rho-GTPases, a primary source of super-oxide radicals [3].

In an unchallenged cell, ROS are products and by-products of normal metabolism and are contained within a physiological range (redox homeostasis) by endogenous antioxidant systems. An excess of ROS, however, will cause cellular damage, ultimately triggering cell death pathways of apoptosis and necrosis. Because tissue levels of endogenous antioxidants can be influenced by external manipulations, ROS mediated hearing loss is a potential target for interventions to prevent or reduce hearing loss.

Prevention of ototoxicity

Aminoglycoside antibiotics, including neomycin, kanamycin, tobramycin, amikacin, and gentamicin and the chemotherapy anti-cancer agent cisplatin (CDDP) are the most common drugs causing hair cell loss and, consequently, hearing loss (ototoxicity). Although this review will emphasize the cochlear effects of ototoxic agents, we should note that aminoglycosides can also cause vestibular toxicity and loss of balance. ROS increase significantly in inner ear tissues following aminoglycoside treatment both in organ explants and *in vivo* [205]. Conversely, cellular antioxidant defense system constituents, such as glutathione, decrease after aminoglycoside treatment [4].

The resulting redox imbalance resulting from increased ROS production and decreased antioxidant function then initiates competing signaling pathways of cell death and survival. involving intracellular organelles such as mitochondria and lysosomes and a network of interfacing signaling systems based on enzymatic actions (e.g., activation of proteases) and changes in gene expression (e.g., via transcription factors or epigenetic modifications). Indicative of this complexity, cell death by aminoglycosides includes both apoptosis and necrosis of hair cells in cochlear and vestibular organs [5–7]; and both caspase-dependent and caspase-independent pathways appear to contribute to hair cell pathology. Caspase-3 has also been implicated in hair cell death, primarily in *in vitro* models of explant cultures and isolated cells or in the vestibular system (e.g., reference [8]). The pro-apoptotic mediator c-Jun NH2-terminal kinase (JNK) apoptotic pathway is often involved, since pharmacological inhibitors (e.g., CEP-1347) can offer some protection *in vitro* from aminoglycosides [9,10]. Like the caspase pathway, involvement of the JNK pathway is reported mostly in cultured explants but may contribute to the overall pattern of cell death *in vivo*. A dominance of caspase-independent cell death emerges in a chronic drug treatment model, where the onset of cochlear deficit is delayed and continues to develop after the cessation of treatment, akin to the clinical situation. In this model, activated calpain and cathepsins are the major mediators of cell death [3]. Most recently, inhibitors of histone deacetylation have emerged as promising candidates for clinical application. Two histone deacetylase inhibitors, trichostatin and butyrate, can reduce gentamicin ototoxicity in experimental models by inhibiting the epigenetic histone modifications induced by aminoglycoside treatment.

The establishment of the mechanisms underlying both apoptotic and necrotic cell death in ototoxicity provides a basis for a rational approach to prevention. For example, small synthetic molecules designed to inhibit one of the many steps in the apoptotic cascade can potentially stave off cell death. For a clinical application, however, a systemic application of such powerful signaling molecules may have far-ranging physiological consequences, particularly when applied to drug treatment, which may last for weeks. Local gene therapy, the process of virally introducing a gene into a tissue may be more suitable in such a situation [11]. However, given the complexity of cell death mechanisms, targeting a single pathway may not be sufficient as the inhibition of one might be bypassed by the activity of others.

The currently most applicable method of prevention is antioxidant therapy, which has become a successful clinical approach to many pathologies that involve free radicals. This type of intervention would also act directly on the ROS upstream of the ensuing cell death pathways and therefore suppress toxic mechanisms at the very onset. A wide variety of antioxidant molecules have been shown to attenuate ototoxicity *in vivo* constituting the most compelling support for ROS as major mediators of aminoglycoside induced hearing loss [1,7,205]. The attenuation achieved in animal models can be dramatic. For example, a gentamicin-induced hearing loss of 60−80 dB could be reduced to a negligible loss of 10 dB or less [12]. Since neither the serum levels of the drugs nor their antibacterial efficacy are compromised, antioxidant therapy provides a promising approach for a clinical application.

A clinically feasible prophylactic therapy requires drugs that by themselves are non-toxic and easily administered to the patients. One such example that emerged from laboratory studies is salicylate, the active ingredient of aspirin [13]. The efficacy of aspirin was tested in a randomized double-blind and placebo-controlled study in patients receiving gentamicin for acute infections [14]. Fourteen of 106 patients (13%) met the criteria of hearing loss in the placebo group, while only three out of 89 (3%) sustained a hearing loss in the aspirin group, for a 75% reduction in the incidence of ototoxicity. Aspirin did not influence gentamicin serum levels or the course of therapy. The protection against gentamicin-induced hearing loss with aspirin was confirmed by a second clinical trial [15]. Although aspirin is widely used in the United States as a daily prophylaxis against myocardial infarction, as well as for treatment of inflammation, fever, and pain, aspirin carries with it a small risk of gastrointestinal side effects. Other antioxidants utilized in clinical trials to date include vitamin E and N-acetylcysteine (NAC). NAC reduced hearing losses in a small study of patients receiving gentamicin for bacteremia and in a study of peritonitis patients receiving aminoglycosides for peritoneal dialysis [16,17]. In contrast and despite success in animal studies, vitamin E did not reduce hearing loss in a clinical study, underscoring the complexity of aminoglycoside responses in humans and the potential pitfalls of translation of animal studies to clinical medicine [18]. Cisplatin (CDDP) also generates ROS as well as reactive nitrogen species in the cochlea and several antioxidants have been shown to provide protection from CDDD induced ototoxicity including L-methionine, NAC, glutathione and ebselen [19]. Susceptibility to ototoxicity can be influenced by factors such as diet (e.g., [4], [20]) as well as genetic influences such as the mitochondrial mutations that can render people hypersensitive to aminoglycosides [21].

Prevention of acoustic trauma

The mechanisms of acoustic trauma-induced pathology are potentially more complex than those of drug-induced ototoxicity, including not only the formation of ROS but also vasoconstriction and direct mechanical damage to the organ of Corti [22]. In addition, the sources of noise trauma can be highly variable, ranging from a single high-energy impact, such as an explosion, to impulse noise to chronic noise exposure at various levels of intensity. Each of these conditions may induce variations in the mechanism of ROS formation or variations on cell death and survival pathways. Furthermore, while a protective therapy can be timed with drug administration, patients may report noise-induced hearing loss days, months,

or years into the trauma. To what extent a delayed intervention may be successful is yet another question that remains to be accurately resolved.

There is good direct evidence that ROS are formed in the inner ear following intense sounds, specifically in the stria vascularis and the organ of Corti. Indicators of ROS formation can be detected at the onset of the noise exposure and may persist for hours or even days following the exposure. Attesting to the importance of endogenous antioxidants, decreased glutathione in the inner ear enhances noise trauma, while dietary supplementation with glutathione attenuates these effects [22,23].

While the precise origin of ROS in the cochlea following noise exposure remains somewhat speculative, there are several sources most likely to contribute. A surge of super-oxide radicals followed by chain reaction formation of other free radicals (via Fenton-type reactions) may be due to increased mitochondrial activity, a prolonged tissue hypoxia due to vasoconstriction and a rebound hyperfusion [24]. Reactive nitrogen species may also arise from the activation or induction of the enzyme nitric oxide synthase. The different isoforms of this enzyme serve a variety of physiological processes in normal tissue metabolism, and the product of the enzymatic reaction, nitric oxide, is an important second messenger molecule. When produced in excess, not only is nitric oxide potentially damaging as a free radical, but it can also combine with super-oxide to produce the highly reactive and destructive peroxynitride. Nitric oxide levels can rise after noise exposure [25,26], perhaps as a consequence of an enhanced release of excitatory amino acids [27,28], and may contribute to the overall pathophysiology of noise-induced hair cell loss.

Multiple cell death pathways — partly independent and partly overlapping — may be triggered by a spectrum of radicals generated in response to noise. The fact that both necrosis and apoptosis are observed in noise trauma attests to the existence of multiple cell death pathways. This notion is also supported by the emergence of caspase-dependent and caspase-independent cell death pathways and the involvement of transcription factors that code for different signaling cascades, such as AP-1, endo-nuclease G, and the calcineurin-related activation of BCL-2 family proteins [22].

Restoring redox balance is therefore one of the potential means of intervention in noise-induced hearing loss, the other being a manipulation and interference with cell death pathways. The latter has been done successfully in animal experimentations *in vivo* [9,29], but similar concerns may arise here as in the prevention of drug-induced hearing loss, in that such systemic intervention in important physiological processes may have unwanted consequences. Other approaches successful in animals may also be impractical for the clinic. For example, the upregulation of antioxidant defenses by systemically administered R-phenyl isopropyl adenosine has adverse physiological effects [30]. A local application may bypass such a problem but is more invasive and affords less control over drug concentrations.

As with ototoxicity, antioxidant therapy is currently the most applicable approach for prevention and/or treatment of noise-induced hearing loss (NIHL), with a variety of drugs as possible protective agents (see reference [31]). NIHL can be attenuated effectively by scavengers such as super-oxide dismutase and allopurinol, antioxidants, and iron chelators and by antioxidant drugs that may have clinical appeal, such as ebselen or *N*-acetylcysteine (NAC) [32]. Multidrug combinations are promising, for example, acetyl-l carnitine with d-methionine or NAC [33,34]. Alternative strategies aimed at vasodilation and providing adequate blood flow to the cochlea during noise exposure may also indirectly relate to ROS formation. Drugs such as pentoxifylline and sarthran improved blood flow to the cochlea and also reduced the temporary effects of noise exposure [35,36]. Magnesium supplementation can maintain cochlear blood flow under noise exposure and reduces the resulting hearing loss [37]. A combination called 'ACE-Mg' consisting of several antioxidants (Vitamins A, C and E) acting by different mechanisms and magnesium as a potential vasoactive agent has proven effective in preventing NIHL in animals [38,39] and is now in clinical trials [39].

The translation from animal models to practical situations in humans has been partially successful in tests on populations exposed to high levels of noise during military exercises and leisure activities. These studies also clearly show the influence of genetic and physiological condition on susceptibility to noise trauma and the limitations that these factors impose on protection. Daily supplementation with magnesium reduced the incidence of permanent threshold shifts (PTS) in military recruits from 21.5% to 11.2% [40] and, likewise, reduced temporary threshold shifts (TTS) in a group of young volunteers [41]. However, the severity of PTS was negatively correlated with monocyte Mg^{2+} content regardless of treatment. As another example, NAC showed significant protection on TTS in subjects with null genotype in glutathione S-transferase (GST) T1 and M1 [42]. In contrast, a group of nightclub visitors taking NAC sustained the same TTS as the control group [42a]. Ongoing trials on US Army and Navy recruits are currently exploring the efficacy of NAC and of ebselen [31] and ACE-Mg [39].

While most strategies for interventions rely on applying the protective treatment prior to or concomitant with noise exposure, recent evidence indicates that the formation of free radicals continues for days following noise exposure and that, consequently, an antioxidant treatment may still be effective for a short period post-exposure [22].

Anti-inflammatory agents

There is increasing evidence that inflammation contributes to the loss of sensorineural elements from both ototoxic drugs and noise and anti-inflammatory agents are therefore another approach to prevention and treatment [43]. Reducing the inflammatory response by controlling the STAT-dependent pathways decreased cisplatin ototoxicity [44]. Likewise, the corticosteroid dexamethasone has been shown to provide protection against several ototoxic agents as well as the trauma from insertion of a prosthesis into the cochlea [45,46] as well as providing protection from noise [47]. It should also be kept in mind that the immune response can play an important and necessary role in response to stresses to the cochlea, when considering prevention and there may be occasions when enhancement rather than reduction provides benefit.

Heat shock proteins

Heat shock proteins (HSPs) provide another natural protective mechanism against various forms of stress. HSPs can achieve their protective role by influencing the stress-related denaturation of proteins (either reducing the denaturation or enhancing renaturation and regaining the correct tertiary structure), as chaperones or through an influence on cell death cascades. Several families of HSPs (commonly designated by their molecular weights) include HSP 25/27, HSP32, and HSP 70/72. Expression of the inducible form of HSP 70 in the cochlea has been shown in response to heat, transient ischemia, noise, and ototoxic drugs [48,49]. This expression is generally found in the sensory hair cells and is transient, peaking three to seven hours following the stress. HSP 27 is found constitutively (without the need for induction by stress) and has a more widespread distribution, not only in sensory hair cells, but in supporting cells and the lateral wall tissues [50]. The levels increase following stress. HSP32 has a small constitutive expression and is also upregulated following stress in hair cells and stria vascularis [51].

A protective role for HSPs in the inner ear is suggested by two types of studies. In one type of study, HSP levels are upregulated by an initial innocuous stress, either low-level noise [52] or controlled heat [53], followed by a noise exposure that would normally cause a significant hearing loss. Either pre-exposure resulted in a significant (30-dB) reduction in noise-induced hearing loss and hair cell loss. Similarly, pre-exposure to a heat stress protected hair cells in the utricle from aminoglycoside induced loss [54]. Geranylgeranylacetone, a drug that can induce induction of heat shock protein 70 (Hsp70) provided protection from noise-induced [55] loss of hair cells and hearing. Celastrol induced upregulation of HSPs was shown to protect

against aminoglycoside induced hair cell loss in the utricle via HSP32 induction [56,57] used transgenic mice that overexpress HSP70 to show protection from aminoglycoside (kanamycin) induced hair cell loss and hearing loss. Heat shock factor 1 (HSF1) is a major transcription factor for the HSPs, present in hair cells and stria vascularis in the rodent cochlea [58], and in mice in which HSf1 has been knocked out there is decreased protection from noise, with increased hearing loss and increased loss of hair cells [59,60].

Neurotrophic factors

Neurotrophic factors play an important role in the development of the cochlea, in regulating differentiation of sensory and neuronal elements, as well as in the formation of afferent and efferent connections (for review see references [61,62]). Many neurotrophic factors and/ or their receptors remain in the mature cochlea, including Glial Cell Derived Neurotrophic Factor (GDNF), Neurotrophin 3 (NT3), and Brain Derived Neurotrophic Factor (BDNF) (see [62,63], for reviews), where they play a role in cellular homeostasis. Neurotrophic factors can have a protective action against stress through a mitigating influence on ROS or intervention in the cascade of events induced by reactive oxygen species (ROS) formation. As a case in point, removal of neurotrophic factors leads to an increase in ROS and cell death through the apoptotic pathways discussed previously. While removal of neurotrophic factors is pro-apoptotic, neurotrophic factors themselves can induce pro-survival pathways and provide protection. Neurotrophic factors may reduce oxidative stress-driven increases in intracellular Ca^{2+} [64] through induced expression of calcium-binding proteins [65] or antioxidant enzymes [64].

Studies have shown that infusion of neurotrophins into the inner ear fluids prior to stress provides protection from hearing loss (for reviews, see references [49,66]). When levels of glial line-derived neurotrophic factor (GDNF) or neurotrophin 3 (NT3) are increased in the inner ear prior to noise overstimulation or the administration of ototoxic drugs there is significant protection, with decreased hearing loss and hair cell loss, [66–71]. This protection is less effective if the neurotrophic factors are provided after the stress rather than before, suggesting that they play a greater role in protection than in repair. BDNF and FGF were less effective in preventing hair cell loss [70,72].

HAIR CELL REGENERATION BY TRANSDIFFERENTIATION

Birds and other non-mammalian vertebrates can regenerate hair cells spontaneously [73,74]; using a mechanism of transdifferentiation, with or without a proliferative stage [75–78]. In contrast, once hair cells of the mammalian cochlea are lost, they will not regenerate spontaneously [79]. Placing new hair cells in ears with severe or complete hair cell loss has been a major goal of tissue-engineering efforts, because the only possible way to restore hearing via biological means is by replacing the missing hair cells. The main approaches for replacing hair cells are introduction of new cells from an exogenous source such as stem cells, or converting endogenous cells that have a non-sensory phenotype to become new hair cells. Methods for generating stem cells and guiding their differentiation to the hair cell phenotype have been recently reviewed [80,81], and the challenges for incorporating these cells into the target tissue (the deaf auditory epithelium) are now being addressed. The discussion of stem cell therapy for inner ear disease is found in a dedicated section of this chapter, below. This section reviews approaches for inducing transdifferentiation of existing (endogenous) cochlear cells to become new hair cells. This approach, once successful, may be applicable to deafness due to most causes, with the exception of hereditary deafness, because the impact of the mutation is not addressed by transdifferentiation therapy. Several extensive and recent reviews on hair cell replacement strategies are available [82–86].

Hair cells and supporting cells develop from common progenitor cells [87]. Their phenotype is determined by a set of genes expressed in a sequence and influencing neighboring cells in an

interactive way, with additional influence of microRNAs [88]. Some of the genes that signal for the differentiation of hair cells have been identified [87,89,90–94]. Knowledge of the developmental genes that regulate the proliferation and differentiation of hair cells during development is essential for designing hair cell regeneration strategies, because reactivation of these developmental signals is likely needed to induce a phenotypic change in the non-sensory cells. Technological means for manipulating gene expression in the mature ear are also necessary. Progress in these two fields, developmental biology and manipulation of gene expression, has allowed for important recent breakthroughs in the advance toward inducing cochlear hair cell regeneration.

The function of several gene families in hair cell development has been characterized by manipulating expression of these genes in mice. In general, these transgenic manipulations use several strategies for increasing or decreasing levels of gene expression, in fashions that can be temporally regulated and cell specific. The Cre-loxP technology has been especially useful for inducing conditional gene expression inhibition in a cell specific manner in the inner ear [95]. With the use of mouse transgenesis, it is possible to eliminate generation of hair cells [90,96] or to induce ectopic hair cell formation next to the organ of Corti [97,98].

One of the genes most commonly used for altering the number of hair cells in a cochlea is *Atoh1*. *Atoh1* is a gene encoding a transcription factor that is necessary and sufficient for hair cell differentiation in the developing epithelial ridges [90,96,99]. Since *Atoh1* is a master regulator gene for hair cell development, manipulating its expression levels has been used to generate new hair cells both in transgenic animals and by overexpression using other methods. Ectopic expression of *Atoh1* is able to induce the formation of clusters of hair cell and supporting cells outside the area of the organ of Corti in explants [90]. Similarly, ectopic hair cells could be found after virally mediated expression of *Atoh1* in non-sensory areas of the auditory epithelium in a mature guinea pig ear *in vivo* [100,101] and in culture [102]. Together, these studies have shown that the non-sensory cells retain the competence to respond to developmental genes and to convert to new hair cells after the onset of deafness, but the ability of the auditory epithelium to respond to overexpression of Atoh1 appears to diminish in mature animals and after the tissue is severely traumatized [103,104].

New hair cells induced by overexpression of *Atoh1* had the surface morphology of hair cells but their detailed morphology was immature [101,105]. Nevertheless, new hair cells that are generated by *Atoh1* overexpression appear to be electrophysiologically active, and several aspects of the maturation process recapitulate the normal development of hair cells [104,106]. These data are encouraging for our future ability to improve auditory thresholds in human ears treated with hair cell regeneration.

The transition from lab work to clinically feasible methods will depend on resolving several difficulties related to:

a) Gene delivery methods,

b) Limited response in mature animals and

c) Lack of response in ears with severe deafness.

The first challenge, gene delivery, has been partly met by the demonstration that adenovirus (or other) vectors that are inoculated into the scala media induce expression of transgenes in the supporting cells, as demonstrated in guinea pigs, rats and mice [107–109]. Still, this route of inoculation is not easily accomplished in the human ear. Designing treatment for ears that have been deaf for some time or suffered a severe trauma that leads to formation of a flat epithelium in place of the organ of Corti is a major challenge, because many of the ears presenting for therapy have some or many areas with a flat epithelium. While treatment with *Atoh1* alone induces no regeneration in the flat epithelium [103], it is possible that combining the transdifferentiation step with forced cell proliferation in the organ of Corti or its

vicinity will yield better morphological and physiological restoration of the tissue. Finally, to advance such therapies toward clinical applications, it will be necessary to test and optimize efficiency and safety, enhance the efficiency in ears that have been deafened for a long time, assess the physiological gains versus side effects (tinnitus, distortion) and design a way to introduce the transgene specifically to the target cells. It is also likely that non-viral methods for inducing gene expression in the cochlea will become available and feasible. Among these methods are nanoparticles that carry genetic material, and siRNA for blocking expression of specific genes at the RNA level [110–115].

Non-viral strategies for manipulating pathways that determine the phenotype of cells in the auditory epithelium have several advantages, especially reduced risk of side effects associated with viruses and increased ease of delivery. For instance, reagents that can mimic Atoh1 action and induce transdifferentiation may be attractive alternatives to viral vectors. Notch signaling molecules are excellent targets for manipulations via viral or non-viral reagents because of their developmental roles in generating the mosaic pattern of hair cells and supporting cells in the organ of Corti [116,117]. Manipulating their levels in developmental stages has resulted in addition of supernumerary hair cells [116,118–120]. Augmentation of the number of hair cells produced by Atoh1 was noted when Notch signaling was inhibited with DAPT 22573692. Blocking Notch signaling in mature animals was not as effective but the ability to induce formation of a small number of hair cells is nevertheless promising [121]. Once we unveil the changes that occur in supporting cells as they differentiate and mature, we could design measures to increase their responsiveness to transdifferentiation therapy. It is possible that combined influence of Notch inhibition and *Atoh1* overexpression will also yield regeneration of hair cells in mature and traumatized ears.

While the first and most basic goal may be generation of new inner hair cells that can depolarize the auditory nerve once an acoustic signal is detected, better hearing could be accomplished if some outer hair cells were also regenerated, and they could improve thresholds and tuning. It appears that the duration of *Atoh1* gene expression may determine whether a cell becomes inner or outer hair cell [122]. There may be cell-cell interactions as well as cell autonomous developmental choices that influence the responses of individual cells in the differentiating tissues, such that introducing *Atoh1* into the tissue may have an outcome beyond the proper cell that expresses the gene [104,123]. Other considerations include the route of gene transfer, choice of vector, limiting gene expression to the target cells and reducing side effects in the ear and elsewhere.

Because hair cell loss depletes the cell population in the organ of Corti, some of the genetic manipulations performed on mice were aimed at influencing proliferation in the cochlea. This is important because it may be possible for some of the new cells to become new hair cells without any further exogenous manipulations. In the normal mature ear, the $p27^{Kip1}$ gene is expressed in non-sensory cells and keeps them quiescent [124,125]. Mice with deficient expression of the $p27^{Kip1}$ gene exhibit an excessive number of hair cells in the organ of Corti. It appears that in the absence of cell cycle arrest, cells continue to proliferate after they normally would have stopped, had $p27^{Kip1}$ been active. Interestingly, mice with this induced mutation have a severe hearing loss, despite the large number of hair cells [125,126]. It is currently unclear whether the hearing deficiencies are due to dysfunction of the hair cells proper, or due to more general cochlear pathologies related to presence of ectopic hair cells. Inhibition of the cell cycle can also be removed by blocking expression of other genes, including Ink4d and Rb1 [127–129], leading to supernumerary hair cells and supporting cells in the tissue. The ability to use genetic manipulation for inducing proliferation in the organ of Corti is a considerable breakthrough that can be combined with transdifferentiation therapy and lead to feasible clinical applications.

The likelihood of treating an ear with severe to complete loss of hair cells and regenerating it to the state of a new, youthful and perfect ear is very low. Would incomplete/partial regeneration

be an acceptable and practical goal? It is likely that placing a population of new inner hair cells that is functional and innervated and large enough to provide useful auditory signals to the auditory nerve would be an outcome superior to that provided by prosthetic electrical stimulation. To reach this goal, it will be necessary to define and design regenerative therapy that is reliable, consistent, permanent and safe.

LOSS OF AUDITORY NERVE CONNECTIONS AND AUDITORY NERVE

The connection between inner hair cells and the auditory nerve is very sensitive to loss from the excitotoxicity generated by over-release of the inner hair cell transmitter, glutamate. This excitotoxicity can be induced by noise overstimulation or trauma to inner hair cells from hypoxia, changes in inner ear fluids or conditions that induce ROS in the hair cells. This can result in a 'bursting' and loss of the unmyelinated portion of the peripheral process of the auditory nerve, normally connecting to the inner hair cell. While there is potential for regrowth and reconnection [28] there is also potential for permanent loss leading to subsequent death of the auditory nerve somata (spiral ganglion neurons) and loss of the central nervous system connection in the cochlear nucleus. A typical inner hair cell may have connections to 10–30 auditory nerve peripheral processes (this varies depending on the position in the cochlear spiral and between species). Studies have shown that a mild to moderate level noise that produces only a temporary loss of hearing and no loss of sensory cells can still result in 20–30% of these connections being lost, reducing the dynamic range of auditory nerve responsiveness [130,131]. Drugs shown to reduce excitotoxicity in the cochlea [28] or in other regions could provide a means to reduce this loss.

When there is loss of inner hair cells, a series of pathophysiological changes follow, including scar formation, a loss of the peripheral processes of the auditory nerve, and, over time, a substantial loss of the auditory nerve itself. This loss of auditory nerve is related to the loss of survival/maintenance factors, including the deafferentation-associated loss of neural activity and the loss of neurotrophic factors that had been released by hair cells and other cochlear elements lost during the scar process. Loss of these survival/maintenance factors causes these auditory neurons to enter into the cell death cycle.

Cochlear prostheses depend on direct stimulation of the auditory nerve, so auditory nerve survival is of major importance. The auditory nerve loss can be blocked or reduced either by blocking the cell death pathway or by replacing the survival/maintenance factors. Thus, activity can be replaced by direct cochlear electrical stimulation E1 with a cochlear prosthesis, and auditory nerve survival is enhanced [132–137]. Stimulation may serve as a survival factor through activation of voltage-gated ion channels and/or through upregulation of autocrine factors, including neurotrophic factors. Neurotrophic factors can be infused into the cochlear fluids, to replace those that are lost and this is even more effective than electrical stimulation in enhancing auditory nerve survival following deafness [66,138–141]. The combination of electrical stimulation and application of neurotrophic factors is more effective than when either is applied singly [142,143].

There is also interest in inducing regrowth of peripheral processes of the auditory nerve. Cochlear prostheses might have reduced thresholds if their target is closer, making them more energy efficient and reducing battery requirements. If regrowth can be directed toward specific sites on the cochlear prostheses, this might also allow a greater number of channels to be used, resulting in more and better frequency separation. Studies have now shown that infusion of neurotrophic factors BDNF, NT-3, and FGF can induce regrowth of the peripheral process of the auditory nerve that regress following inner hair cell loss [52,144–146]. Thus, neurotrophic factors can serve not only to enhance survival of the auditory nerve but also to restore its peripheral processes, although different factors may be necessary for most effective treatment(s). Directed regrowth remains a challenge.

GENETIC DEAFNESS

One in 1,000 newborns suffers from hearing impairment. In developed countries, more than half of the afflicted babies are deaf for genetic reasons. Genetic inner ear impairments can be non-syndromic, affecting only hearing, or syndromic, with multiple defects occurring in several body systems. Many of the genes for deafness, as well as their products, have now been identified, with mutation of connexins being the most common cause of non-syndromic deafness in people. Many stereocilia-related mutations have also been found, including in several myosins and adhesion proteins [147]. Identification of the genes that are mutated in families with genetic inner ear impairments is important not only for diagnostic purposes, but also for the potential for prevention and cure. Probst et al. [148] demonstrated that in transgenic 'shaker-2' mice destined (genetically) to become deaf, insertion of the wild-type (correct) myosin MYO15 gene sequence into the fertilized egg corrected the genetic deficit, leading to normal inner ear structure and function in the adult mouse. This demonstrated that addition of the correct copy of the gene can rescue the inner ear from genetic deficits. Another approach is to silence genes when a genetic hearing disorder is a consequence of gain of function mutations and siRNA can be delivered to the cochlea to accomplish this function (see [149] for review). Mutation of a gap junction protein (GJB2) will result in deafness in humans and in mouse models, delivery of SiRNA to suppress expression of the mutated protein resulted in prevention of loss of hearing [150].

METHODS OF THERAPEUTIC INTERVENTION

With effective treatments for protection from acquired deafness and treatment following deafness identified, the next challenge is the methods for providing these interventions.

Cochlear prostheses

Auditory prostheses can provide direct stimulation of the auditory pathways for return of hearing following deafness. In severe and profound sensorineural hearing loss, the sensory cells of the cochlea are destroyed. Cochlear prostheses bypass the damaged receptor epithelium and electrically excite the auditory nerve fibers directly. The development of the cochlear prosthesis has been the success story of the field of neuroprotheses. It has provided a therapy for the profoundly deaf, where none previously existed. The first FDA-approved inner ear implants, approximately 30 years ago, were single-channel devices that provided crude input and were an aid to lip reading. Now multichannel devices, combined with many advances in signal-processing strategies, routinely provide intelligible speech perception with open set speech discrimination and even use of the telephone in the majority of implant recipients (e.g., references [151,152]). With the success of cochlear implant performance and the low risk associated with its placement, the age at which implantation is considered has decreased to where very young children receive implants and show benefit from early placements, taking advantage of critical periods for auditory system development. There is also increasing bilateral placement to allow binaural hearing, important for spatial localization [153,154,206]. Recently the candidate pool for cochlear implants has expanded to include people with remaining hearing. Shorter implants provide electrical stimulation in the basal cochlea and acoustic stimulation remains possible in the more apical cochlea. These 'hybrid electrodes' allow acoustic – electrical hearing with improved speech recognition, particularly in noise and subject also report better appreciation of sounds such as musical melodies [155].

Central auditory prostheses

Central auditory prostheses can be advantageous when the cochlea is not suitable for implantation, when there is insufficient auditory nerve survival, or when auditory nerve must be removed in patients with VIII nerve tumors (acoustic neuroma). While a peripheral cochlear implant allows for more 'normal' auditory pathway processing, the central implants can take

advantage of the tonotopic organization of auditory nuclei for frequency separation. Central auditory prostheses can also have reduced thresholds because of the close proximity to neurons as well and increased dynamic range. Cochlear nucleus implants have been successfully applied in many subjects, with excellent performance possible [156,157] in non-tumor patients however when there is a tumor (the most frequent need for central auditory prostheses) the complications of the tumor and its removal can compromise the long-term function of the cochlear nucleus implant (e.g., reference [158]). Auditory prostheses have also been applied in the inferior colliculus in both humans and in animal models [207] with animal models showing improved thresholds, increased dynamic range, and better frequency discrimination than cochlear implants [159].

Gene therapy

Gene therapy is used to manipulate levels of specific proteins in cells and tissues. It can also be used to inactivate specific proteins or to overexpress them. In most cases, this is accomplished either by introducing a foreign gene into the target cells or by using siRNA approaches. Viral vectors are the most efficient vehicles for gene transfer. Adenovirus, herpes simplex virus, adeno-associated virus, and lentivirus can mediate gene transfer into cells of the inner ear [82,160–165]. For example, mice lacking vesicular glutamate 3 (VGLUT3) are born deaf and adenoassociated virus mediated gene transfer of VGLUT3 into inner hair cells can partially restore normal cochlear morphology and hearing [166].

Gene transfer protocols can be used either for influencing the target cell proper, or to accomplish secretion of diffusible factors that act in a paracrine way. Example for the former is the overexpression of Atoh1 for inducing transdifferentiation of supporting cells to new hair cells. Examples for therapy with genes encoding secreted and diffusible proteins include neurotrophin therapy, used for protection of epithelial and neural elements in the cochlea [11,71,167,171].

One of the main goals of gene therapy is finding treatment for hereditary deafness. This would involve introducing the wild-type gene into the cells that are affected by the mutation, or using siRNA for treating dominant negative mutations. Use of siRNA has been used successfully in mouse models to silence dominant negative mutations (see [149] for review) and use of bovine adenoassociated virus restored *connexin26* gene expression and protein in cochlear organotypic cultures from mice with a dominant negative form of a Connexin26 mutation, providing proof of concept for *in vivo* application [172].

Biopolymers and scaffolding

Biopolymers can be synthesized that contain both the protein sequence for silk, to provide strength and flexibility, along with sequences of functional domains that naturally occur in the extracellular matrix protein to confer specific biological properties [173]. These can be 'spun' in microstructured thin films on the surface of prosthetic devices. Biopolymers can be produced that contain the sequence of fibronectin or laminin to provide a biological 'glue' (for neurons and epidermal cells, respectively), which can then be coated on implants. This can, in turn, be used to influence and stabilize the neuroprobe-tissue interface or to attach transformed cells for *ex vivo* gene transfer (see later). The biopolymer can also be used for timed release of specific factors. Thus a prosthesis could be providing electrical stimulation while releasing one or more chemical factors through microchannels (see later) and other factors through diffusion out of the biopolymer. Specific placement of biopolymer and microchannels can also provide different factors to different regions. A timed release can also be accomplished using nanoparticles that can be placed into the cochlear fluids (e.g., [208], [174]). These can be designed with different sizes, shapes and release properties and can also be designed to target specific cochlear elements. They can also be placed directly on prostheses.

Microcannulation — miniosmotic pumps

The fluid spaces of the cochlea provide for a closed environment well suited to receive local delivery of chemicals. Local delivery provides improved access and avoids the side effects that systemic delivery could entail. The model of miniosmotic pumps with cannula into the inner ear fluid of scala tympani or into the middle ear with access through the round window has been developed in animals models (e.g., [175]) and effectively utilized for local application of chemicals that provide protection from acquired deafness or enhanced survival of auditory nerve (see earlier sections). While devices for fluid delivery to the inner ear can be developed for human application [176–178], most recent efforts have focused on combining fluid delivery with cochlear prostheses (see later section).

Middle ear drug release

The middle ear more easily accessible than the inner ear fluids and drugs can be delivered into the middle ear through the tympanic membrane (ear drum). This can be for middle ear infections but can also cross the round window and diffuse into the cochlear fluids. Slow release formulations have been developed that can be placed close to round window for this purpose [179].

Combinations: prostheses with microchannels

Studies now show that combining electrical stimulation and chemical delivery is more effective in enhancing auditory nerve survival following deafness than either applied by itself [142,143]. Moreover, more patients with some residual hearing have been shown to benefit from cochlear implants and more patients with surviving hair cells are now considered candidates for cochlear implants. These subjects would benefit from protection of these remaining hair cells from the trauma of cochlear prosthesis insertion. There is also the potential of inducing regrowth of peripheral processes toward the stimulation sites, which could lower thresholds and enhance selectivity and separation. Therefore recent efforts have been made by cochlear implant manufacturers and research groups to develop cochlear prostheses capable of both electrical stimulation of the auditory nerve and delivery of pharmaceuticals into the cochlear fluids [180]. These have been successfully applied in animal studies [143], and clinical application is beginning.

Prostheses with biopolymers and *ex vivo* gene transfer

Another approach to delivery from prosthesis is to place cells capable of secreting the substance of interest directly onto the prosthesis, where they can deliver to cochlear fluids after placement into scala tympani. With *ex vivo* gene transfer, cultured cells are transformed to produce a specific gene product and placed into a specific area of the body for region-specific release. One problem has been that the transformed cells can migrate to a different region. With the use of biopolymers with fibronectin or laminin, transformed cells (e.g., fibroblast and fibronectin; Schwann cells and laminin) can be securely attached by the biopolymer to a neuroprobe or prosthesis, which is then inserted into the region of interest. The cells will then remain in place and have their highly localized action. One such study has recently shown the ability of BDNF, secreted by cells attached to the implant, to enhance survival of spiral ganglion neurons in a deafened guinea pig ear [169]. It may also be possible to place stem cells (discussed in more detail in the next section) on a prostheses, either to release drugs of interest or as new neurons close to the stimulation sites that can create a new connection either directly to the central auditory system or to remaining spiral ganglion neurons.

Stem cells

The use of stem cells, exogenous or endogenous, provides the potential to treat deafness by replacing lost hair cells or lost auditory nerve. Embryonic stem cells or stem cells derived from

bone marrow can be induced to reach a hair cell-like phenotype [181–184] or an auditory nerve-like phenotype [185] *in vitro*. In addition *in vivo* studies have shown that mouse embryonic stem cells can be placed into the cochlear fluids of scala tympani or directly into the auditory nerve, survive, differentiate into a neuronal phenotype [186–190], and can migrate into the remaining spiral ganglion cell population [191]. Stem cells that reach a neuronal phenotype will send processes that re-innervate hair cells [192,193] and have recently been shown to make connections in the cochlear nucleus and improve auditory brain stem responses [209]. Differentiation into an auditory nerve-like phenotype typical of SGN can be improved by genetically engineering the stem cells to produce factors that guide auditory nerve differentiation during normal development and/or by providing neurotrophic factors that influence development of the SGN phenotype [189]. Patch clamp physiological studies of these cells *in vitro* demonstrate depolarizations within only days with spiking and appropriate K^+ and Na^+ currents within five days [194].

Induced pluripotential stem cells (IPSCs) provide another option for generating sensory cell and/or auditory nerve replacement and recent studies have shown success in inducing IPSCs into generating sensory hair cell-like characteristics [81,195]. Finally, endogenous progenitor/stem cells have now been identified in the inner ear of both animals and human which may be induced to regenerate tissues of the inner ear. In animals endogenous pluripotential cells have been identified in regions of both the sensory cells and auditory nerve cell bodies in both the auditory and vestibular regions of the inner ear [196–201]. In human temporal bones, endogenous mitogen responsive cells have been identified in regions of auditory nerve cell bodies [197,202]. In animal and humans these cells demonstrate the properties of stem cells to form spheres and self-renewal [202,203]. In animal studies these cells have been demonstrated to give rise to hair cells, neurons and glia [204]. However, the ability of endogenous cells to form spheres and to spin off new stem cells decreases with postnatal age, particularly for cells from the organ of Corti [203]. Isolated adult human cells *in vitro* are induced to form spheres, proliferate, and differentiate into neuron-like phenotype by neuronal growth factors. This provides the potential that these cells can be cultured and placed into the cochlea to replace hair cells or auditory nerve, or perhaps they can be induced to differentiate *in vivo* to provide replacement. Indication that this later may be possible has recently been show by Watanabe [200], who demonstrated that in young adult mice, *in vivo*, exposure to damaging noise levels induced a proliferation of nestin expressing cells in the region of the organ of Corti. These new cells did not go on to become new mature hair cells; the challenge is to identify the factors that will drive that final step.

CONCLUSIONS

Major advances in our understanding of the molecular mechanisms underlying deafness and of the factors that influence and modulate its expression and progression have occurred since the mid-1990s. We can expect, as progress in these areas continues, that this knowledge will form the basis for *molecular otology*. Novel tissue-engineering-based therapeutic interventions may become a major part of the practice of otolaryngology in the twenty first century.

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PART 16

Ophthalmic

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CHAPTER 66

Stem Cells in the Eye

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INTRODUCTION

The eye is a complex organ consisting of epithelial, mesenchymal, connective and neural tissue. Vision is dependent on carefully regulated structural and functional integration of these tissues. Over the last two decades it has become increasingly apparent that there are a number of stem cell niches in ocular tissues which are important in maintenance and repair. This review will consider our current knowledge of ocular stem cells and reflect on their therapeutic potential in repairing damage to ocular tissue.

CORNEAL EPITHELIAL STEM CELLS

Limbal stem cells (LSCs) are crucial to corneal epithelial tissue repair and regeneration throughout the life of the adult cornea (see [1–3]). The ability of LSCs to self-renew and generate daughter cells, which undergo progressive differentiation until they are shed from the surface as terminally differentiated cells, supports the concept of 'stemness' in the cornea.

The corneal epithelium is the outermost layer of the cornea, whose functions include transparency, and protection from the external environment. The corneal epithelium is essential to the corneal functions of transparency, refraction and protection. It consists of a stratified multilayered (5–7 layer) epithelium, that is continually renewed from a population of relatively undifferentiated cells that reside in the basal cell layer of the corneal limbus. The limbus is defined as a narrow ring of tissue situated between the cornea and conjunctiva, terminating anteriorly at Bowman's membrane. The limbus, as is appropriate for all stem cell niches, offers both physical protection and nourishment to the stem cells that reside there. The deep undulations of the Palisades of Vogt ensure that the LSCs are hidden and protected from any hostile external environment, while the nearby limbal blood vessels provide nourishment. In addition, melanin in LSCs is thought to protect against ultraviolet light and reactive oxygen species. LSCs are slow-cycling cells that self-renew and produce transient amplifying cells (TACs). The TACs differentiate into postmitotic cells as they move centripetally toward the central cornea and upwards from the basal corneal epithelial cell layer, before being shed from the epithelial surface as terminally differentiated flattened superficial cells [4,5].

Interestingly, a recent study by Dua et al. [6] suggests that the limbal stem cell niche may extend beyond that originally thought. They termed this novel anatomical structure, which formed cords of ABCG2-positive epithelial cells, extending from the limbal palisades; *'the limbal epithelial crypt'*. Further studies are required to determine the 'stemness' of cells derived from this structure.

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The limbal stem cell niche

Evidence in support of the limbus as a niche for stem cells that renew the corneal epithelium has been described in several studies. The migration of limbal pigmented cells towards the central cornea in a wound healing response provided evidence for the existence of LSCs in the Palisades of Vogt [7]. The fact that cells, in the limbal niche, are slow-cycling [8], relatively undifferentiated cells [9,10], and when cultured *in vitro* have a higher proliferative potential than those of cultured central or peripheral corneal epithelial cells [11–13] provided further evidence. The distribution of LSCs does not appear to be uniform; the population of LSCs differs according to region, with the greatest number being in the superior and inferior cornea [14]. A single, unipotent LSC self-renews and produces a TAC. The TACs proliferate to produce more TACs dependent on requirement (e.g., proliferation will be increased during corneal regeneration following wounding). Each stem cell is capable of producing TACs throughout ones lifetime but it is likely that a proportion of LSCs remain in the resting state of Go for at least part of their lifetime [8,15,16]. Following wounding, LSC proliferation rate can be upregulated by as much as 8–9 fold [8,17,18] within 12 hours compared to a two-fold increase in TACs following central epithelial injury [8]. Epithelial cell proliferation rate is highest in the peripheral cornea in comparison to both central cornea and limbus [11,19]. The change in frequency and number of TAC divisions represents a shortening of cell cycle time triggered by cell injury and loss upon wounding [8,17].

As for other stem cell niches, the adjacent stromal environment plays a critical role in defining stem cell destiny. The precise molecular mechanism by which the stromal niche regulates limbal stem cell fate is unclear, although it is likely to be due to intricate interactions between the stem cell and its microenvironment [20,21], as well as short and long range signals.

Differential regulation of LSCs and TACs

The regulation of stem cell homeostasis typically involves various short and long range, internal and external factors. The environments that encompass LSCs and TACs differ in various ways, and are important in determining cell fate in each region [22,23]. Important definers include the differences that exist between the basal substrate that underlies these cells in the limbus and peripheral corneal peripheral cornea. Differences include distinct laminin isoforms, collagen IV α-chains [24,25] and the basement membrane-related AE27 BM antigen [26].

Cytokines are well known to modulate both corneal epithelial and stromal wound healing responses [27], through a number of mesenchymal-epithelial interactions [28–30]. It is almost certain that they play similar roles in the regulation of LSCs and TACs. Dependent upon the mesenchymal-epithelial interaction they are involved in, cytokines can be assigned to groups. Epithelial cells secrete Type I cytokines (transforming growth factor-β (TGFβ), Interleukin 1β (IL1β), Platelet-derived growth factor-BB (PDGF-BB)) to modulate fibroblasts, mediators of both epithelial and fibroblasts are Type II cytokines (Insulin-like growth factor 1 (IGF1), TGFβ1, TGFβ2, bFGF), and corneal epithelial cells produce Type III cytokines (keratinocyte growth factor (KGF), hepatocyte growth factor (HGF)). Interplay between epithelial cells and stromal fibroblasts in the cornea and limbus is likely to influence cell behavior and phenotype [28–32]. A good example is the presence of TGFβ 1, 2 and 3 and receptors in the limbal epithelium. TGFβ inhibits LSC proliferation [31] and may therefore serve to influence stem cell maintenance in the limbus. Stem cell regulation by many cytokines/growth factors has been reviewed and reported by various investigators [33–39]. In addition we have recently shown that Notch/Jagged/Delta interactions play a key role in TAC differentiation [40].

Evidence of corneal epithelial cell plasticity

The plasticity of adult corneal epithelial cells is implied by their ability to alter phenotype in response to signals from the embryonic dermis [41]. These findings suggest that corneal epithelial cells can be reprogrammed. The 'screw' model of stem cell behavior suggests that adult TACs, once differentiated, under certain signals, can be induced to revert to their original 'stemness' [42,43]. In the above study, adult corneal epithelial cells appear to have matured (after de-differentiation) into cells that express epidermal specific keratin 10. The corneal epithelial cells responded to signals and directions of their new environmental niche, such that their fate was redirected to allow participation in hair-follicle morphogenesis [41,44]. The mechanisms that control these processes are still to be determined.

The potential for epithelial cell differentiation into a neural cell phenotype has also been suggested. However, since the embryonic corneal epithelium is derived from the neural ectoderm, it is not surprising that a subset of epithelial cells express the neural marker, nestin [45] or that Notch 1 expression can be induced in cultured limbal epithelial cells [46].

The pursuit of a corneal stem cell marker

General stem cell characteristics that are exhibited by corneal LSCs define their stem cell status. The slow-cycling nature of LSCs was first recognized by the detection of label-retaining cells in the basal cell layer of the limbus [8]. The lack of differentiation of a subset of cells within the basal cell layer of corneal limbus adds to their potential for being stem cells. The relatively undifferentiated status of LSCs has been demonstrated by their lack of the epithelial differentiation markers, 64 kDa cytokeratin 3 (CK3), and the cornea-specific cytokeratin 12 [10, 47,48] in the limbal basal epithelium, compared to localization to all layers of the corneal and suprabasal cells of limbal epithelium. In addition, LSCs are small and round, and have features suggestive of a more primitive phenotype (reviewed by Schlotzer-Schrehardt and Kruse [49]).

The TACs are rapidly-cycling cells, with limited proliferative activity, which, like stem cells, can be upregulated in corneal wound healing. At the corneal margin, basal cells co-express vimentin and cytokeratin 19 [50], and as the TAC migrates across the limbo-corneal margin, the cells express differentiation features: cytokeratin 3 and 12 [9,23,47]. Lavker et al. [51] identified peak proliferative activity in this zone.

As with many tissues that are derived from stem cells, to date, a definitive marker which characterizes the limbal stem cell is yet to be identified. However, a number of negative differentiation markers and putative stem cell-specific markers have been investigated and are comprehensively reviewed by Wolosin et al. [52] and Schlotzer-Schrehardt and Kruse [49]. Table 66.1 demonstrates some of the markers described that may be used to discriminate basal limbal epithelial cells from basal corneal epithelial cells. Used, in combination, these markers have potential in limbal stem cell isolation and characterization of differentiation state.

A number of putative stem cell markers have been suggested, but most are not solely expressed in a subpopulation of cells in the basal cell layer of the limbus, or are highly expressed in

TABLE 66.1 Positive and negative markers of limbal basal epithelial cells

Negative markers of LSCs	Limbal basal cell markers
K12/K3	ABCG2
Connexin 43	K19
P-cadherin	Vimentin
Involucrin	Integrin α9
Integrins α2, α6, β4	KGF-R
nestin	metallothionein

the limbal basal epithelium (i.e., also positive for corneal basal cells). Alpha-enolase is present in basal cells of the embryonic cornea [18] and was observed in basal limbal epithelial cells [53,54]. However, its potential as a stem cell marker was low, as alpha-enolase is expressed in basal cells (non-stem cells) of several stratified epithelia and was also detected in basal cells of the peripheral cornea. Pellegrini et al. [55] indicated the transcription factor p63 as a potential keratinocyte stem cell marker. However p63 did not appear to be exclusive to stem cells, as low levels were occasionally observed in peripheral corneal basal cells. Also, p63 was identified at low levels in meroclones (young TACs) compared to holoclones (stem cells) [55].

Epithelial tissues are suggested to consist of around 0.4% adult stem cells. In the limbus, estimations for the stem cell population that maintains the corneal epithelium indicate less than 10% of the basal limbal epithelial cell population [51] or 100 cells in the rodent cornea. Like other stem cells, a subpopulation (known as side population cells) of limbal epithelial cells, approximately 0.4%, are able to efflux Hoescht 33342, a property attributable to the ATP-binding cassette transporter G2 [52,56–58]. Interestingly, de Paiva et al. [57] and Umemoto et al. [58] demonstrated conflicting results in the potential of side population cells to proliferate and form colonies *in vitro*. Umemoto et al. [58] reported that the discrepancy in findings was due to the non-colony forming side population cells being arrested in G0/G1, having no telomerase activity and thus representing a quiescent stem cell population in the basal cell layer of the limbus. A study of rabbit limbal epithelial side population (SP) cells concurred: the SP cells represented 0.73% of the population, were small, undifferentiated and non-cycling, and could be induced to enter the cell cycle upon wounding [59].

The potential for tissue engineering of LSCs in ocular surface disease

In corneal pathologies such as traumatic injuries (chemical and thermal burns), contact lens-induced keratopathy, Stevens Johnson syndrome and ocular pemphigoid [60,61], the absence or depletion of LSCs results in failure of corneal epithelial formation. Instead, persistent epithelial defects, neovascularization, scarring, ulceration, and in some instances corneal perforation, results. In such cases, corneal transplantation, which involves full-thickness transplant of a central corneal button (without inclusion of the limbal region), will not restore vision.

Several investigators have harnessed the proliferative potential of corneal and limbal epithelial cells for therapeutic purposes [13,37,62–64]. Limbal epithelial cells have a higher proliferative potential than cells in the peripheral and central cornea and clonogenicity studies have confirmed that holoclones (derived from stem cells) have the greater capacity for clonal expansion than meroclones and paraclones [13]. Clinically, cells derived from this region have led to successful limbal transplantation with the formation of a stratified corneal epithelium [63–65]. In the absence of the limbal epithelium, conjunctivalization of the cornea results in an abnormal corneal surface [66,67].

Initially, autologous conjunctival transplantation was performed to treat limbal deficiency-related ocular surface disease [68–70]. However, since the discovery that 'conjunctival transdifferentiation' does not occur [67,71,72], other methods were investigated. Keratoepithelioplasty describes the transplant of lenticules of peripheral cornea [5], and in 1989 the first human conjunctival limbal autograft was performed [73]. Kinoshita et al. [74] then introduced the concept of transplantation of LSCs in rabbits. Human limbal stem cell autografts utilise the limbal cells of the healthy contralateral eye, whereas bilateral disorders necessitate stem cell allografts isolated from living tissue-matched eyes or non-matched cadaver eyes.

Next, the field progressed to *ex vivo* expansion of LSCs. This concept minimized the risk of limbal stem cell deficiency in the donor eye [74], since only a small biopsy was required and allograft rejection was reduced due to the elimination of Langerhan cells during culture [75]. Pellegrini et al. [62] reported the first successful autologous graft for unilateral severe

ocular surface disease, using cultivated corneal epithelial stem cells. A number of substrates for the culture of epithelial stem cells have been introduced, including fibrin, amniotic membrane and thermo-responsive plastic. The later allows cultivated epithelial cells to be released on change of temperature so that only the epithelial sheets are transplanted [76]. The fibrin gel, used by Rama et al. [77] as a carrier, is degraded following transplant, whereas the amniotic membrane appears to persist following transplantation [78].

Preserved amniotic membrane has been used for several years, in combination with limbal transplantation to promote ocular surface reconstruction in patients suffering from severe ocular surface disease. The benefits of transplanting amniotic membrane in transplantation include: provision of growth factors important in re-epithelialization, anti-inflammatory effects, inhibition of conjunctival fibrosis [79] and anti-bacterial properties. It is also postulated that the amniotic membrane (AM) provides the LSCs with a new niche [80]. A number of studies have evaluated the use of intact AM versus denuded AM (epithelial cell removed) as a substrate for limbal stem cell culture [8–83]. Limbal epithelial cells have been shown to demonstrate stem-like properties when cultured on intact AM, including slow-cycling and lack of expression of the differentiation markers CK3, CK12 and connexin 43. However, cultivation on a denuded AM showed increased limbal epithelial cell migration and produced a better stratified epithelium [83]. If denuded AM is used to cultivate epithelial cells, growth arrested 3T3 feeder layers are required to synthesize the growth factors that were previously produced by AM epithelial cells.

Bio-engineered epithelial tissue equivalents have been derived from both limbal explants and cell suspensions isolated from limbal epithelium [83]. Both techniques generate epithelial cell sheets that express CK3 and CK12, although more evident in superficial cells of the cell suspension-derived sheets. The latter also resulted in the formation of a greater number of desmosomes, smaller basal intercellular spaces and secure attachment via hemidesmosomes to the underlying basement membrane. The use of the air-interface method of culture, whereby the level of media is reduced to below the epithelial cell surface ensures that a well differentiated, stratified epithelial tissue is available for transplantation.

Ex vivo expanded cells have been used, in both allograft and autografts, to promote repopulation of the recipient cornea by donor-derived cells to facilitate restoration of epithelial integrity and vision [63,64,81,82,84,85]. In the case of allografts, immunosuppression is required to prevent epithelial rejection.

Donor epithelial cells have been identified in the recipient bed for up to 30 months after limbal allograft transplantation [86], but the long-term duration of donor-derived epithelial stem cell viability remains uncertain [86–88]. Although successful long-term clinical outcome has been described in limbal allografts, donor epithelial cell survival could not be detected [89]. Whether donor limbal stem cell survival is sustained or activation of resident recipient stem cells occurs on re-establishment of niche signals has yet to be confirmed.

Tissue-engineered stem cells from other tissues as an alternative to limbal epithelial cells

ORAL MUCOSA

Nakamura et al. [90] pioneered the idea of using *ex vivo* expanded oral mucosal cells in autologous grafts to reconstruct the corneal epithelium of a rabbit. As with limbal epithelial cells cultured *ex vivo*, oral epithelial cells were enzymatically isolated from oral mucosal biopsies and cultured on denuded AM using the air-lift culture system. The epithelial tissue equivalent, which was generated after 2–3 weeks culture, showed cornea-like properties with expression of CK3 (but not CK12), and the formation of a 5–6 layered stratified epithelium with desmosomes, hemi-desmosomes and tight junctions. Subsequently, the feasibility of using oral mucosa to treat corneal epithelial pathology in humans has been confirmed [91–93].

BONE MARROW STEM CELLS (BMSCS)

Bone marrow-derived CD34+ progenitor cells have been identified in the cornea, particularly in the stroma [94]. This suggests that BMSCs may play a role in stromal maintenance and repair. Transplantation of bone-marrow-derived human mesenchymal stem cells (cultivated on human amniotic membrane) onto the ocular surface of a chemically damaged rat cornea demonstrated a novel idea for therapeutic reconstruction of the ocular surface. However, it appeared that the mesenchymal stem cells did not differentiate into corneal epithelial cells, but served to suppress inflammation and angiogenesis [95].

The bioengineered cornea

The idea of a bioengineered cornea has arisen with corneal equivalents being reconstructed from corneal cell lines. Griffith et al. [96] reconstructed a human cornea from immortalized cells. The resultant corneal equivalent behaved similarly to a normal cornea with respect to morphology, transparency, ion and fluid transport, and gene expression. However, bioengineering for the production of a replacement implant for wounded or diseased human corneas is still at the investigatory stage and requires further development in order to produce a bioimplant with the same tensile strength and durability as a normal cornea. Perhaps using LSCs, hematopoietic stem cells (HSCs) or combinations thereof in the future will overcome some of these problems and obviate the need for immortalized cells lines. The ideal artificial implant is likely to be a composite of biomaterials and cells, to provide a transparent, flexible, but strong biocompatible implant, which can withstand surgical procedures, as well as normal day to day mechanical stresses. To this purpose the chemically cross linked collagen-glycosaminoglycan biomatrix [97] and naturally occurring biomaterials (e.g., coral or sponges) have been suggested.

Stem cells in the corneal endothelium and conjunctival epithelium

While the first part of this chapter has concentrated on LSCs, there is evidence for other stem cell populations in the anterior segment of the eye. The conjunctival epithelium, like the corneal epithelium, undergoes constant renewal. The source of the population of stem cells is controversial, with different reports localizing stem cells to the fornix, palpebral and mucocutaneous zones. Evidence demonstrating slow-cycling cells with higher proliferative capacity in these regions [72,98,99], together with clinical observations, supports this concept. Unlike the stem cells of the corneal epithelium, conjunctival stem cells are not unipotent, as they differentiate into either a mucin secreting goblet cell or epithelial cell. Wolosin et al. [52] suggest that the different epithelial phenotypes of the conjunctiva and cornea are under the control of genes present in only one of the two epithelia. In addition, Whikehart et al. [100] proposed the existence of a stem cell niche in the posterior limbus between the endothelium and trabecular meshwork that supplies both trabecular meshwork and corneal endothelium. Furthermore, Kawasaki et al. [101] have described clusters of corneal epithelial cells residing ectopically in human conjunctival epithelium.

RETINAL PROGENITOR CELLS

It has been extensively reported that stem cells exist in the retina of fish and amphibians and that these cells add to the retina throughout their lifetime [102]. Furthermore, these cells, which are located to the ciliary-retinal interface, are also able to regenerate a complete retina, including retinal pigment epithelium (RPE), under appropriate experimental conditions [103]. By contrast, the neural retina and RPE in mammals are largely developed by the early postnatal period and show no evidence of the adult regeneration observed in fish and amphibians [102,104].

Surprisingly, retinal progenitor cells were not identified in mammals such as mouse, rat, cow and human until 2000 [105,106]. These retinal progenitor cells which are located at the

ciliary marginal zone represent only about 0.2% of pigmented cells in the ciliary margin. These progenitors display many of the properties associated with stem cells;

1) They are multipotent,
2) They can self-renew,
3) They are proliferative and express the neuroectodermal marker nestin.

Other potential markers can include CD133, CD15, Notch, Numb and FGFR4 (see Young, 2005 [107]). These retinal progenitor cells can clonally proliferate *in vitro* to form spherical colonies of cells that exhibit differentiation markers for a variety of cell types including photoreceptors, intermediate neurons and Muller glia [105,108]. Thus it would appear that these progenitors have the potential, given the right environment, to be engineered into the morphological and functional layers associated with the retina.

The inability of these ciliary margin progenitor cells to renew retinal cells in the postnatal period in mammals indicates that the progenitor cells find themselves in an inhibitory environment. Little is known about what regulates retinal progenitor cells, but transcription factors such as Pax6, Six3, Rx1, Chx10 and Hes1 [105,109] are strongly implicated, as well as the cyclin-dependent kinase inhibitor protein, $p27^{Kip1}$ [110]. Growth factors such as FGF2, EGF and IGF-I have all been reported to regulate proliferation but their precise role remains equivocal [104–106,110]. Furthermore, it appears from studies in the chick that Muller glia have the potential to become neurogenic retinal progenitor cells [92]. Furthermore, Muller glia cells appear to play an important role in the generation of multipotent precursor cells from embryonic retinal cells [108].

The identification of adult retinal progenitor cells opens the possibility that these cells can, given the appropriate cues, be engineered for transplantation in retinal degenerations such as retinitis pigmentosa and age-related macular degeneration [111]. Due to the limited number of retinal progenitor cells and the difficulty in isolating them, investigators have concentrated on investigating the incorporation and differentiation in the retina of transplanted brain-derived neural progenitor cells [112–114], embryonic retinal progenitor cells [115], bone marrow-derived stem cells [116] and embryonic stem cells from the inner mass of the mouse blastocyst [117]. While these studies, largely carried out in rodents, provide proof of principle for stem cell transplantation, differentiation and integration in the host retina, they do emphasize that the correct inductive cues are essential for a successful outcome. Studies in the pig have shown that retinal progenitor cell xenografts to the pig retina can result in integration and differentiation [118]. Furthermore, recent research has shown that retinal progenitor cells can be isolated and greatly expanded *ex vivo*. When grafted into the degenerating retina of dystrophic mice, a subset of retinal stem cells developed into mature neurons, including cells expressing photoreceptor markers [107,119]. Not only was rescue of photoreceptors observed but there was also integration of donor cells and animals showed improved light responsiveness. Despite these successes cell delivery, survival of transplanted cells and regulated differentiation of grafted cells need to be optimized if we are to regenerate a fully functional adult retina.

BONE MARROW STEM CELLS

Although not resident in the eye it appears that bone marrow stem cells (BMSCs) play an important role in retinal homeostasis and repair. Adult BMSCs are mostly quiescent cells that comprise ~0.05–0.1% of the bone marrow [120–122]. Adult HSC expand and differentiate exclusively in the bone marrow, from which they can be mobilized into the bloodstream. At the same time, BMSCs may divide to yield undifferentiated progeny with identical BMSC characteristics, fulfilling the two criteria for a stem cell: self-renewal and the ability to give rise to differentiated progeny. Cell-cell and cell-matrix interactions are crucial to the proliferation and differentiation of HSCs in the bone marrow niche. Depending on the circumstances,

HSCs can travel from the marrow into the circulation in large numbers, a process termed mobilization [123]. However, Kucia and Ratajczak [124] propose that in addition to BMSCs, the bone marrow also harbors versatile subpopulations of tissue-committed stem cells (TCSCs) and perhaps even more primitive pluripotent stem cells. Exogenous administration of granulocyte colony stimulating factor (G-CSF) is the standard method of inducing stem and p 123 cells. The chemokines interleukin 8 (IL-8) [125] and stromal-derived factor 1 (SDF-1) [126] both participate in G-CSF-mediated stem cell mobilization. SDF-1, as well as VEGF and PlGF (vascular endothelial growth factor and placental growth factor, respectively), induce osteoclasts to secrete the metalloprotease MMP-9, whose action results in the shedding of the membrane-bound cytokine stem cell factor (SCF) from the bone marrow, releasing it into the circulation.

Upon activation, BMSCs proliferate and differentiate into progenitor cells which can be found in the peripheral blood [127]. These progenitor cells may then differentiate further as required. BMSCs have been reported to have the potential to differentiate into a variety of other tissues, including liver [128], muscle [129] and neuronal cells [130–132], thus demonstrating a role for stem cell plasticity in tissue maintenance and repair. Recent studies support the possibility that BMSCs may also have broad potential to differentiate into various ocular cell types [133,134]. Lethally irradiated mice were reconstituted with HSCs from mice homozygous for green fluorescent protein (GFP+) and subjected to laser-induced Bruchs membrane rupture. GFP+ retinal pigment epithelial cells, astrocytes, macrophages/microglia, pericytes and vascular endothelial cells were observed in or adjacent to the wound site, confirming that HSCs have the capacity to differentiate into cells expressing different ocular phenotypes.

Postnatal neovascularization has previously been considered synonymous with proliferation and migration of pre-existing endothelial cells resident within parent vessels, i.e., angiogenesis [135]. However, Asahara et al. [136] identified cells derived from bone marrow that were capable of differentiating into neovasculature as $CD34^+$ expressing cells. Thus, these cells presumably are key mediators of endothelial repair. We and others have demonstrated that HSCs provide functional hemangioblast activity during retinal and choroidal neovascularization [137–141] and that HSCs give rise to a variety of lineages including the $CD34^+$ endothelial progenitor cells (EPCs). This mechanism of endothelial repair represents a significant source of cells for neoangiogenic vessels. Defects in EPC function appear to be responsible for the development and persistence of acellular capillaries in diabetic retinopathy, since lack of repair of acellular capillaries contributes to the development of retinal ischemia and represents an irreversible step in the progression of this disease [142,143]. EPCs isolated from patients with Type 1 diabetes have a decreased rate of migration and incubation with a nitric oxide (NO) donor alters the EPC cytoskeleton, normalizing their rate of migration. EPC migration can be stimulated by activation of growth factor receptors [120,144–148] such as vascular endothelial growth factor 1 and 2 (VEGFR1 and VEGFR2) and cytokine receptors such as CXCR4 (receptor for SDF-1 [147,149–151]. Inhibiting SDF-1 has been shown to reduce the degree of stem cell involvement in induced neovascularization [152]. It also appears that autologous bone marrow-derived lineage negative HSCs containing endothelial progenitors stabilizes and rescues retinal blood vessels in two mouse models of retinal degeneration [153]. Interestingly, this regeneration of retinal vessels was accompanied by neurotrophic rescue of the retina and preservation of cone vision.

THE POTENTIAL FOR STEM CELLS IN OCULAR REPAIR AND TISSUE ENGINEERING

The realization of the existence of undifferentiated cells, which self-renew and show plasticity, in adult tissues offers great potential in the treatment of both ocular and non-ocular diseases. It is now becoming evident that both intraocular and extraocular stem cells have the ability to play a critical role in the maintenance of ocular tissues. Furthermore, the plasticity

of these cells means that they can be engineered to repair injured or diseased ocular tissues. Corneal LSCs are now routinely used in the restoration of the injured ocular surface and BMSCs hold great promise in the repair of retinal damage.

Ocular stem cells are an ideal starting point for tissue engineering of ocular tissues and offer a means of therapeutic intervention in a variety of pathologies involving cell/tissue loss (e.g., age-related macular degeneration, retinitis pigmentosa, glaucoma and diabetic retinopathy). The future will tell if these stem cells can be used to deliver normal genes to correct genetic abnormalities in specific ocular tissues.

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CHAPTER 67

Corneal Replacement Tissue

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INTRODUCTION

It is estimated that over 10 million people globally were visually impaired or blind due to the lack of available treatment for corneal diseases or corneal injury in 2010 [1]. Over 90% of all untreated eye diseases are found in developing countries. The gold standard of treatment for many corneal diseases is transplantation of either a partial or full thickness donated cornea. Treatment for corneal diseases in countries with well established health care systems is routine. However, the need for donated corneas outpaces the supply, and the need is likely to increase as the population ages.

Obtaining high quality donor corneas in countries such as the United States relies on an extremely well run eye banking system that is not universally available. Adding to the cost and constraints of donor cornea transplantation is the need for extensive and expensive toxicological testing that is necessary to prevent the spread of pathogens. This is further compounded by the problem of donor quality, which decreases as the donor population ages due to the decline in corneal endothelium function. Ironically, treatments such as Laser-Assisted in situ Keratomileusis (LASIK), used to surgically improve vision by altering the shape of the cornea, currently make the cornea ineligible for donation. Lastly, religious and cultural factors limit the scope of organ donation and therefore the availability of donor corneas available for transplantation. In an effort to overcome this shortage of donated tissue, researchers and clinicians are using stem cells and tissue-engineering methods to manufacture corneal layers or even completely bioengineered corneas.

CORNEA: OVERALL STRUCTURE

Why is the cornea so critical for vision? The cornea protects the entire eye from the harsh external environment and acts as the first 'lens' of the eye. The cornea and tear film provide over 80% of the focusing power to the retina, with the lens providing the remainder [2]. Light bends or refracts as it enters the tear film and cornea. The light is then refocused by the lens onto the retina. The retina converts the light rays into nerve impulses which are then transmitted to the brain in the process of visual transduction. To understand how the cornea provides these important functions for vision, it is necessary to first explore the structure of the cornea.

The cornea is a dome shaped structure at the front of the eye that appears to be relatively simple when initially examined (Fig 67.1a). However, closer inspection reveals a complex structure that consists of three cell layers and two membranes that are optically transparent (Fig. 67.1b). The first outermost cell layer is the epithelium, the middle is the stromal layer,

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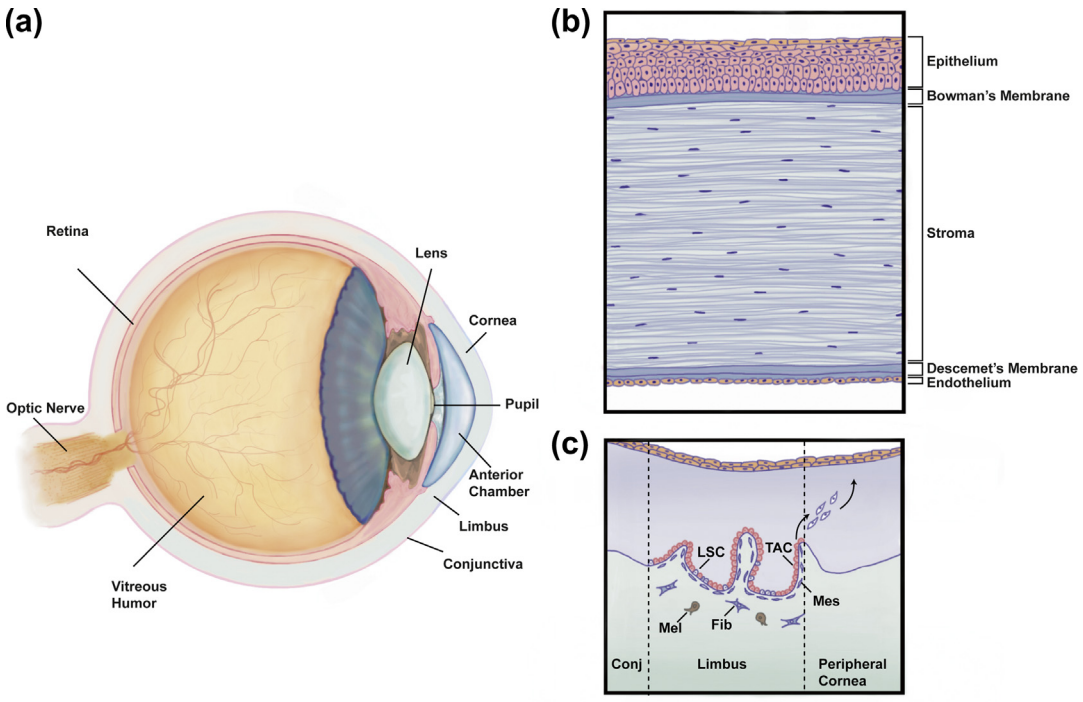

FIGURE 67.1

The anatomy of the eye, cornea, and limbus. a) The cornea forms a clear, dome structure at the front of eye necessary for protection and refraction of light. b) The cornea is an optically transparent multilayered structure consisting of three cell layers and two membranes. The outermost cell layer is the epithelium, Bowman's membrane is in between the epithelium and the stroma, followed by the thickest layer, the stroma. Descemet's membrane lies between the stroma and the innermost cell layer of the cornea, the endothelium. c) The limbus is the niche where the limbal stem cells reside, which is responsible for replenishing the epithelium. The limbus is at the junction of the conjunctiva and the peripheral cornea. Limbal stem cells undergo asymmetric division to produce the transient amplifying cells (TACs) that have a high proliferative capacity. TACs migrate from the limbus to the peripheral cornea to reside in the epithelial cell layer. The progeny of the TACs go onto make the cell types of the epithelium, which are sloughed off and replaced. Conj=conjunctiva; Fib=fibroblast; LSC= limbal stem cell; Mel=melanocyte; Mes=mesenchymal cell; TAC= transient amplifying cell.

and the deepest is the endothelium. The epithelial and stromal layers are separated by Bowman's layer, a collagen-containing membrane. Similarly, Descemet's membrane can be found separating the stroma layer from the endothelium. These three cell layers and Bowman's and Descemet's membranes, along with sensory innervation, are the first and critical interface of the process of visual transduction and also protects the eye from environmental insult and injury.

EPITHELIUM: PROTECTS EYE FROM NOXIOUS STIMULI AND THE ENVIRONMENT

The epithelium consists of a superficially multilayered structure of 5–6 layers of cells that are replenished on an approximately weekly basis. The corneal epithelium has many characteristics that differentiate it from other types of squamous epithelium. The corneal epithelium is a smooth, wet, apical surface that is regular in thickness and must be transparent to be functional. It is highly innervated to sense and prevent damage to the cornea or other eye structures, yet needs to perform routine functions common to other epithelia, such as be resistant to abrasion, prevent fluid loss and restrict pathogen access, as well as respond rapidly to wounding. Structurally the outer layer contains 3–4 cells layers of flattened squamous cells, 1–3 layers of mid-epithelial cells such as wing cells, and a single layer of columnar basal

cells. The basal cells are anchored by a complex system of collagen links to the basement membrane and the underlying stroma [3]. The corneal epithelial cells are replenished by the limbal epithelium stem cells. The corneal epithelium thus provides challenges to the tissue engineer who must replicate a complex biological structure, addressing the need for continuous replacement of the epithelial cells due to wear and tear, and maintaining integrity as a barrier, and being optically transparent.

STROMA: PROVIDES STRENGTH AND TRANSPARENCY

The stroma makes up about 90% of the thickness of the cornea and is relatively acellular, with only 3–10% of the volume consisting of stromal keratocytes. Structurally, the stroma is comprised of tightly packed parallel collagen fibrils that have a small, uniform diameter. Other extracellular matrix components can be found in the stroma such as glycosaminoglycans and fibronectin. There is a global as well as a local organization of the fibrils that are regular in size and shape and aligned within individual lamella. Scarring of the stroma is common after injury because of the disruption of the orderliness of the collagen as well as the transformation of distal keratocytes that can become motile and mitotically active fibroblasts [4]. Optical transparency is lower when the local organization of collagen fibrils is disturbed, and can result in loss of vision. The stroma provides the tissue engineer two major challenges that can at times be mutually exclusive: high tensile strength with optical clarity.

ENDOTHELIUM: MAINTAINS PROPER DEHYDRATION AND NUTRITION

The endothelium is the thinnest of all the corneal layers, but is critical for the function of the cornea. The relative dehydration of the cornea is necessary to maintain optical clarity. The endothelium is thought of as a 'leaky' pump, where some substrates are allowed to leak from the stroma due to the swelling pressure while others are actively pumped out to keep the cornea relatively dehydrated. The pumping function of the endothelium has several requirements including $Na^{+}K^{+}$ ATPase, $HCO3^{-}$, Cl^{-}, and carbonic anhydrase activity. The majority of the nutrients of the cornea are supplied by the corneal endothelium, but the aqueous humor and tear film also provide nutrition to the cornea. Glucose transporters in the endothelium selectively allow nutrients from the aqueous chamber to feed the stromal keratocytes and the epithelial cells. The pumping functions are very metabolically taxing, thus the endothelium has a high concentration of mitochondria to power these functions. The density of the mitochondria should cause opacity, however, the endothelial cells are very thin, such that the optical path length is short, so little light is scattered as it passes through the endothelium [5].

APPROACHES TO ENGINEERING CORNEA

The cornea is an incredibly well-engineered structure that has both high refractive power and high tensile strength all while being optically transparent. Its two major functions, light refraction and protection of the eye, are difficult to achieve simultaneously from engineered sources. Therefore, researchers have recently taken two main approaches that utilize tissue engineering (Fig. 67.2). The first is to use tissue culture to expand or create the desired cell population, and the second is to provide a support structure for the cornea to repopulate with native cells. The first approach of tissue culture can use cornea cells grown in a sheet and then transplanted as a sheet. Alternatively the cells can be dissociated and injected into the anterior chamber or desired cornea layer (Fig. 67.2a). The second main approach provides a biomimetic or a biosynthetic device that allows the native cornea cells to slowly stimulate endogenous corneal regeneration (Fig. 67.2b). These types of devices should not be confused with the fully artificial corneas or keratoprostheses, such as the Boston keratoprosthesis. Keratoprostheses are used in restricted cases such as where human donor tissue has failed

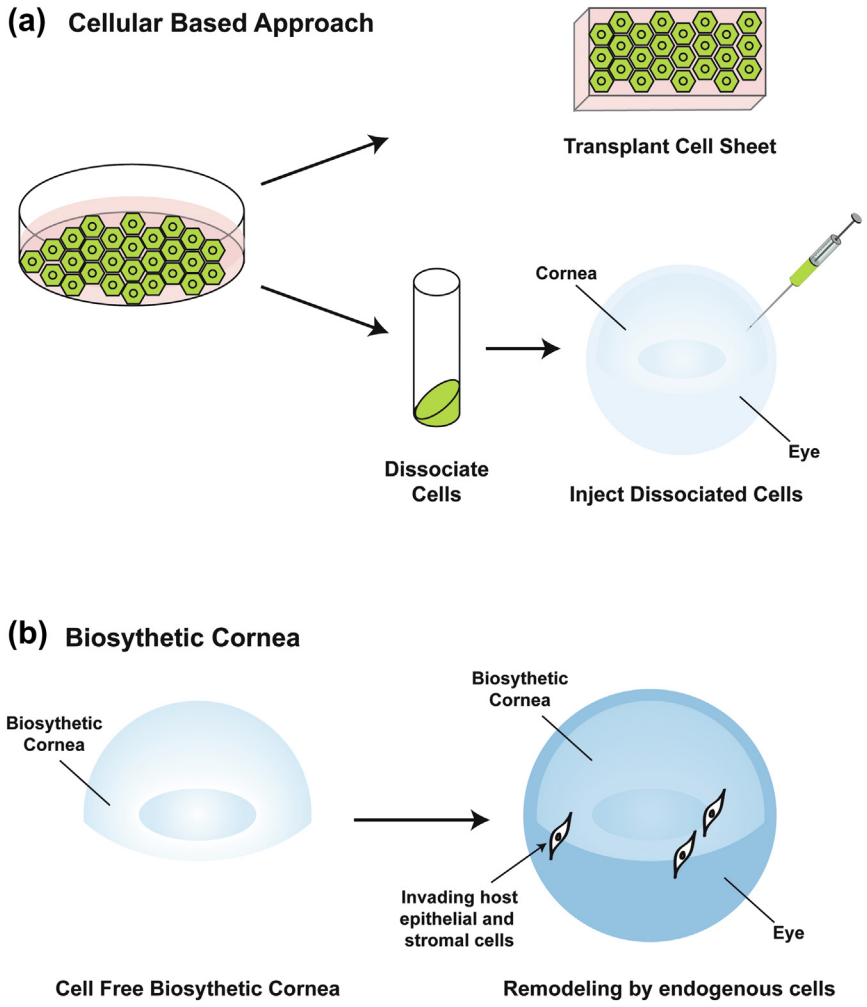

FIGURE 67.2
Two major approaches to utilize tissue engineering for corneal replacement. a) The cellular based approach employs cell culture to expand and then differentiate adult or embryonic stem cells into the desired population of corneal cells. Cells can then be transplanted as a sheet or injected into the cornea as dissociated cells. b) The biosynthetic cornea is a cell free construct that allows for slow regeneration of the epithelial and stroma layers from the patient's own cornea. The endogenous cells invade the biosynthetic cornea and remodel the matrix to make it a hospitable environment for the host cells.

repeatedly or the cornea can no longer be supported by the pathological eye. Keratoprostheses can be valuable tools for improving vision, but the high complication risks make them unsuitable for the majority of indications and will not be discussed further in this chapter [6]. Each of these approaches may prove useful in different cornea disease indications [7]. Additionally, the combination of cell culture derived cells with a biosynthetic device may one day result in a completely bioengineered cornea.

CURRENT PROGRESS: EPITHELIUM

Of all the cornea layers, the most progress has been made with the corneal epithelium. Work done by Pellegrini and colleagues [8] first demonstrated the power of tapping into the natural abilities of adult stem cells residing in the eye for treatment of limbal stem cell disease. Under normal conditions, the outer layer of the cornea, the corneal epithelium, is continuously replaced as cells are shed. Although there is some controversy regarding whether there is a single (limbus) or multiple locations (limbus and central cornea) of the corneal epithelial stem cells [9–11]. The consensus is that the majority of the stem cells required for regeneration of the epithelium reside in the limbus [12]. These are referred to as limbal stem cells (Fig. 67.1c). The limbal stem cells can replicate themselves as well as provide the highly proliferative but restricted potential transient amplifying cells. The transient amplifying cells in turn give rise to the basal cells. The basal cells will then mature to become the wing cells and eventually the outermost epithelial layers, which are shed as needed. If the limbus is

affected by disease or injury, the limbal stem cells are lost or depleted, making the normal regeneration of the corneal epithelium impossible. In limbal stem cell disease, transplantation of a cornea is not indicated since the improvement will only be temporary. Without resident limbal stem cells, recovery of the epithelium will only be transient, since the epithelial cells are normally continually replaced, and the transient amplifying cells have a limited capacity to do so, and the transient amplifying cells need to be replaced. Once the existing proliferative potential of the existing transient amplifying cells are depleted, the epithelium will be lost, which results in a recurrence of vision loss and pain.

Pelligrini and colleagues set out to test in human if their methods of expanding limbal stem cells *in vitro* could be utilized in the treatment of limbal stem cell diseases. Expansion of an adult stem cell population can be difficult, such that progenitor cells are amplified but the stem cells either make a very small percentage of total cells, or are lost entirely. Therefore it is critical to be able to identify the percentage of stem cells in the *in vitro* expanded culture. Like other adult stem cell populations, the identification of limbal stem cells by molecular markers remains elusive and inexact. Limbal stem cells are typically identified by the expression of positive stem cell markers such as ABCG2, negative for a cell differentiation markers like Cytokeratin 3, small cell size, and a high nuclear to cytoplasm ratio. Although there were several other groups that have utilized similar techniques in a clinical setting [13], the use of high expression of ΔNp63α as an important quality control measure has allowed for greater success in the clinic [14]. Not surprisingly, the higher the percentage of true limbal stem cells as opposed to progenitors in the expanded cultures resulted in a higher success rate. Additionally, strict patient inclusion was also a reason for increased success, since a hostile microenvironment is not conducive to successful transplantation [15]. Currently, there are several clinical trials using limbal stem cells worldwide, with anticipation of this technique being tested in the United States in the next few years pending regulatory review.

Other cell sources for corneal epithelium

There are many cases where using an *in vitro* expansion of an autologous graft is not indicated such as bilateral limbal stem cell deficiency, bilateral chemical scarring of the limbus, or where the risk of damaging the contra-lateral eye during the harvest is too high. Other research groups have explored alternative sources to limbal stem cells including cells derived from patient derived oral mucosal cells [16,17] and limbal stem cells derived from embryonic stem cells [18–20]. In particular, clinical trials using oral mucosal cells have been completed, with ongoing improvements resulting in enhanced patient outcomes [21]. However long-term studies will be necessary to judge the efficacy of these types of techniques in replacing endogenous limbal stem cells.

CURRENT PROGRESS: STROMA

The stroma makes up about 90% of the thickness of the cornea and is mostly acellular. From an engineering prospective, it is a highly organized transparent collagenous-based scaffold with cells scattered throughout. The stromal keratocytes are required to maintain the structure by providing the appropriate replacement proteins and enzymes. From a clinician's view, it is a tissue that responds to injury by usually scarring. Although stromal injury can heal without scarring, it is not uncommon for the resolution of a scar occurs over a long period of 1–3 years, and there is a high probability that any scarring is permanent.

It is not clear why the stroma only sometimes heals scar free. Perhaps it is dependent on the response of the resident population of the adult keratocyte stem cells, and when those cells are properly activated, scarring is minimized. In a developing chick embryo, there is limited evidence that the developing stroma may have a larger capacity to heal scar free [22]. Other epithelial systems such as embryonic cutaneous tissue have a well established ability to heal scar free [23]. One might speculate that the higher number of stromal keratocyte stem cells

in the embryo could account for the apparent discrepancy between embryonic and adult methods of injury resolution. A key question for tissue engineers is whether the power of adult keratocyte stem cells can be harnessed to heal a scarred stroma or perhaps into generate an entirely new stroma.

The quest to identify an adult keratocyte stem cell population was taken up by several groups in recent years. The majority of attempts resulted in the observation of multipotent keratocyte progenitors in a variety of species by utilizing a sphere forming assay, rather than the desired keratocyte stem cells [24–26]. However, Du and colleagues [27] took an alternative approach, in which the cells were cultured and then sorted by flow cytometry to isolate the stem cells. In this way, they were able to find a 'side population' of cells that expressed ABCG2, a common marker of presumptive stem cells. They were able to concentrate the cells, and then adjust the culture conditions to maintain that particular small subpopulation of cells. The keratocyte stem cells were stable as stem cells as indicated by their expression of stem cell markers and a large number of population doublings. When the keratocyte stem cells were challenged by appropriate differentiation conditions, stromal keratocytes were generated in large numbers. Additionally, the stem cells maintained their embryonic nature by showing their neural crest origin, such that when the cells were exposed to neural or chondrocyte differentiation media, the cells could generate neurons or chondrocytes respectively [27].

The next step was to identify the potential of the multipotent stem cells derived from adult corneal stroma. The initial studies indicated that the cells when exposed to keratocyte differentiation media would produce keratocan, aldehyde dehydrogenase 3A1, and keratan sulfate. However, it did not appear that the cells were making a stromal collagenous matrix in a typical 2D culture. The thought was that the keratocytes would require a three-dimensional structure in order for the cells to start producing the collagenous matrix. The cells grown as pellets without rigid scaffolding were able to express a large set of genes similar to stromal keratocytes and produce an extracellular matrix (ECM) that resembles that made by keratocytes [28].

The complexity of producing a collagen-based multilayered lamellae to generate a strong transparent stroma may take some time and technological breakthroughs. A smaller, but important hurdle was to determine whether adult keratocyte stem cells could remodel a disorganized stroma. To test the function of the keratocyte stem cells, the cells were injected into the stromal cell layer of a lumican null mouse. Lumican is a proteoglycan linked to about half of the keratin sulfate in the stroma and is thought to regulate fibril growth and diameter. In the absence of lumican in the mouse, large collagen fibril aggregates form, which both alter lamellar structure and increase light scatter, with opacity changes that mimic stromal scarring in humans. Interestingly, the injection of keratocyte stem cells can rescue this phenotype, such that the stromal thickness and collagen fibril organization is indistinguishable from wild type mice [29]. The authors argue that this remodeling of the stroma is not a wound healing response; rather it recapitulates the initial formation of the stroma. The authors temper the idea that a direct cell therapy approach may not be feasible in the human due to two important differences between the mouse and human cornea. Notably, the mouse stroma is considerably thinner, thereby making it more experimentally accessible, and more importantly, the human stroma contains extensively cross linked collagen which may make it more resistant to remodeling [29]. The complexity of the global and local organization of the collagen fibrils in the human stroma may make it one of the last layers to be bioengineered in the cornea and the feasibility will be further discussed in the biosynthetic cornea section.

CURRENT PROGRESS: ENDOTHELIUM

The corneal endothelium provides the tissue engineer with several challenges to overcome: an extremely limited supply of endogenous endothelial progenitors, the need to have the layer function for decades, a complex multi-component endothelial pump function, and the

transparent Descemet's membrane. Some species, such as cows and rabbits, have a corneal endothelium that can regenerate, however the human and non-human primate endothelium do not. When human corneal endothelial cells die, the adjacent cells are thought to migrate to the wound and expand their cell diameter to repair the damaged endothelium without replicating. The limited capacity of the human and non-human primate corneal endothelial cells to proliferate has only been observed *in vitro*. The peripheral endothelium appears to have a greater (but still limited) potential to divide *in vitro* compared to the central corneal endothelium [30–32]. Additionally, this limited *in vitro* capacity to proliferate is generally restricted to younger human endothelium (<30 years old) [30]. Overcoming the lack of proliferation has been a major focus for many laboratories addressing the need for providing corneal endothelium for transplantation.

The endogenous human corneal endothelial cell has a finite lifespan, such that the density and number of corneal endothelial cells naturally declines with age with an approximate 0.6% decrease each year [33]. There is an average density of 3,500–4,000 cells/mm^2 at birth [34] which declines to 2,300 cells/mm^2 by age 85 [33]. Other factors such as disease, eye surgery, UV exposure, and contact lens use can all speed the decline of the corneal endothelium. The minimum density of corneal endothelium for function is thought to be around 500 cells/mm [2]. Below that amount, the endothelium does not have enough pumping power to maintain the relative dehydration of the cornea, which leads to it becoming cloudy. Therefore, the tissue engineer also needs to consider the natural effects of aging as well as external factors such as surgery to ensure that the endothelial cell density and the pumping function of transplanted cells is high enough to have a lasting impact.

Another confounding aspect to consider when engineering the corneal endothelium is Descemet's membrane. This membrane is a collagenous fiber layer between the corneal endothelium and the stroma that is first formed during development. In the embryo, it is about 3 μm thick with periodic banding of collagen fibers bound in a latticework of 110 nm intervals. Postnatally, the structural appearance changes and the membrane is continually, but slowly formed over the person's lifetime, to approximately 10 μm in thickness in adults [35]. In general the overall composition of the proteins of embryonic and adult Descemet's membranes are very similar, with some proteins changing their apical or basal localization in the adult [36]. Interestingly, two proteins that are only expressed embryonically, Tenascin C and Fibrillin-1, appear to also be present in disease states such as bullous keratopathy and Fuch's endothelial dystrophy [37,38]. Alterations in the protein composition can disrupt the transparency of the membrane, such is the case in Fuch's disease, where extracellular matrix deposits called guttae along with endothelial cell death results in loss of vision [39]. In the current corneal transplantation method, the host's endothelium and Descemet's membrane is removed and replaced by the donor tissue. Will the tissue engineer need to provide a Descemet's membrane when replacing the corneal endothelium? In some corneal endothelial diseases, it may be possible to leave the host membrane intact while removing the diseased endothelium. However, in Fuch's disease where there are multiple central guttae, it would be expected that the diseased membrane would also need to be removed to effect sufficient restoration of vision.

The concept of expanding and replacing the corneal endothelium in animal transplantation models was explored by Jumblatt [40] and shortly after by Gospodarowicz [41,42]. Both groups tested whether the cultured rabbit or bovine endothelial cells were functional in an *in vivo* model where the endothelium had been removed. The entire cornea was removed from the donor and then seeded with the cultivated corneal endothelial cells. Then the entire cornea with the exogenously derived endothelium was transplanted into a new donor animal. These experiments showed the potential feasibility of expanding an adult endothelial cell culture, until it was realized that these species are not representative of primates. The true sticking point of the method for clinical use is to find a reliable and sustainable source of human corneal endothelial cells.

The corneal endothelium of several species has the capacity to divide and expand in culture. However, human and non-human primate corneal endothelia have a very limited capacity for division if the phenotype and function are to be maintained. To override the lack of expansion, approaches have included transformation of endothelial cells with viruses to promote immortalization [43,44], disturbing the balance of cell cycle regulators [45,46], finding spontaneous transformations that result in immortalized human corneal endothelial cell lines [47], and optimization of culture conditions [48–50]. The first three types of approaches have serious drawbacks for use in the clinic, such that viral transformations, overexpression of exogenous genes, or aberrant karyotypes, since approval by regulatory committees may prove to be overly burdensome and have a real chance of causing cancer in humans. Therefore, either corneal endothelial cells should be generated from another cells source or the methods used for *in vitro* expansion of adult corneal endothelial cells need vast improvement.

Progress from the initial promising studies in the 1970s has been understandably slow, due to the low proliferative capacity as well as the limited supply of human corneal endothelium for experimentation. Experiments done by the Kinoshito and Koizumi laboratories have made significant progress; getting closer to an engineered endothelium by utilizing the non-human primate as a model system. By applying the lessons learned from the non-human primates, improved human culture techniques have made it feasible to test these expanded cultures for transplantation into humans. One such improvement is use of the small molecule, Y27632. Y27632 is known for one of its ability to inhibit rho-associated protein kinase (Rock) but may also affect other signaling pathways. Y27632 can improve survival, proliferation, and attachment of corneal endothelial cells *in vitro* [49]. Separate experiments have shown that the inhibitor can help heal corneal endothelial wounds when applied as an eye drop as well as improve the survival of transplanted corneal endothelial cells [51–53]. The progress they have made is quite substantial; however, there are real limitations in the ability to scale cells up to clinical grade production, due to the restrictions in the expansion from high quality donor tissue, donor age, donor tissue availability, and performing quality control between relatively small lots of cells.

Another approach to solving the problem of generating enough corneal endothelium in large lot size is to convert human embryonic stem cells into corneal endothelial cells. Although still in the research phase, it is possible to convert large numbers of human embryonic stem cells into corneal endothelium that express typical markers (i.e., Col8A1, AQP1) and phenotype of corneal endothelial cells in culture, such as the tight junction marker, Zo-1 (unpublished data, Fig. 67.3). Further experimentation is needed to test the function and safety of human embryonic stem derived corneal endothelial cells *in vivo*. It is likely that the culmination of work from many laboratories in the last 40 years will soon result in the clinical testing of corneal endothelial cells for transplantation.

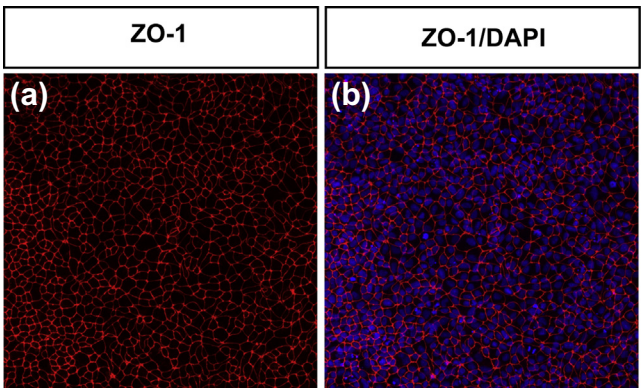

FIGURE 67.3
Corneal endothelial cells can be derived from human embryonic stem cells. a) Confluent monolayer of cells express the tight junction marker, Zo-1 (red), typical polygonal and hexagonal corneal endothelial cells. b) Merge of Zo-1 (red) and the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI) (blue). *(Unpublished data, McCabe, 2012).*

CURRENT PROGRESS: BIOSYNTHETIC CORNEAS

A goal of several laboratories is to ultimately generate a fully bioengineered cornea that would replace or repair the corneal epithelium, stroma, and endothelium. Work done by the Griffith laboratory has been ground breaking in the advancement in finding alternatives to the traditional corneal transplant. The endogenous limitations of repair and regeneration of the quiescent endothelium, as discussed above, has led to the focus for partial thickness bioengineered corneas for the time being, namely for the epithelium and stroma. The proof of concept for a biosynthetic cornea begun in 1999 with the use of a cross linked porcine collagen-chrondroitin sulfate substrate was performed *in vitro* [54]. Subsequently, improvements on the scaffold used included pure human source collagen to eliminate the possibility of introduction of prions [55]. Their efforts resulted in the first human phase I clinical trial for a biosynthetic cornea. The 500 μm thick biosynthetic corneal substitutes were composed of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide that was cross linked recombinant human collagen [56,57]. The implants were stably integrated into the host in all 10 cases with no adverse complications such as neovascularization, inflammation, or rejection being noted. Most of the patients had regeneration of the epithelium within 1−3 months. Interestingly, the presence of fine superepithelial nerves were observed along with a concomitant partial return of sensitivity within 3−6 months in 6 out of the 10 operated corneas. Significant re-innervation was still occurring 12−24 months post-surgery. These studies showed the tolerability of such implants, however, the remodeling of the collagen in the implant was significantly variable, which led to unpredictable clinical outcomes, where visually acuity either increased, remained unchanged, or decreased.

Further research is needed to produce a transparent stroma of even thickness. Saeidi and colleagues [58] may have pinpointed a problem with utilizing a disorganized collagen scaffold as a foundation for building a stroma. The essence of the idea is that in the stromal layer naturally forms in the absence of a scaffold, so by letting the biosynthetic stroma develop in a scaffold free system there may be improved outcomes. They argue that in embryo, the formation of collagen scaffold in the stroma occurs in a rapid manner, in direct opposition to scar formation which occurs over a much longer time-scale. Most approaches have attempted to generate of the stroma by capitalizing the stromal repair process. The inherent problem with this approach is that the repair process normally results in scarring. It has been suggested to take a different approach by attempting to mimic the *development* of the cornea, rather than the imperfect repair process.

Understanding the nature of collagen deposition in the stroma is likely to provide the breakthrough needed to generate a flawless biosynthetic stroma. There are two main concepts concerning the way that the keratocytes go about collagen deposition; the fibripositor and the liquid crystal collagen models. In the fibropositor model, the monomers of collagen are formed into fibrils within the cells, which are released to form pre-organized extracellular matrix. The liquid crystal collagen model relies on the confinement and concentration of the collagen monomers into a liquid crystalline pattern that condenses into regular arrays of fibrils. Regardless of the actual mode, whether fibripositor or liquid crystal, new collagen deposition is likely to be disrupted by introducing a disorganized scaffold as the foundation to be remodeled by newly invading stromal keratocytes. These types of studies demonstrate the need for the tissue engineer to go beyond the initial idea of simply generating large numbers of the cells that make up the three cell layers of the cornea, but to also take into consideration the potential roles of the environment (i.e., growth factors, artificial matrix, cell-cell contact) as well as the timing of the formation of the layers in the hope to generate a bioengineered cornea.

SENSORY INNERVATION: IMPORTANT FOR CORNEAL HEALTH

The cornea is one of the most highly innervated tissues in the body. Corneal innervation is important for the proper functioning of the cornea and impacts the cornea in a variety of ways,

including sensation, proliferation, wound healing, epithelial integrity, secretion of the tear film, and protection of the eye as a whole [59]. The innervation of the epithelium and stromal layers allows the cornea to sense temperature, pain, and pressure. Without proper sensory innervation, the cornea and the eye are at increased risk of damage by noxious stimuli. Alterations of innervation in the cornea from neurotrophic keratitis may lead to a reduction of the tear film, and the resultant disease of dry eye where the patient can suffer excruciating pain. Additionally, it is important to remember that corneal nerves are needed to help maintain the ocular surface integrity by releasing factors such as nerve growth factor (NGF) and Substance P to promote epithelial health. In fact, new data suggests that the innervation of the limbal epithelium helps maintain limbal stem cells and their niche [60], thus adding to the long list of important functions of corneal innervation.

The loss and deterioration of corneal innervation can result in neurotrophic corneal diseases caused by corneal nerve injury and disease, trauma to ocular nerves either by injury or surgery, and brainstem diseases. Patients who have had cornea transplantations have altered and limited re-innervation of the cornea. Additionally, patients who chose to use laser treatment to alter corneal shape to improve vision also have alternations, and potentially, complications due to poor re-innervation. It is clear that innervation of the cornea is important for protection and maintenance of a healthy cornea, but the knowledge of how to alter absent or abnormal innervation is limited [59]. This leaves the tissue engineer with a dilemma: should re-innervation be guided by the slow endogenous process in adults or increased to the speed found during development, or perhaps prevented from occurring? The approach taken should be tempered with what is known about the role of innervation of the formation of cornea.

Many developmental biologists would argue that if you want to replace a structure in an adult, one should first understand how that structure was built in the first place. The origins of the majority of corneal nerves are sensory, and are derived from the neural crest component of the trigeminal ganglion [61]. Additional sympathetic and parasympathetic inputs are from the superior cervical ganglion with some minor contributions from the ciliary ganglia [62–64]. Studies in adult mammals have revealed no major differences in innervation of the cornea [59,65–67]. Until recently, it was assumed that cornea innervation in the mouse embryo occurred in the same manner of the developing chick and human. However, McKenna and Lwigale [61] have shown that there are key differences in the process; namely the nerve bundles entering the cornea enter through four quadrants, rather than the typical entrance through the pericorneal ring found in human and chicken embryos. There are combinations of secreted factors and their receptors such as Semaphorin/Neuropilin as well as Slit/Robo interactions that are important for the formation of the pericorneal nerve ring, such that the cornea initially repulses the axons until the proper entry time [68–71]. The tissue engineer may want to mimic the development of innervation by strategically placing growth factors on the biosynthetic cornea to guide the re-innervation in cases where pre-existing conditions exist or complications arise.

CONCLUSIONS

Complex economic, ethical, and religious factors prevent millions of people from receiving much needed treatment for blinding corneal diseases and injuries. The standard treatment for many forms of corneal blindness is partial or full thickness transplantation of donor cornea. The shortage of donor corneas has and will continue to limit the number of patients who can be treated for corneal disease worldwide. Researchers and clinicians have turned to bioengineering and regenerative medicine to find alternatives to the donor cornea. The use of adult limbal stem cells in the treatment for limbal stem cell disease will likely alter the vision of patients in the near future. The potential is present for the use of stromal keratocyte stems to alter scars, however, a fully engineered stem cell derived stroma is likely in the more distant future. The use of biosynthetic corneas for partial thickness transplantation also shows

significant promise. And finally, the use of either expanded adult corneal endothelial cells or stem cell derived corneal endothelium provides hope for those who suffer from corneal endothelial diseases. It seems likely that these technologies will be used either alone or in combination to replace the need for donor derived cornea transplantation in the coming years.

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CHAPTER 68

Retinal Degeneration

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Definitions

Age-related macular degeneration (AMD) AMD is a condition that affects older adults which results in a loss central vision due to damage of the retina, specifically the macula. It is classified into two forms. The dry (non-exudative form) is characterized by drusen and changes to the retinal pigment epithelium (RPE), including hyperpigmentation and geographic atrophy. In the wet (exudative) form, blood vessels grow up from the choroid underneath the retina.

Stargardt's Macular Dystrophy (SMD) SMD is the most common inherited juvenile macular degeneration that causes progressive loss of central vision in adults under the age of 50. It can be associated with several different genes and inheritance patterns, but the vast majority of cases are autosomal recessive due to a defect in a transporter protein expressed by the rod outer segments.

Retinitis Pigmentosa (RP) RP is a group of hereditary disorders that diffusely involve photoreceptor and RPE function characterized by progressive visual field loss and abnormal electroretinogram (ERG), which shows loss or marked reduction of both rod and cone signals.

Diabetic Retinopathy (DR) DR is a frequent cause of blindness in the United States. Its prevalence increases with the duration of diabetes and patient age. Potential vision loss in patients with DR is due to macular edema (capillary leakage), macular ischemia (capillary occlusion), and the sequelae from ischemia-induced neovascularization.

Orphan Diseases An orphan disease is any disease that affects a small percentage of the population. There is no specific cutoff that defines a disease as an orphan disease.

EPIDEMIOLOGY — PREVALENCE AND CAUSES OF LOW VISION

In 2010, an estimated total of 733 million people worldwide were visually impaired (VI); defined as best-corrected visual acuity (BCVA) worse than 6/12 or 20/40. Worldwide, the five major causes of blindness are uncorrected refractive error (50%), cataract (23%), glaucoma (6%), age-related macular degeneration (AMD) (5%), and diabetic retinopathy (3%). The total worldwide financial cost of VI was estimated to be $3 trillion in 2010. AMD caused $0.3 trillion (12%) of the total financial cost of VI. It is projected that by 2020 there will be approximately 929 million people with VI worldwide — an increase of 27% from 2010 [1].

An estimated 937,000 (0.78%) of Americans older than 40 are blind ($\geq$ 6/60 or $\leq$ 20/200 BCVA in their better-seeing eye). An additional 2.4 million Americans (1.98%) are visually impaired. The leading cause of blindness among Caucasians is AMD (54.5% of the cases)

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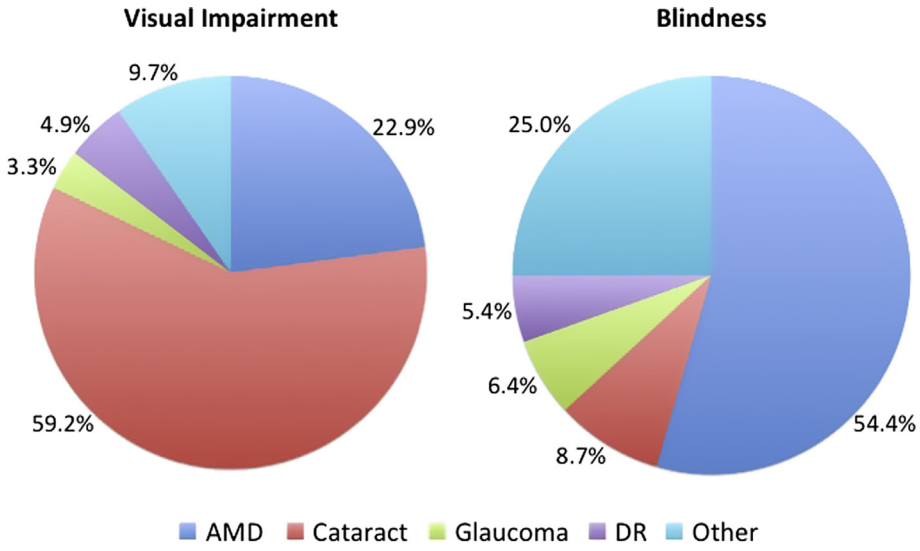

FIGURE 68.1
Causes of visual impairment (best-corrected visual acuity < 6/12 or < 20/40) and blindness (best-corrected visual acuity < 6/60 or < 20/200) in Caucasians.

while among black persons, cataract and glaucoma accounted for more than 60% of blindness (Fig. 68.1). The number of blind persons in the United States is projected to increase by 70% to 1.6 million by 2020, with a similar rise projected for low vision [2].

AMD affects 10–20% of people older than 65 years and is the leading cause of severe visual impairment in the elderly population in industrialized nations [3]. It is estimated that 1.75 million individuals have AMD. Of these, 1.22 million have neovascular AMD in at least one eye, 973,000 individuals have geographic atrophy (GA) in at least one eye, and 7.3 million individuals have large drusen in either one or both eyes. The estimated number of individuals in the United States with AMD is projected to increase by more than 50% from 1.75 million in 2000 to 2.95 million in 2020. Australia is estimated at present to have 130,000 cases of AMD; Western Europe has 3.35 million cases of AMD [4].

Hereditary retinal degenerations, such as retinitis pigmentosa (RP), are major causes of blindness in the Western world, with a prevalence of 1 in 2,000 individuals [5]. The progressive loss of vision is due to mutations in more than 100 identified genes and affects different cellular compartments in either the photoreceptor cells (PRCs) themselves or the underlying RPE [6].

While anti-angiogenesis therapies are available for neovascular AMD, no treatment exists for GA due to AMD and hereditary retinal degenerations.

NEUROSENSORY RETINA AND RETINAL PIGMENT EPITHELIUM — ANATOMY AND BACKGROUND

The neurosensory retina is a light-sensitive tissue lining the inner surface of the eye. Originating as outgrowths of the developing brain, the retina is the only part of the central nervous system (CNS) that can be visualized non-invasively. The embryogenesis of the neuroretina occurs during the first month of life. The optic vesicle extends from the forebrain, which consists of a single layer of neuroectodermal cells. This invaginates to form a bi-layered optic cup aligned apices to apices. Posteriorly, the inner layer of neuroectoderm becomes the sensory retina while the outer layer becomes the RPE. The macula appears at the end of the fourth week, but the foveal pit forms late in embryonic life and continues to mature in early childhood. A normal foveal pit is required to obtain 20/20 visual acuity and the induction of the foveal pit requires normal RPE.

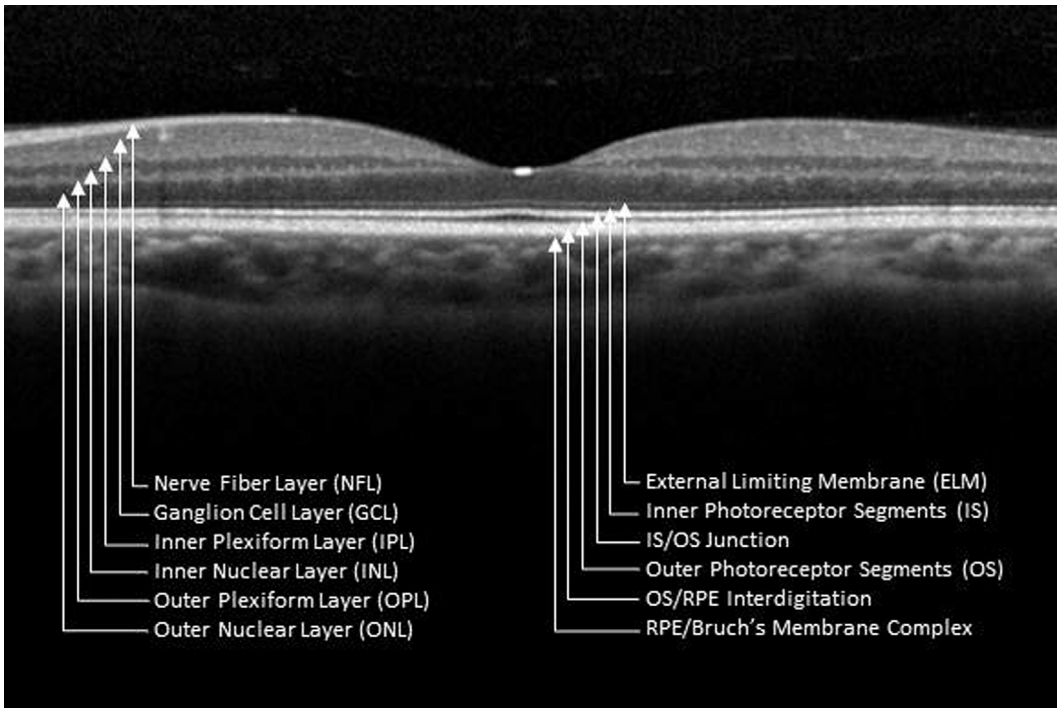

FIGURE 68.2
Retinal layers on Optical Coherence Tomography (OCT).

The neural retina consists of the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer, inner plexiform layer (IPL; synaptic processes between bipolar cells and ganglion cells), inner nuclear layer (INL; nuclei of bipolar, horizontal, amacrine, and Muller cells), outer plexiform layer (OPL; synaptic processes between bipolar cells and photoreceptors), outer nuclear layer (ONL; nuclei of the rods and cones), the external limiting membrane (ELM; formed by cell junctions between photoreceptors and the terminal optical processes of Muller cells), and photoreceptor (PR) layer (rod and cone cells) (Fig. 68.2).

The macula is the specialized region of the retina responsible for high-resolution visual acuity. This region is about 5.5 mm in diameter and is centered approximately 4 mm temporal and 0.8 mm inferior to the center of the optic disk. Anatomically, it can be defined as the central part of the posterior retina that contains xanthophyll pigment and two or more layers of ganglion cells. At its center, the umbo, the neural retina consists only of the ILM, Henle's fiber layer, the ONL, the ELM, and the PR outer and inner segments.

The retinal pigment epithelium (RPE) is a monolayer of polarized hexagonal cells densely adherent to one another as well as to the underlying Bruch's membrane, and is adjacent to the PR layer. The RPE has many functions, including phagocytosis of outer-segment disks in photoreceptor renewal, transportation of metabolic waste from the retina into the choriocapillaris, absorption of light and heat, and secretion of growth factors essential for proper differentiation of photoreceptors during development.

In the macula, RPE cells appear smaller in diameter with a higher cell density and broader and flatter in the periphery [7]. Macular cell densities also seem to increase throughout life [7], despite higher central apoptotic activity [8], suggesting the migration of peripheral RPE cells may compensate for the death of macular RPE cells. RPE cells of the macula have a higher melanin content and higher activity of lysosomal enzymes acid phosphatase and cathepsin D [9]. Whether this phenomenon is a functional adaptation to local microenvironments or an irreversible epigenetic process is unclear. This will be an important point to elucidate, for if the RPE mosaic were predetermined, then autologous peripheral cell transplantation would not be ideal.

The retinal blood vessels supply the inner half of the retina and the choriocapillaris supplies the RPE and outer retinal layers. Both have a blood-retinal barrier analogous to the blood-brain barrier. The outer blood-retinal barrier is maintained by the RPE; it blocks the inward migration of small molecules from the choriocapillaris into the subretinal space via tight junctions (zonula adherens and adjacent zonula occludens), both situated near the apex of the cell and encircling it. Specialized tight junctions (zonulae occludens) between individual retinal vascular capillary endothelial cells constitute the inner blood-retinal barrier. The subretinal space is therefore considered an immune-privileged site [10].

THE EYE AND THE RPE

Our significant dependency on sight makes good vision directly related to quality of life. This makes the eye and vision-threatening diseases an area of interest. The eye is unique in that there are *in vivo* endpoints, such as visual acuity and imaging modalities, which are measureable to assess successful engraftment of transplanted cells. Spectral-domain optical coherence tomography (OCT) allows *in vivo* histopathologic visualization of the retinal layers, while autofluorescence imaging allows *in vivo* assessment of the health and function of the RPE cells. The anatomy of the eye also makes it an ideal target organ for cell transplantation and tissue engineering. As mentioned above, the distinctive vascular system of the retina allows the subretinal space to be considered an immune-privileged site.

In the eye, degeneration of neural cells in the retina is a hallmark of such widespread ocular diseases as AMD and RP. In these diseases, the primary cause of blindness is the loss of photoreceptors, which can result from dysfunction in either the PRCs or the underlying RPE that supports their survival.

Damage to the RPE resulting in atrophy is a critical feature of AMD. RPE cell death occurs presumably via apoptosis associated with the loss of cell attachment [11]. Data suggest that widespread oxidative damage occurs in the retina of patients with advanced GA [12]. Therefore, oxidative damage is thought to play a major role in advancement of RPE loss. Additional reports support a role for the immune system, including complement activation in the development of drusen and the pathogenesis of AMD. Degenerative changes within the neurosensory retina and/or choriocapillaris occur simultaneously with RPE atrophy [13].

The RPE cell exhibits an apico-basal polarity necessary for transportation of water, ions, and nutrients [14]. Water and ions are constantly pumped from the apical to basolateral surface of the RPE [15], thus maintaining retinal adhesion [16]; nutrients such as glucose, retinol and fatty acids are actively taken up by the RPE from the blood and either utilized within the cell or transported to the PRCs [17]. As previously mentioned, the RPE is a key metabolic coordinator for PRCs in the phagocytosis of PR outer segments, which in turn enables regeneration of rhodopsin molecules in the visual cycle of vitamin A [18,19]. Proper epithelial polarity is also essential for transport of glucose, retinol, and other nutrients from blood to the photoreceptor, and electrolytes from the subretinal space to the choroid [20]. Thus, success of RPE transplantation also depends on the ability of the transplanted cells to establish polarity after transplantation and perform the complex functions of the RPE. It is a logical target cell for transplantation as the RPE layer is anatomically isolated and is a self-contained monolayer that can be readily assessed with a retinotomy to the subretinal space.

In retinal degeneration, the replacement of diseased RPE would be critical to protect or rescue the adjacent PRCs and vice versa. Kwan successfully transplanted photoreceptors in a mouse model of retinal degeneration, which reconstituted outer segments and allowed these mice to perform simple light-dark discrimination testing [21]. Replacing photoreceptors is far more challenging than replacing RPE cells because the connections between photoreceptors and neurons are lost, and also because of the potential scarring response.

RPE TRANSPLANTATION

The concept of RPE transplantation evolved out of improved human RPE cell culture experiments by Flood [22]. This was followed by several studies in which cultured human RPE cells were transplanted into monkey eyes [23,24]. Later, the therapeutic potential of RPE transplantation was demonstrated in the Royal College of Surgeons (RCS) rat model in the late 1980s [25]. These rats have a mutation in the MERTK, an enzyme necessary for RPE-specific phagocytosis of the photoreceptor segments. Their impaired ability to perform phagocytosis leads to photoreceptor dysfunction and death; retinal atrophy occurs within two months of birth, and by three months of age these animals become fully blind. Transplantation of cultured immortalized human RPE has been shown to improve photoreceptor survival and also prevented the onset of secondary degenerative events in these animals [26]. Dissociated retinal epithelial cells obtained from the retinas of normal, congenic pigmented strain of rats were transplanted onto Bruch's membrane in the subretinal space of RCS rats and were also shown to be fully capable of phagocytosing host outer segments, thus preventing subsequent retinal atrophy [25].

Attempts at RPE transplantation in humans were then performed by Peyman in two cases of terminal AMD [27]; the first case utilized an autologous flap and demonstrated visual improvement, whereas the second used allogeneic homologous RPE cells with no report of visual improvement.

Allogeneic RPE cells and sheets for AMD

Transplantation of both intact sheets of RPE and suspensions of isolated individual RPE cells have been attempted as treatments for AMD. Among the earliest attempts, Algvere et al. treated five AMD patients with a patch of cultured human fetal RPE placed in the foveal area after membrane excision [28]. At three months follow-up, four of five cases lost fixation and developed macular edema, suggesting rejection. At 12 months follow-up, most of the patients had experienced mild visual loss compared to their preoperative vision [28]. A subsequent experiment transplanted circular patches outside the fovea in four eyes of atrophic AMD with the intention that the fovea might become overgrown with RPE or a rescue effect may occur. At one year, there was no adverse or beneficial effect on visual function [29]. Finally, a cell suspension of concentrated dissociated fetal human RPE has been used in seven cases of atrophic AMD. Rejection developed with 50,000 RPE cells (after 8–12 months) and with 500,000 cells (after three months). Two eyes, which received 20,000 and 200 cells, did not show rejection at one year but there was also no visual improvement [30].

Other studies have shown that allogeneic RPE cells do not attach to senescent Bruch's membrane efficiently and do not undergo proliferation and spreading to fill in the defect [31,32]. Even under circumstances in which allogeneic RPEs do attach and proliferate on aged Bruch's membrane, their ability to survive long-term is compromised [33].

Graft rejection is a serious concern in allogeneic transplants. Initial reports on RPE allografts in the RCS rat suggested that there was no rejection in eyes at one year follow-up [34]. Subsequent studies, however, demonstrated chronic rejection mediated through MHC II expression [35]. It was also reported that the immunologic response was related to the number of transplanted cells and increases over time [36,37]. Consequently, strategies to improve RPE graft survival were attempted with disappointing results [38,39].

Autologous RPE cells and sheets for AMD

Autologous transplantation, in cell suspension or as a patch of RPE and choroid, seems an obvious solution to avoid graft rejection. Autologous transplantation with iris pigment epithelial cells has been documented for use in patients with AMD and RP. Preliminary results suggest use of these cells may help preserve but not improve visual function [40–43]. In one trial, 20 patients with exudative maculopathy underwent surgical removal of choroidal

neovascularization combined with transplantation of autologous iris pigment epithelial cells to the subretinal space [43]. After three years of observation, one patient had significant visual acuity improvement, 13 were unchanged, and three patients had vision loss. Vascular membranes did not recur. In 1997, Rezai attempted to use autografts of iris pigment epithelium (IPE) to replace defective RPE [44]. IPE has the ability to phagocytose PRC outer segments [45] but at a much slower pace [46]. The use of IPE autografts is especially appealing due to the ease of harvesting the cells from a peripheral iridectomy. Transplantation can then be scheduled at an optimal time, even years later, as it has been shown that cryopreserved IPE does not lose function during long-term storage in liquid nitrogen [47]. Some degree of visual improvement has been reported as well as prevention of further vision loss [41,42,48]. However, IPE has also been shown to be incapable of expressing essential enzymes of the retinoid visual cycle [49].

Several studies have also investigated harvesting of RPE cells from the posterior retinal areas. In a prospective study of 14 eyes (13 patients) with AMD, Binder et al. applied a subretinal technique with gentle mobilization and aspiration of RPE cells from the nasal retina and reported improvement in BCVA of two or more lines in 57.1% [50]. Similar results were shown in a larger prospective study of 53 eyes in patients with foveal choroidal neovascularization, who were not eligible for laser or photodynamic therapy [51]. Improvement of visual acuity by two or more lines was seen in 53.8% of patients receiving an autologous transplantation of RPE cells compared to 21.1% of patients who had excision alone. This study demonstrated total cell numbers harvested ranging from 11,000 to 29,000 with constant harvesting efficacy (6165 $\pm$ 746 cells/DD) and high cell viability (82.0 $\pm$ 6.9%). This established that reasonable numbers of highly viable RPE cells suitable for autologous RPE transplantation can be efficiently harvested from small retinal areas [52]. Light and electron microscopy of the debridement site revealed extensive disruption of the ONL with only remnants of inner segments remaining. The choriocapillaris was replaced with extensive collagen deposition. Some areas had no RPE while others had thickened Bruch's membrane. In contrast, microscopy of the transplanted site showed the presence of RPE with normal shape, polarity, and pigmentation with basal infoldings and apical villi surrounding PR outer segments. The cells were arranged in a monolayer in some areas, while other areas revealed multilayers. The choriocapillaris was present and appeared normal [53]. Binder also described a surgical technique for transplantation of the RPE suspension via subretinal cannula. In a pilot study, 14 eyes underwent subretinal surgery for foveal choroidal neovascularization (CNV) with simultaneous transplantation of RPE harvested from the nasal subretinal area of the same eye. After a mean observation of 17 months, BCVA improved two or more lines in 57.1%, remained the same $\pm$ one line in 7.1%, and useful reading acuity between Jaeger 1 and 4 points was achieved in 21% [50]. Van Meurs et al. described a different approach, wherein 80,000–160,000 RPE cells were mechanically removed from the retinal periphery and translocated under the macula following the submacular injection of 2 μg poly-L-lysine to promote cell adhesion. Of the eight cases studied, vision remained unchanged in five eyes but deteriorated in three because of retinal detachment (RD) and proliferative vitreoretinopathy (PVR) [54].

Transplantation of intact RPE sheets has also been described, with mixed results. The benefit of sheets over suspension is that RPE sheets provide a means of transplanting an organized polarized patch of RPE. Several studies reported disappointing results due to formation of multilayers with folding and contraction of the sheet following transplantation [55,56]. Van Meurs reported stabilization of vision or mean gain of one line in patients for whom he performed full thickness RPE-choroid transplantation after CNV removal [54]. A major limitation of the autologous RPE patch graft is obtaining a desirable graft size. Further, the RPE-choroid sheet method is very traumatic and predisposes to the observed high PVR complication rate [57]. MacLaren et al. examined long-term results of AMD patients who underwent surgery for (CNV) and simultaneously received a macular RPE translocation. Although RPE choroidal grafts survived five to six years post transplant, visual acuity

continued to decrease in three of four patients followed and all patients lost foveal fixation [58].

Autologous RPE transplantation may be limited as these cells carry the same genetic information to that which may have led to the AMD manifestation. Whether the transplanted cells can adhere to damaged Bruch's membrane in AMD patients, which is a process that is essential for survival and function, remains another area of concern [32,59,60].

STEM CELLS IN RETINAL DEGENERATION

Tissue replacement in the body occurs by two physiologic mechanisms. One is the replacement of differentiated cells by newly generated populations derived from residual cycling stem cells (SCs). The second mechanism is the self-repair of differentiated functioning cells, thus preserving their proliferative activity.

SCs are of interest because of their plasticity and the capacity to self-renew and give rise to specialized cell types. They remain uncommitted and self-renewable until they receive a signal to develop into distinct cell types. Retinal SCs may restore vision in patients who have degenerative retinal disease by two possible means: 1) repopulation of damaged retina, and/or 2) rescue of retinal neurons from further degeneration [61]. Recent reports have demonstrated the ability of human neonatal photoreceptor precursors to differentiate and integrate into the ONL in both the intact and the degenerating retina of mature mice [62,63]. Evidence has shown that photoreceptors and embryonic retinal tissue, when transplanted into the subretinal space, can form new synapses with existing host neurons. MacLaren et al. showed that timely selection of the progenitors is essential for success; the optimal result occurred when selected cells were biochemically committed but not yet morphologically differentiated [63].

Adult stem cells

Adult SCs are committed SCs or progenitor cells from various tissues in both adult animals and humans. For cell replacement therapy in the ocular system, the isolation and characterization of murine retinal SCs (RSCs) in 2000 represented an important step forward [64,65]. These cells were localized in the pigmented ciliary margin and were able to clonally proliferate *in vitro* to form spherical colonies that differentiated into retinal-specific cell types, including rod photoreceptors, bipolar neurons, and Muller glia. Similar cells were later identified in other mammalian species, including pigs and humans, but proliferated to a lesser extent than fetal or ESC-derived retinal stem cells [66,67]. Retinal stem cells were also discovered within the iris epithelium as described above.

Adult bone marrow-derived stem cells

Bone marrow (BM)-derived SCs (BMSCs) are multipotent SCs that differentiate into cell types from the germ layer of their origin. However, unlike embryonic stem cells, which are pluripotent, or can give rise to any cell type of the body, BMSCs have a limited range of differentiated derivatives. The following subsets of SCs can be found in the BM:

1) Hematopoietic SCs (hSCs), the source of all blood cells;
2) Mesenchymal SCs (MSCs), non-hematopoietic stromal cells that differentiate into mesenchymal tissues; and
3) Tissue-committed SCs (TCSCs) that are already committed to neural, cardiac, and other lineages [68,69].

A mouse model study showed that adult BMSCs could upregulate some of the RPE markers *in vitro* and can home onto focal layers of induced RPE damage in a mouse model and form a monolayer of MITF-positive cells on the Bruch's membrane [70].

Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent cells that can give rise to all of the cell types in the body. Thus, ESC could be seen as a universal cell source for regenerative medicine, and multiple studies have shown their ability to produce functional cells of various types, which were successfully used in different animal disease models. Human ESCs show the following characteristics:

1) They can be isolated from blastocysts, morula-stage embryos, or even from a single blastomere without embryo destruction;
2) They proliferate indefinitely *in vitro*;
3) They maintain a normal euploid karyotype over extended culture;
4) They differentiate into derivatives of all three germ layers;
5) They express high levels of markers of pluripotency such as Oct-4, NANOG, Rex-1, TRA-1−60, TRA-1−81, SSEA-3, SSEA-4, alkaline phosphatase, and
6) They show telomerase activity [71].

Numerous differentiated cell types have been derived from ESCs, including neural tissue, endothelial cells, blood cells, cardiomyocytes, and hepatocytes. Schraermeyer and colleagues were the first to demonstrate that subretinal injection of mouse ESCs into RCS rats can prevent PR degeneration [72], yet these transplanted mouse ESCs produced teratomas when transplanted into various adult tissue sites [73,74]. Klimanskaya et al. demonstrated reproducible generation of RPE from human embryonic stem cells (hESCs) [75], which were capable of extensive photoreceptor rescue in the RCS rat model [76,77]. The hESC-derived RPE (hESC-RPE) cells were transplanted into the subretinal space of RCS rats at six weeks of age, when the photoreceptors were still alive as assessed by various visual tests (optomotor response, luminance threshold, ERG). While the control (sham-injected) animals were completely blind, hESC-RPE recipients showed a remarkable rescue of the photoreceptors as demonstrated by vision tests and also confirmed histologically. In control animals, the ONL of the photoreceptor was gone or diminished to 1−2 cell layers thick, whereas in the operated rats, multiple (5−7) layers of photoreceptors were preserved. The cells integrated into the host's RPE layer and stained positive with two RPE-specific markers, RPE65 and bestrophin. They also stained negatively with human-specific proliferating cell nuclear antigen (PCNA), indicating lack of continued cell division among the donor cells. They demonstrated long-term survival by remaining viable for over 100 days after transplantation. The photoreceptor rescue effect in RCS rats was seen with as few as 20,000 cells and became more pronounced at 50−100,000 [77].

hESC-RPE also provided a similar rescue of the photoreceptor in a mouse model of Stargardt's disease (ELOVL4 mutation) [77]. In untreated ELOVL4 mice, visual acuity deteriorated as photoreceptor degeneration progressed. Subretinal transplantation of hESC-RPE cells improved visual acuity compared to controls at all time points, which was found to be statistically significant. No tumor formation or abnormalities were observed in these studies.

In 2012, Schwartz et al. transplanted the first human patients with hESC-RPE in the course of Phase I/II clinical trials [78]. The cells used for the trial were produced under Good Manufacturing Practices (GMP) and passed multiple safety criteria. They were shown to be free of human and certain animal pathogens, have normal karyotype, and were free of contaminating pluripotent cells. When injected into immune-deficient NIH III mice [78], hESC-RPE did not proliferate beyond three months after transplantation as demonstrated by staining for Ki-67, integrated into the host's RPE layer, and exhibited characteristic morphology and baso-lateral organization of bestrophin, typical for mature polarized RPE (Fig. 68.3); no tumors or other abnormalities were detected after nine months post-transplantation. These data indicated that transplanted hESC-derived RPE could be safe and efficacious in a host. Two patients, one with dry AMD and one with Stargardt's disease, were injected subretinally with 50,000 viable hESC-RPE cells in suspension. The patients underwent pars plana

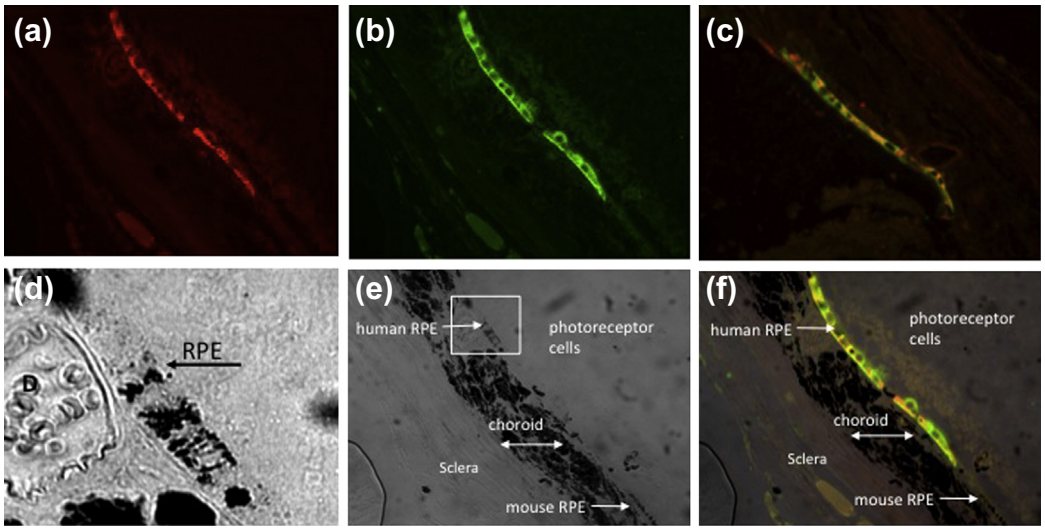

FIGURE 68.3

Survival and integration of RPE generated from hESC-MA09 into NIH III mouse eye after nine months. Section stained with anti-human mitochondria (a) and anti-human bestrophin (b). Notice precise co-localization of human mitochondria and bestrophin staining in the same cells (c: a and b merged) and absence of staining in mouse RPE (f: a, b, c, and e merged). Frame on the bright field image (e) is enlarged in (d) to show morphology of human RPE. Magnification 200x (a—c, e, f) and (d) is additionally magnified 4.5x.

vitrectomy with induction of a posterior vitreous detachment at the optic nerve. The hESC-RPE cells were injected into the subretinal space with a 38-gauge cannula, creating a subretinal bleb. Initial observations revealed no adverse events, including no signs of hyperprofileration, tumorigenicty, ectopic tissue formation, or apparent rejection.

DELIVERY OF CELLS

Currently, the two most popular methods of delivering stem cells into the eye are the intravitreal and subretinal routes. The intravitreal route delivers stem cells into the eye through an injection using a small-gauge needle. This method is technically easier and less invasive than the subretinal route. However, the cells have to migrate through the vitreous and inner retina to the outer retina. There are also no means of directing the cells toward the target treatment area.

Subretinal transplantation strategies include grafting RPE sheets on a BM prosthesis and subretinal injection of cell suspension. As mentioned earlier, RPE cell suspensions may fail to survive or function on the damaged BM [32, 59,60]. Formation of multilayered clumps that damage the overlying neural retina has also been reported [79,80], which could also be a concern for hESC-derived RPE cell suspensions.

The use of biological and tissue-engineered membranes

It was been shown that the survival of donor RPE *in situ* is impaired when cells are plated onto aged Bruch's membrane or Bruch's membrane from AMD donors [33,81]. Thus, many groups have tried to address this issue by providing a RPE carrier substrate to serve as either a temporary or permanent BM prosthesis. Amniotic membranes appear to provide a suitable growth surface for RPE and IPE, both *in vitro* and *in vivo* [82—85]. Human RPE cultured on dispase-treated amniotic membrane [83] showed differentiated phenotype and upregulated gene expression for RPE65, Retinaldehyde-binding protein 1 (CRALBP), bestrophin, and Tyrosinase-Related Protein 2 (TRP-2) as well as production of vascular endothelial growth factor (VEGF), thrombospondin-1 and pigment epithelial derived factor (PEDF). Morphology of cadaver human RPE was retained when cultured on amniotic membrane [85]. Interestingly, when IPE was cultured on amniotic membranes, it showed an upregulation of genes important for RPE function, such as PEDF, bestrophin, and RPE65, and subretinal transplantation of IPE sheets on amniotic membranes

showed a significant rescue of photoreceptor in RCS rats [84]. RPE maintained its phenotype in culture on ILM [86], elastin-like recombinamer (ELR) films, which have an integrin-binding domain RGD [87], and Bombyx mori silk fibroin (BMSF). RPE cultured on anterior lens capsule was also successfully transplanted *in vivo* [88].

Artificial membranes need to be inert to prevent rejection, porous to allow diffusion of small and large molecules, and have a suitable RPE attachment surface. One such membrane is a synthetic mesh-supported submicron parylene-C membrane (MSPM) [89]. The permeability of parylene-C membranes to macromolecules is similar to the permeability of Bruch's membrane. hESC-RPE cells cultured on these membranes, adhered, proliferated, and formed a monolayer of polarized epithelia typical for RPE with microvilli. A study comparing several matrix candidates performed in pig eyes showed that parylene-C membranes as well as polyethileneglycol did not produce any significant disturbance to the retinal anatomy [90]. Other substrates which hold promise as a matrix include cross linked collagen [91], cryoprecipitate [92], and synthetic matrices such as poly-L-lactic-acid/poly-lactic-co-glycolic-acid (PLLA/PLGA) [93,94] and surface modified poly(hydroxybutyrate-co-hydroxyvalerate) thin films [95]. Corneal endothelial cell-produced extracellular matrix and conditioned medium improve survival of human RPE on aged Bruch's membrane, which suggests another possible tool to boost the efficacy of RPE transplantation for AMD patients [96,97].

An important observation we made in the course of experiments for optimization of hESC-RPE preparation for human clinical trials was the correlation of RPE pigmentation, i.e., maturity, with its post-thaw attachment and survival *in vitro* as well as with survival after passing through the injection needle [78]. Cells with higher pigmentation level had a lower overall survival rate, and an optimal melanin level was found that correlated with high survival of the cells *in vitro* [78] and the efficacy of photoreceptor rescue *in vivo* [77]. The low rate of success of transplantation of human RPE – both fetal and adult, allogeneic and autologous – could be partially attributed to the phenotype of freshly harvested cells that are highly pigmented and have mature tight junctions, which may take too long to disrupt during cell isolation, and therefore damage the cells prior to transplantation. An important advantage of hESC-RPE cells is that its phenotype can be modulated in culture to the desired standard by harvesting them when they are still 'underpigmented' to ensure optimal cell survival.

CONCLUSIONS AND FUTURE DIRECTIONS

As the rates of visual impairment and blindness continue to rise, so does the importance of finding a feasible therapy. Regardless of the underlying etiology of the retinal degeneration, the common endpoint is loss of the photoreceptors and underlying RPE. Cellular replacement strategies offer a promising solution in the treatment of retinal degeneration. Success of these strategies is contingent on finding a viable source of replacement cells, establishing a safe technique for delivery, survival of transplanted cells within the host, restoration of normal retinal architecture, and stabilization or improvement in vision. Over the past two decades, there have been many advances in RPE cell replacement strategies. These studies have elucidated potential mechanisms, as well as challenges and drawbacks. Recent advances in stem cell therapy, though early in their development, offer a promising and exciting prospect for replacement therapies.

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CHAPTER 69

Vision Enhancement Systems

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INTRODUCTION

This chapter provides an overview of visual system properties in health and disease, with an emphasis on the stages in visual-signal processing that are most commonly affected by disease and dysfunction. It then provides an overview of current approaches to vision restoration and of approaches that promise to migrate from the lab to the clinic in the coming years. In a third section, the potential roles for engineered cells and tissues in this restoration process are discussed. The chapter closes with an estimate of the developments that can be expected to occur in the next 5–10 years.

Twenty years ago, the best hope vision researchers had for the enhancement of impaired vision was to build a better magnifier, by integrating optics and electronic image processing. Since then, restoration of vision to functionally blind individuals has become not just an engineering target, but a realistic goal for which engineering milestones are being set and reached. The role of tissue engineering in this field may as yet be modest, but it has great promise, as will become clear in the following pages.

In this chapter's overview of approaches to the restoration and enhancement of impaired vision in human patients, inspired by tissue-engineering principles and related technologies, we argue that the nature of eye disease and the fragility and complexity of ocular tissues such as the neural retina do not (yet) lend themselves to application of the techniques emerging in the repair of other tissues. Thus, while some of the approaches presented here can function independently of tissue engineering as presented elsewhere in this volume, they at least complement those engineering approaches and lend themselves to future integration. This is particularly true in the areas of cell transplantation and neural prosthesis development, presented in the later part of this chapter, but to a lesser extent also for the optical and optoelectronic aids presented.

VISUAL SYSTEM, ARCHITECTURE, AND (DYS)FUNCTION

Human vision is mediated by one of the most highly developed sensory systems found anywhere in nature. Its capacity to combine high spatial resolution near the center of fixation with a wide peripheral field of view, accurate depth perception, color discrimination, and light-dark adaptation over 12 orders of magnitude is unparalleled. Every stage in the system is organized to accomplish this. The photoreceptor layer in the retina provides the high signal

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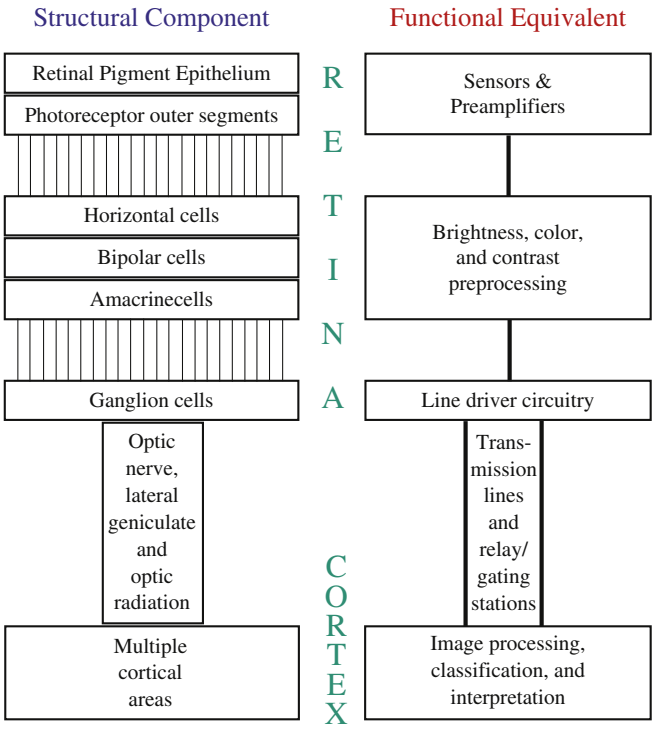

FIGURE 69.1
Schematic representation of the visual system: The outer retina (RPE and photoreceptor layers) form the sensor array, followed by several inner retinal preprocessing stages, the ganglion cell transmission stages, and further central processing stages in subcortical and cortical brain centers.

amplification of the rods required for night vision and the dense packing of three cone types required for detailed central and color vision. The intricate local preprocessing performed by subsequent retinal cell layers augments these functions by performing brightness and color comparisons and helps to condense the information stream acquired by over 100 million photoreceptors, to allow transport across a mere 1 million fibers in the optic nerve to the visual centers in the brain, where further parsing and interpretation of the image takes place.

From a functional point of view, the visual system can be understood as depicted in Fig. 69.1: with sensors, preamplifiers, preprocessors, transmission lines, and several central processor stages. The two most crucial stages in this process — the conversion from light into chemical and electrical signals, and the signal transmission from the eyes toward the brain — are also the most vulnerable ones. Light conversion and signal amplification in the photoreceptors require a highly complex interplay between the molecules inside these cells and ion channels and other permeable structures in the cell membrane. These components participate in interlocking cycles of conversion and regeneration and are assisted by surrounding cells — in particular the retinal pigmented epithelium (RPE) cells, which provide nutrients to, and digest cell membrane discarded from, the photoreceptors. Any step in this intricate process can easily be disrupted by nutritional deficits, overexposure to short-wavelength light (presumably causing oxidative changes), attacks by pathogens, and especially genetic miscoding of one or more participating molecules. Since the mid-1980s, the list of mapped (>225) and identified (>185) gene loci has grown at an ever-increasing rate. For each identified gene there may be multiple mutations, often leading to distinct disease phenotypes; for an up-to-date list of the known mutations leading to loss of outer retinal function, see the Retnet website [1]. Not only do such mutations directly cause impaired signal transduction, but the additional energy demand, presence of abnormal molecules, and excess shedded cell membrane may exceed the RPE cells' support capacity, which inexorably leads to degeneration of both photoreceptors and RPE cells. The most common group of disorders caused by genetic miscoding of molecules involved in the phototransduction cycle is jointly known as retinitis pigmentosa (RP), while another group is caused by a breakdown of RPE function in the central retina due to either a genetic defect (Stargardt macular dystrophy) or

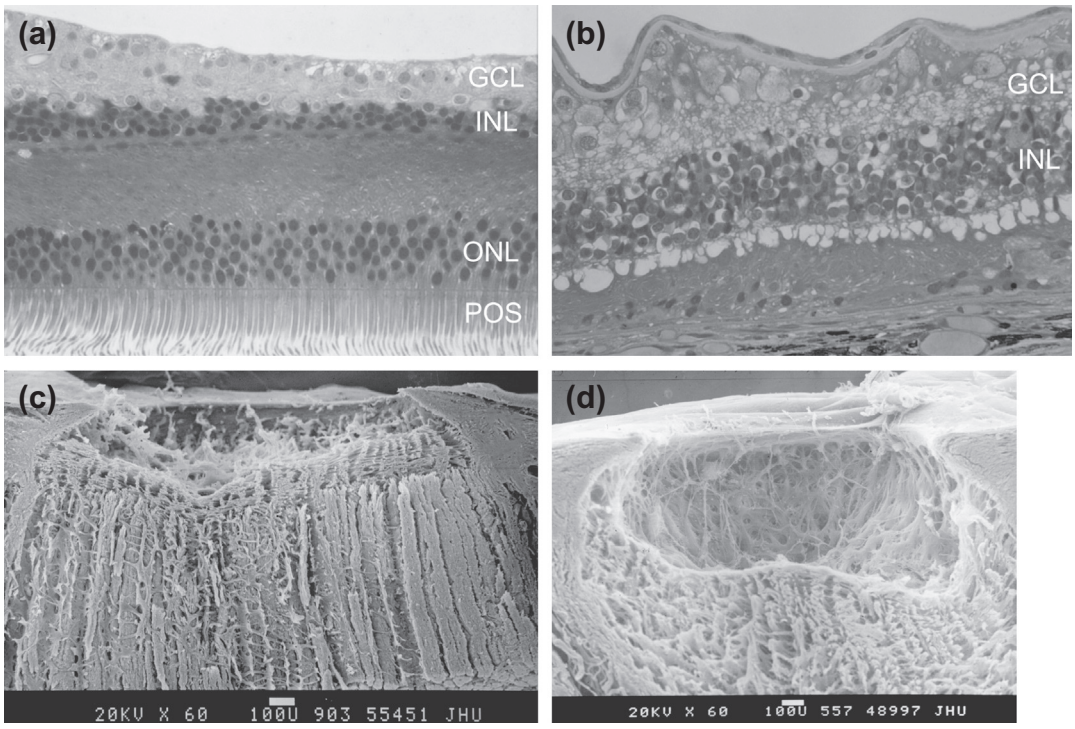

FIGURE 69.2

Representative samples of ocular morphology in healthy and diseased conditions. (a) Cross section through the retina near its center (fovea), showing healthy photoreceptor outer segments (POS), multiple layers of photoreceptor cell nuclei in the outer nuclear layer (ONL; labeled black), bipolar cell nuclei in the inner nuclear layer INL) and ganglion cell bodies in the ganglion cell layer (GCL). (b) Retina of a patient with a long history of retinal degeneration, and bare light perception in the last years of life, showing lack of photoreceptor outer segments and cell bodies in comparison with (a). (c) Scanning electron microscope cross section of the optic nerve head, showing healthy appearance of the support structure, the lamina cribrosa. (d) Optic nerve head from a patient with long history of glaucoma, showing compression of the lamina cribrosa and embedded nerve fibers (RGC axons). *Micrographs C and D courtesy of Harry A. Quigley, M.D., the Johns Hopkins Univ., Baltimore, MD.*

a combination of genetic predisposition and environmental factors (age-related macular degeneration, AMD). Jointly, all these disorders are known as retinal degenerations (Figs. 69.2a and b).

Retinal neurons communicate in a variety of ways, with ion-gating channels fulfilling a role in reaching and maintaining an operating level (also known as the adaptation state) and neurotransmitters and gap junctions fulfilling the principal messenger roles in carrying information in chemical and electrical form, respectively. The loss of outer retinal function, and therefore of neurochemical and electrical signal transmission to the inner retina, will affect secondary retinal cells (horizontal, bipolar, amacrine, and ganglion cells) but not necessarily threaten their survival. Until a few years ago, morphometric studies of donor retinal tissue such as that shown in Fig. 69.2b suggested survival of bipolar and ganglion cells at rates of 80% and 30% in the macula [2] and 40% and 20% at eccentricities up to 25° [3], respectively, in retinas practically devoid of photoreceptors. In recent years, a series of highly detailed and elegant microanatomical studies using a technique called computational molecular phenotyping (CMP) [46] has demonstrated that the connectivity patterns of these inner retinal cell populations are fundamentally altered by the degeneration process (Fig. 69.3). Survival of these cells is predicated on their continued activity, and this they accomplish through the formation of new axonal and dendritic branches and through self-organization into clusters called microneuromas. These new and functionally random connections allow spontaneous oscillations to occur: in the absence of meaningful input signals from the erstwhile photoreceptors, RP patients may experience such spontaneous activities as photopsias, or 'light shows' [4].

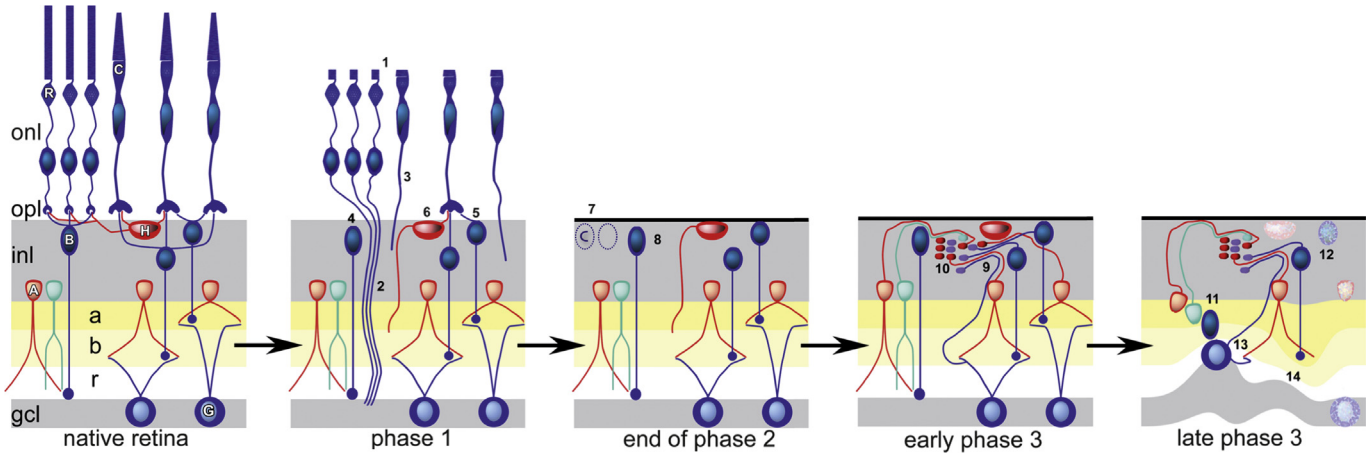

FIGURE 69.3

Simplified schematic of possible alterations during retinal remodeling. The first panel shows a representative 'native' mammalian retina with its basic complement of rods (R) and cones (C) driving bipolar (B) and horizontal (H) cells. In turn, bipolar cells drive amacrine (A) and ganglion cells (G) of the proximal retina to form the major cone OFF (sublayer a), cone ON (sublayer b) and rod ON (sublayer r) zones of the inner plexiform layer (IPL). Remodeling occurs in phases, as indicated under each panel. In phase 1, rod and cone stress lead to truncation of outer segments (1) and, in some instances, extension of rod (2) and cone (3) axons deep into the inner nuclear (inl), inner plexiform and even ganglion cell (gcl) layers. Both rod and cone bipolar cells truncate their dendrites (4,5) and some rod bipolar cells may transiently switch to surviving cones (not shown). Horizontal cells also send axons into the inner plexiform layer (6). Phase 2 is a complex period of cell death, ablation of the outer nuclear layer (onl), and resolution of the distal margin of the neural retina into a largely confluent glial seal (7) formed by the distal processes of Müller cells. Surviving neurons may continue to alter their phenotypes by changing receptor expression patterns (8) and some cone cells may even escape cell death (C). Neural remodeling becomes even more extensive during phase 3, with formation of complex axon fascicles (9) and new synaptic complexes termed microneuromas (10). As remodeling continues throughout life, some neurons begin to migrate along glial columns (11), others die (12), and the inner plexiform layer becomes transformed through new synapse formation (13) and laminar deformation (14).

At the retinal output signal transmission stage, where retinal ganglion cells (RGCs) convert the neurotransmitter signals into electrical spike trains that travel along RGC axons toward the thalamic relay nuclei and other brain areas, the system is vulnerable to mechanical insults rather than to genetic dysfunction: injury to the optic nerve (trauma), increased pressure inside the eye (crushing the fragile axon fibers at the optic nerve head [glaucoma]), and inflammation (optic neuritis) or impaired blood supply (ischemic neuropathy) of the optic nerve itself. Each of these can impair or interrupt the signal-carrying capacity of optic nerve fibers (Figs. 69.2c/d).

In addition to damage occurring at these distinct stages, more generalized damage to the retina can occur. Common mechanisms for this are the leakage of capillaries in diabetic retinopathy and the interruption of the blood supply to the inner retina (retinal vascular occlusive disease), especially in individuals with a predisposition toward the development of blood clots. Each of these can lead to widespread cell death in the inner nuclear and ganglion cell layers throughout the affected area. Vascular occlusive disease provokes angiogenesis: New retinal vessels sprout in response to growth factors signaling ischemia, but these vessels tend to leak and damage the retina's fragile structure. Diabetic retinopathy is a major cause of preventable blindness in the developed world and increasingly in developing countries as well.

CURRENT AND NEAR-TERM APPROACHES TO VISION RESTORATION

As in all biomedical engineering, approaches to restore function can be based on interruption of the disease process and tissue regeneration (autologous or grafted), or on hybrid techniques combining biological tissue with synthetic materials and devices. To support

the cells affected by disease or injury, neuroprotective substances, growth factors, and genetic modifications of cell function may be used, postponing or preventing further loss of function. In addition, as long as usable function remains, one can strengthen the stimulus and internal response signal, counteracting visual impairment as much as possible.

If little or no sensor function remains, one can seek to restore it in limited form through newly grown or transplanted photoreceptors and/or RPE cells, through a prosthetic device that will electrically stimulate the remaining secondary retinal cells, or through man-made tissues that mimic photoreceptor function. At the RGC level, substitution for lost signal transmission may be sought through protection of any remaining cells and administration of factors promoting axon regeneration. This may require the use of synthetic tissues and factors enabling reconnection of axons with central structures — or through *in situ* growth of new cells, promoted to differentiate into RGCs and to send new axons through the optic nerve.

In preliminary form, many of these approaches exist in the laboratory or are entering clinical testing, while others are merely ideas. In the following sections, both existing and prospective methods are presented.

Enhancing the stimulus through optoelectronic and optical means

Most of these devices are based on a combination of optical and electronic image enhancement techniques. Their common principle of operation is to improve visibility of the image to the diseased retina, through magnification, contrast and/or color enhancement, and filtering or feature extraction. Early incarnations, such as the low vision enhancement system — LVES; no longer in production; see Fig. 69.4a and Massof [5] — used optical magnification, zoom, automatic focus, and — for applications limited by available contrast, such as face recognition — analog image enhancement. These techniques have been adopted by more lightweight and cosmetically attractive video visors, such as the Jordy (Fig. 69.4b; Enhanced Vision Systems, Huntington Beach, CA) that enjoy considerable popularity among

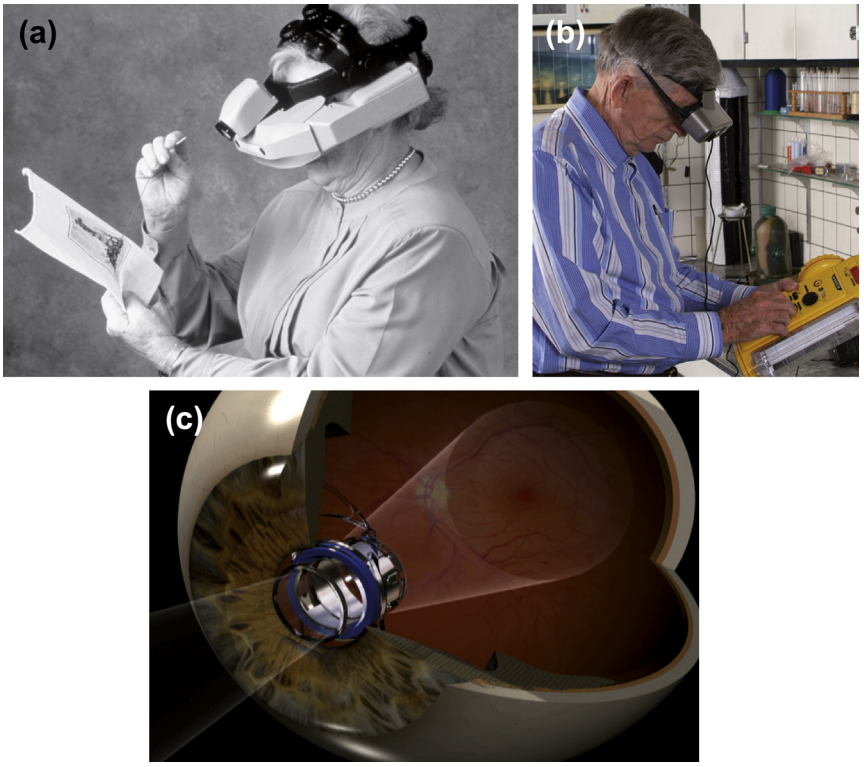

FIGURE 69.4
(a) The Low Vision Enhancement System (LVES), developed at the Johns Hopkins University's Lions Vision Center, with support from NASA and the Department of Veterans Affairs, is a characteristic example of optoelectronic vision enhancement systems. This system features binocular orientation cameras, a centrally placed 1.5—10× zoom camera with automatic focus, and a binocular projection path with 36°×48° field of view and built-in refractive correction and alignment for the wearer. (b) The Jordy video visor has a narrower field of view, but is much lighter thanks to its LCD screens, and has a full color display. Photo courtesy of Enhanced Vision Systems. (c) The Implantable Miniature Telescope (IMT™) provides monocular 3× magnification, to benefit patients with large central scotomas. *Diagram courtesy of VisionCare Ophthalmic Technologies.*

patients with severe visual impairment. More advanced image processing techniques, such as digital feature enhancement and gaze-contingent remapping, will allow wearers to avoid losing crucial information in blind areas of the visual field. Such image processing techniques are now feasible, but cost and cosmetic considerations have thus far limited their application. Another limitation of current versions is the product of field of view and angular resolution, i.e., the number of pixels across the screen. The expectation is that the widespread penetration of high definition video and telepresence applications in industry and entertainment will enable cost-effective application of this technology to low vision aids. Similarly, cosmetic and weight factors will improve as smaller, lighter, and more luminous flat panel displays become available.

A useful property of the cameras in portable video visors is their built-in automatic gain control, resulting in constant internal image brightness over a wide range of environmental illumination levels. Among other properties, this makes the image acquisition and processing stages of these systems highly suitable as sensors and preprocessors, respectively, for prosthetic and tissue-based image enhancement systems.

Optical systems can also be used to increase the visibility of the stimulus. One such system, approved by the United States Food and Drug Administration (FDA) for clinical application in 2011 and aimed at patients with a large central scotoma (blind spot) in both eyes due to advanced AMD, is an implantable miniature telescope (IMT™, Fig. 69.4c; VisionCare Ophthalmic Technologies, Saratoga, CA), placed behind the iris in the location of the crystalline lens. It provides one eye with a magnified view of the central visual field, while the other eye is not implanted, to preserve the wide peripheral view of the fellow eye.

Following an adaptation and rehabilitation training period of three to six months, most wearers have no difficulty switching between the magnified view in the implanted eye and the unenhanced view in the fellow eye. Note that this is a true prosthetic device, since it is permanent.

Visual prostheses based on electrical tissue stimulation

As was already mentioned, retinas with severe degeneration of the sensor layer retain high numbers of secondary cells. In analogy with the principle of operation of the cochlear prosthesis, i.e., restoring limited hearing through stimulation of spiral ganglion cells in the cochlea [6], this provides the opportunity to convey rudimentary vision to the degenerated retina by a prosthetic device stimulating remaining secondary cells with a two-dimensional array of microelectrodes, reminiscent of the images produced by dot matrix printers a few decades ago [7]. From an engineering point of view, one can envisage a range of possible approaches (stimulation electrodes under vs. over the retina; fully integrated photosensing, image processing and stimulating systems vs. external image capture and processing linked to an intraocular stimulating matrix, to name a few) but also a host of questions arise concerning biocompatibility, signal processing, and power management.

The most pressing question, regarding the feasibility of conveying visual imagery to patients blind from retinal degeneration, has been answered affirmatively. In a series of experiments started at Duke University in 1992, continued from 1993 until 2000 at Johns Hopkins, and since then confirmed through similar experiments at Harvard University and in several German university clinics, volunteers with end-stage RP – whose remaining vision was limited to, at best, light perception – as well as several patients with advanced AMD have participated in tests where, during a surgical procedure under local anesthesia, the inner surface of the retina was electrically stimulated with small and brief biphasic current pulses applied through a single electrode or multiple electrodes [8–10]. Among the most salient findings are the subjects' ability to see small punctate light flashes (phosphenes), whose perceived location corresponds exactly to that of the stimulation, and the ability to see simple

patterns of multiple phosphenes when multiple electrodes are activated simultaneously. Stimulation at rates greater than 40–60 pulses per second is perceived as continuous stimulation, and perceived stimulus intensity increases with pulse duration and amplitude as well as repetition rate. Independent tests in blind volunteers and in amphibian retina [11] have demonstrated that stimulus pulses 1 ms or longer in duration preferentially stimulate the (deeper) bipolar cells rather than the (more superficial) RGCs.

Over the past few years, research in this field has moved from acute experiments to chronic implant models, and academic research has joined forces with start-up companies and in some cases government labs to develop dedicated technologies enabling chronic implants. A good example is the relocation of the implant group formerly at Johns Hopkins University to the Doheny Retina Institute at the University of Southern California, the founding of Second Sight Medical Products LLC (SSMP; Sylmar, CA) as a corporate partner, and the collaboration of these two entities with laboratories at state universities (e.g., the University of California in Santa Cruz (Fig. 69.5a), San Diego, and Berkeley) and in the US Department of Energy (Oak Ridge, Sandia, Livermore). The SSMP-led consortium, with cumulative private and public funding levels of $100M each, introduced the 16-electrode Argus 16 to clinical testing in 2003, and the 60-electrode Argus 2 in 2007. Following a clinical trial with 30 end-stage RP patients in the US and Europe, the Argus 2 was approved for routine clinical implantation in Europe in 2011, with a more limited approval in North America, under a humanitarian device exemption by the US Food and Drug Administration, granted in early 2013. Recipients of the device during the clinical trial have demonstrated the ability to localize and track objects [12,13] and recognize high-contrast letters and short words [14].

Simultaneously, several other groups are pursuing intraocular prosthetic devices. Such devices come in two variants. Stimulating 'photodiode arrays', typically placed under the retina (subretinally) and stimulating electrode arrays with external image capture and preprocessing, placed either subretinally or epiretinally (adjacent to the nerve fiber layer). The latter, more common, prosthesis type is being pursued by an additional group in the United States – Harvard/MIT [9] – and by two groups in Germany: Intelligent Medical Implants AG (Fig. 69.5b) and a consortium of German universities [15]. All three groups have designed prototypes with 50 or more electrodes and wireless transmission to the freely moving eye, but only the IIP group has been able to test several such prototypes in a series of clinical pilot studies [16]. A number of other groups, most notably in Japan, Korea, and Australia, are experimenting with similar implant designs. Some of these groups seek to stimulate the retina from outside the eyeball, to avoid the risk associated with retinal surgery, while accepting the reduced resolution inherent in the greater separation between electrodes and target cells. Others are testing electrodes that penetrate through the scleral wall of the eye, in order to bring the stimulus charge as close as possible to the target cells.

The less common type of retinal prosthesis, a subretinal chip that uses photovoltaic elements for image capture in the location once occupied by the native photoreceptors, is conceptually simpler but in practice more challenging. In a primitive version [17] (Fig. 69.5c) of the subretinal array, a 2 mm-diameter silicon chip with approximately 5,000 small photodiode units was placed under the retina in 20 late stage RP patients with remaining central vision. These devices were located near the arcades, well away from the area of functional photoreceptors. The photodiodes converted incident light into small DC electrical currents and, while the precise mechanism of action of these currents remains to be elucidated, it appears that they may have caused secondary release of neurotrophic factors in the surrounding retina, which in turn exerted a beneficial effect on the remaining photoreceptors. This implant may therefore have preserved some remaining vision, but it did not substitute an image by stimulating the secondary neurons, as a prosthetic implant would.

A true prosthetic, photosensitive, subretinal implant does exist, however, by virtue of additional circuitry to provide signal amplification and pulse generation [18] (Fig. 69.5d). When

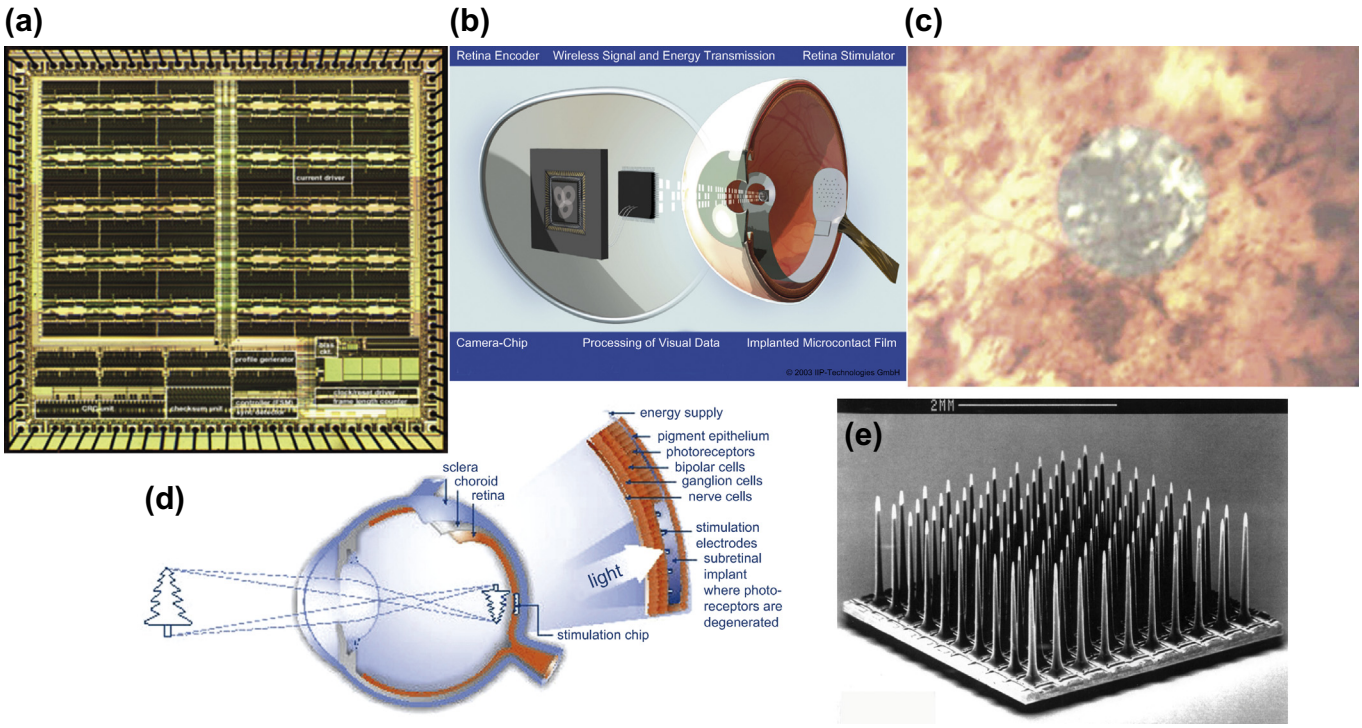

FIGURE 69.5

(a) Photomicrograph of Die3 chip, developed at the University of California Santa Cruz for reception, decoding and driving of 60 intraocular multielectrode signals. Each driver unit contains circuitry to drive five electrodes. For design considerations and other information [18a]. Photo courtesy of Wentai Liu, Ph.D., UCSC, Santa Cruz, CA (b) Schematic epiretinal implant with transmission of electrode control signals (IR) and energy (RF) to the intraocular decoder chip. Diagram courtesy of IIP Technologies GmbH. (c) Optobionics Artificial Silicon Retina (ASR™) chip *in situ* under the retina of a late-stage RP patient. Photo courtesy of Optobionics Corporation. (d) Schematic subretinal implant with external energy supply and on-chip image capture and signal amplification and conditioning. Diagram courtesy of Retina Implant AG. (e) Silicon multielectrode designed by Normann et al. (1996) for penetrating stimulation of the visual cortex or retina. Interelectrode distances between 200 and 400μm can be manufactured. The 1—2mm long electrode shafts are insulated, and the tips can be metallized with Pt black or other suitable metals. *Image courtesy of Richard A. Normann, Ph.D., Univ of Utah.*

placed at the level of the missing photoreceptor/RPE layer, it collects a sharp image of the outside world, mediated by the eye's optics, and generates localized electrical impulses that stimulate nearby (bipolar) cells in the overlying retina. Such an integrated prosthesis has great simplicity and elegance, but its feasibility hinges on three important premises: that nutrients and oxygen from the inner retinal vasculature will suffice to nourish the retina overlying the implant; that external energy can be provided to drive the amplifier and pulse generation stages; and that the heat generated by the implant's electronics can be safely dissipated into the underlying choroid without appreciably heating the overlying retina. Developers of these systems have successfully demonstrated that solutions for these problems can be found, albeit that existing prototypes of the implant still use an external power supply. They have also designed surgical methods to safely insert large arrays of such units under the retina [19]. Clinical implantation for periods of up to a year have been carried out in several dozen patients, with positive functional outcomes reported [20]. A novel, integrated, subretinal prosthesis, developed by a consortium centered at Stanford University, circumvents the need for an external power supply by driving the implant with high intensity pulsed infrared light projecting a 'monochromatic' image of the scene onto the implant [21].

In addition to retinal stimulation, three other approaches to electrical stimulation of the visual system are being pursued. One of these is direct stimulation of optic nerve fibers, either through a set of electrodes mounted on the inside of a cuff placed around the optic nerve [22],

or through an array of penetrating electrodes placed on the inside of such a cuff [23]. Implantation around the optic nerve is surgically less invasive than placement of a device inside the eye, but it has the drawback that either large numbers of fibers are stimulated simultaneously (with the simple cuff), or correspondence between electrode location and retinal origin of the axon (and therefore location of the elicited phosphene in the visual field) cannot be predicted. Thus, selective stimulation of individual optic nerve fibers will require a sophisticated mapping system to establish the correspondence between visual field locations and individual electrodes. A second approach to an electronic visual prosthesis is through stimulation of cells in the lateral geniculate nucleus, an important relay station along the visual pathway. Access to the *lgn* is problematic, however, since in addition to the need for small openings in the skull, placement will have to be performed stereotaxically, similar to the placement of deep brain stimulating electrodes placed to reduce the effects of Parkinsonism [24]. Preliminary tests in rodents have shown the feasibility of this approach [25], but it is unlikely that major developments of this approach will occur in the near future.

The oldest attempts at vision restoration involved stimulation of the visual cortex through intracranial electrodes, controlled by external image acquisition and signal-conditioning circuitry. Several decades ago, Brindley and Lewin [26] took the first steps in this direction by implanting a set of electrodes over the visual cortex of a blind volunteer; the phosphenes described by this volunteer were similar to those elicited by intraocular stimulation. More recently, a team at the National Institute for Neurological Disorders and Stroke (NINDS) performed tests with over 30 electrodes penetrating the cortical surface [27], and scientists at the University of Utah experimented with implantation of denser intracortical electrode arrays in cat and monkey visual cortex [28] (Fig. 69.5e). The same group was also the first to perform simulation studies to establish minimum requirements for prosthetic vision [29], an approach followed by several other groups (for a review, see Dagnelie [30]). The cortical work that originated at NINDS is being continued through primate studies at the Illinois Institute of Technology [31] and through evaluation of unsuccessful previous attempts [32,33], in preparation for human implants in the near future.

The cortical prosthesis bypasses both retinal and optic nerve problems and might therefore be seen as a universal approach to vision restoration. Admittedly, it may be the only viable approach for patients whose inner retina and/or optic nerves have been destroyed by glaucoma or trauma. However, cortical stimulation requires complex surgery of an otherwise-healthy brain; moreover, the convoluted layout of the visual cortex complicates mapping of objects and locations in the outside world into a pixelized image that can be understood by the prosthesis wearer. Algorithms to establish such a map have been tested in primates [31] and through simulations in sighted volunteers [34], but thus far only for small sets of simulated phosphenes. Finding an efficient approach to mapping hundreds of phosphenes will be a crucial aspect of cortical implant research.

A final point of consideration for all visual prostheses for which the input image is acquired outside the eye is the altered role of eye movements. In natural vision, the intent of most eye movements is to bring an object of interest to the center of the retina, where it can be resolved with greater detail and receive directed attention. If a head-mounted or handheld camera is used for prosthetic image acquisition, movement of the camera rather than eye movements will recenter the image. To compensate for this difference, either the external portion of the visual prosthesis must be equipped with eye movement monitoring and processing capability to shift the image accordingly, or the prosthesis wearer must learn to suppress eye movements as a means to acquire visual information, and use camera (i.e., head or hand) movements instead.

Detailed reviews of visual prosthesis development and simulation studies can be found in Dagnelie [30,35,36].

Retinal cell transplantation

Cell transplantation in the neurosensory system can, in principle, restore function through two mechanisms: rescue of threatened cells (either by restoring a failing support system, or through trophic factors secreted by the transplanted cells) and replacement of degenerating cells and functional integration of the transplanted cells into the host tissue. Both mechanisms may play a role in retinal cell transplantation, and the distinction is not always obvious, as will become clear.

There are two important distinctions in the choice of transplant modality. First, there is the distinction between autologous cells — harvested from the same individual who is to receive them, typically following amplification and/or differentiation in tissue culture — and allografts — harvested from a different donor, who is typically unaffected and immunologically matched. Second, the developmental stage of the transplanted cells may range from pluripotent stem cells, to organ-specific undifferentiated cells, to postmitotic (e.g., photoreceptor precursor) cells. Since the range of possible approaches far exceeds the scope of this chapter, only major developments will be indicated; a recent review of stem cell approaches to retinal degeneration can be found in Tibbetts et al. [37].

Some important categories of retinal disease are mediated by loss of RPE function, and both transplantation of autologous pigment epithelium and allografts have shown success in rescuing photoreceptor function. In the Royal College of Surgeons (RCS) rat, rescue of photoreceptor function can be accomplished by replacement of the degenerating RPE with a wide range of transplants, from RPE xenografts (harvested in a different species) [38] to autologous iris pigment epithelium [39]. In human retinal disease, macular translocation surgery in AMD has demonstrated the feasibility of rescuing threatened vision by relocating the neural retina over a relatively healthy area of RPE [40], although visual outcomes of this surgical approach have been mixed and appear to be highly dependent on the surgeon's skill and experience. Immunologically mismatched RPE allografts and xenografts may not provide viable treatments in human retinal disease: Contrary to the neural retina, where immune response appears to be muted or absent, a slow inflammatory response to mismatched transplanted RPE tissue is commonly observed [41].

Such an inflammatory response is less likely to occur when the transplanted cells are grown *in vitro*. In the last few years, RPE replacement has shifted toward experimental procedures involving cultured RPE cells grown from pluripotent embryonic stem cells [42]. These cells, which can be grown in the laboratory under conditions meeting the standards of Good Manufacturing Practice, have now been awarded an Investigational Device Exemption (IDE) by the USFDA, and in 2011 entered clinical studies as a potential treatment for Stargardt macular dystrophy and dry AMD (http://www.clinicaltrials.gov: NCT01344993/ NCT01345006, date accessed July 10, 2013).

Retinal diseases that originate in a photoreceptor cell defect do not lend themselves to treatment with autologous cell transplantation, since the same defect is likely to plague the transplanted cells, unless these can be genetically modified prior to implantation. Since the mid-1990s, transplants with cell suspensions and organized sheets containing mature photoreceptors or photoreceptor precursors (with or without RPE) have been used therapeutically, with varying degrees of claimed efficacy. In a light-damaged rat model, morphological evidence of synapse formation [43] and behavioral evidence of regained function [44] were among the earliest indicators that transplanted photoreceptors may be capable of assuming visual function. More recent evidence in the same direction from other laboratories suggests that functional synapse formation is possible in some animal models, while in others a gliotic seal under the retina effectively inhibits integration of the transplanted tissue with the host retina [45]. The mechanisms responsible for such widely different results remain to be elucidated, but are most likely associated with varying

patterns of reorganization occurring in response to different retinal degeneration genotypes [46].

Fetal tissue has thus far proven more successful than fully developed retina or stem cells in forming synaptic connections and retinal morphology resembling that of intact retina, and it has become the tissue of choice in most transplantation attempts. This tissue also carries a lower risk of rejection by the host immune system, at least in photoreceptor grafts; indeed, rejection does not appear to occur in rat or mouse photoreceptor transplantation [47]. Admittedly, some caution in extrapolating this finding to other species is warranted, for immune reactions in rodents tend to be less severe than in humans. The use of fetal tissue for research and transplantation purposes is subject to legal and ethical concerns in a number of countries, and this is an important reason for continued efforts to develop transplantation techniques that use adult donor tissue.

Preliminary attempts at retinal cell transplantation in blind volunteers, performed primarily to demonstrate safety, have yielded mixed, and at best modest, results. Allografts of cultured RPE appear to provide protection to functional AMD through trophic factors, but in exudative AMD the graft is quickly overwhelmed by an inflammatory reaction [48]. Photoreceptor transplants appear to convey an improvement in vision in some cases [49], but whether this is mediated by graft-host synapse formation or trophic support to the few remaining photoreceptors cannot be distinguished [50].

The ciliary body in the human eye contains pluripotent stem cells that will, under appropriate conditions, differentiate into retinal neuron precursors [51]; hence, the potential for photoreceptor cell replacement from tissue culture does exist. At this time, however, any attempts to use bone marrow and retinal stem cells to replace degenerating photoreceptors in human patients through injection into the vitreous or subretinal space, as repeatedly claimed in the popular media, seem highly premature. Without thorough research in animal models of retinal degeneration, and until photoreceptor cells can be cultured with the same level of control as has been achieved for RPE cells, it is unlikely that such attempts will lead to effective and reliable vision restoration, or even to long-term survival of the remaining photoreceptors.

Optic nerve protection and regeneration

RGCs behave as central nervous system neurons in their inability to recover from severe injury: RGCs whose axons are damaged by cutting or crushing the optic nerve undergo apoptosis within days following injury, although this particular mechanism of cell death may be restricted to certain classes of RGC [52]. And while the axons of peripheral (motor or sensory) neurons can regenerate following surgical reconnection of nerve fascia, CNS neurons appear to lose such plasticity once their original outgrowth during the organism's development is completed, due to changes in both their internal makeup and their environment. Oligodendrocytes that form the protective myelin sheath around optic nerve axons and astrocytes appear to play a major role in preventing axon regeneration following injury [53]. It has been known since the 1980s, however, that this environment can be effectively modified. Cut optic nerve axons in the rat and cat will not form new neurites in their natural optic nerve environment, yet they can be made to regenerate into a peripheral nerve graft and reach the superior colliculus [54–56]. Two basic steps are thus necessary for therapeutic intervention in advanced optic nerve disease to become a reality: protection of RGCs from apoptosis following severe damage to the optic nerve, and axon regeneration, including formation of functional connections at the thalamus and other midbrain structures. In recent years, a third aspect, functional enhancement of remaining RGCs, has been added to the basic glaucoma research repertoire [57].

Studies in the last decade have provided encouraging indications in both areas. Intravitreal injections of anti-apoptotic drugs and neurotrophic factors can limit the loss of RGCs

and prolong the window for application of other therapeutic modalities [56]. Most of these therapies are aimed at preserving the RGC soma so that it can sustain a partially damaged axon, but there is still a need for targeted therapies that can either repair damaged axons or stimulate new axon growth [58]. Some progress is being made in stimulating axonal growth: Oncomodulin has been identified as a factor that is particularly effective in promoting growth following optic nerve crush [59]. In addition, there is increased interest among researchers in free radicals, immune response, and nerve growth factors in the retina remaining unbalanced even after the primary causes of glaucoma (such as increased intraocular pressure) have been treated [60].

Drug delivery

Since 1995, our understanding of photoreceptor and RGC death has changed dramatically. While it was previously thought that these cells die because a functional part — the photoreceptor outer segment and the axon, respectively — becomes dysfunctional, both events are now widely understood to trigger cell death through apoptosis and similar mechanisms. Better understanding of these mechanisms has led to the search for pharmacological interventions that may prevent cell death.

The process of RGC degeneration following transsection or crushing of the optic nerve can be halted, at least in animal models, with the use of neuroprotective agents such as neurotrophin-4/5 (NT-4/5) [61]. It turns out, however, that this protection has only a limited duration. Most RGCs die within two to four weeks, even with sustained administration of NT-4/5 [62]. Various other neuroprotective strategies have been attempted in animal models, but their long-term efficacy as well as the feasibility of long-term delivery near the threatened site remains to be demonstrated.

In the case of photoreceptor degenerations, a variety of nerve growth factors and neuroprotective agents have been used, both *in vitro* and in animal studies, and several of these have shown promise [63]. What remains to be worked out for most of these substances is the optimal delivery route. Systemic administration is not an option, since many of these agents do not easily pass the blood-retina barrier and/or have unwanted systemic side effects. Administration as an eye drop is not effective, because the active substance would need to diffuse through the cornea or sclera, and most of it would be washed away in the tear film. Repeated injection into the vitreous is unattractive to the patient, and it is not clear how long the active substance would persist in the eye and reach the outer retina.

Three relatively recent delivery techniques have all been successfully applied to ocular drugs in recent years: delivery by macromolecules, slow-release implants, and encapsulated-cell technology. The first of these approaches caused a revolution in the treatment of exudative AMD about 15 years ago, and is now widely known as photodynamic therapy (PDT). Liposomes with an embedded anti-angiogenic substance (verteporfin) are injected into the bloodstream, and irradiation of the retina with a low-energy laser beam is used to release verteporfin in the choroidal space under the retina. The resulting oxygen free radicals attack vascular endothelial cells and stop angiogenesis [64]. Unfortunately, this endothelial-cell cytotoxicity is not an effective long-term treatment, so, following the demonstrated safety of locally administered vascular endothelial growth factor (VEGF) inhibitors, PDT has now been replaced as the treatment of choice by intravitreal injections of such substances (notably Avastin, and Lucentis) [65], even though such injections have to be repeated, in most cases several times over a six month period. The longer-term outlook for these substances is very good. They can be embedded in poly(lactic-glycolic) acid (PLGA) microspheres to create slow-release implants that could be inserted into the eye through a small incision and attached to its inside wall, or they may even be effective when placed on the outside of the eyeball [66]. The third delivery method, encapsulated-cell technology (ECT), is elegantly exemplified by the NT-501-permeable-membrane delivery system developed by Neurotech

SA (Lincoln, RI). When loaded with a transgenic cell line producing ciliary neurotrophic factor (CNTF) and implanted in the vitreous cavity, it will release a flow of CNTF for many months [67]. Applications are sought in the area of photoreceptor protection in retinal degenerations; a Phase I trial in retinitis pigmentosa has been completed successfully [68], follow-up studies in RP and AMD have shown modest positive results, and several other clinical trials using NT-501 as a treatment for retinal diseases are planned or on-going.

In some diseases, even the simplest of drug delivery may be effective. In retinal ischemia, just as for other vascular blockages, 'clotbuster' drugs, such as tissue plasminogen activator, can be intravenously injected. If this is done soon (hours or even days) after the ischemic event, much of the damage may be prevented; experimental therapy with these drugs has had promising results [69].

Genetic interventions

Delivery issues also play a role in the introduction of new genes into the degenerating retina. Most retinal degenerations are genetic in nature, either inherited from one or both parents or caused by a new mutation. The premise of gene therapy is that intervention at an early stage may restore normal function to the cell and prevent the degeneration process that might otherwise ensue. Several strategies are needed to combat inherited retinal degenerations.

- In recessively inherited diseases, both copies of a gene are defective, and introduction of a third copy may be sufficient to achieve adequate production of a functional protein to replace the defective one encoded by the mutated gene. A virus is used to introduce a healthy copy of the gene. Following a number of demonstrations of successful gene transduction into RPE cells and photoreceptors *in vitro* and feasibility studies in rodents, a highly publicized study of vision rescue in a canine model of Leber congenital amaurosis (LCA) [70] provided proof that gene therapy for some human retinal degenerations may soon be a reality. Since then, several small trials in children with LCA have demonstrated some improvements in ambulatory vision, but limited long-term treatment effects; for a review, see [70a].
- In X-linked disease, males carry only one copy of the X-chromosome, including the defective gene; here, too, introduction of a healthy copy of the gene may be enough to achieve normal function.
- In dominant disease, a single bad copy of the gene suffices to 'poison' the delicate balance of the cellular machinery, or, at the very least, to prevent its proper function. To reduce or prevent this, a therapeutic intervention must block a step along the transcription pathway from the defective gene to its product protein but not inhibit expression of the good copy of the gene; a gene for such a blocking agent could, in principle, permanently neutralize the defect. This technique has been used in a transgenic rat model, using ribozymes to destroy mRNA produced by the P23H mutant gene defect responsible for one form of autosomal dominant RP [71]. In a rat model with a naturally occurring degeneration, a gene for ribozyme production was successfully introduced into photoreceptors and proved effective even when introduced at a time when many photoreceptors had already succumbed to the effect of the defective rhodopsin gene [72], which bodes well for therapeutic interventions in dominant RP. These forms of RP tend to preserve substantial levels of vision into middle age, and this remaining vision might thus be rescued.
- An entirely separate class of retinal degenerations, typically asymptomatic until middle age and often associated with multisystem disease, is caused by mutations of the maternally inherited mitochondrial DNA, responsible primarily for cellular energy supply. Mitochondrial DNA has multiple copies, and it remains unclear whether single or multiple copies are responsible for these disorders. Moreover, the mitochondrial DNA does

not lend itself to normal viral transduction techniques, which adds a level of complexity to potential gene therapeutic approaches. Recent reports suggest that proteins may be used to deliver expression-blocking factors or new functional genes into the mitochondrial milieu [73].

Vision-related gene therapy research is concentrated around RP, Stargardt macular dystrophy, LCA, and related diseases with known inheritance patterns. Due to the multiple genes that can lead to these disorders and the enormous number of specific mutations that have already been identified, many gene therapeutic variants will be needed to address a substantial proportion of these disorders. This is an extremely active area of research; recent reviews can be found in Farrar et al. [74] and Smith et al. [75].

Still, this means that a true cure may become available for these retinal degenerations. AMD and optic nerve diseases like glaucoma and ischemia, on the other hand, have no known genetic causes, though some genes associated with predisposition for such diseases have been identified. Genetic interventions may assist in preventing some forms of these diseases, but the prospects of other therapeutic approaches are more promising in the near term than those of gene therapy.

EMERGING APPLICATION AREAS FOR ENGINEERED CELLS AND TISSUES

Of the current approaches to vision enhancement considered earlier, strictly speaking only the use of stem and transgenic cells falls within the realm of tissue engineering. The complexity of ocular structures such as the retina and optic nerve poses daunting challenges to anyone seeking to recreate their function. While this may explain the lag in progress when compared to other organ systems, it should not keep the researchers who are working to restore vision from drawing on the remarkable progress of tissue-engineering approaches. We next briefly consider four application areas, corresponding to the aforementioned three processing stages in the early visual system where severe vision loss may occur and the supporting retinal infrastructure.

Photosensitive structures

Current efforts in the areas of retinal cell transplantation and intraocular prosthesis design, while promising, are in no way guaranteed to lead to reliable — i.e., long-term and stable — restoration of useful (let alone high-quality) vision. As was explained earlier, each of the approaches currently being explored has inherent drawbacks. Implanted electrode arrays require external image acquisition and preprocessing, which may necessitate real-time eye movement tracking and compensation. Integrated implants require signal amplification and thus external energy supply. Both implant types may be limited in resolution to ambulatory vision due to the 100 to 200 μm distance separating the electrodes from the target cells. As a rule of thumb, resolution in the underlying tissue will be no better than this distance. Retinal cell transplantation efforts are likely to be limited by the spotty record of transplanted cells in making functional contacts with the native inner retinal circuitry. And all three approaches are limited by microneuroma formation, destroying the functional diversity of the inner retina and limiting its resolution to 50–100 μm, i.e., at best 20/200 visual acuity — legal blindness.

To achieve improved retinal prosthetics, electrodes will have to make more intimate contact with the target cells. If successful, this would improve resolution to the limit imposed by the repatterned inner retinal neuronal circuitry. One can envisage such a penetrating array as the Normann microelectrode in Fig. 69.5e, but the damage such an array might do to the delicate microvasculature of the retina is a distinct disadvantage. As an alternative, one might envisage inserting or growing, *in situ*, an array of parallel 'neurites'. These might branch out of cells

grown on the surface of implanted stimulating chips, penetrating the retina until they reach a specific target environment, e.g., the inner nuclear layer, where they would make synaptic contacts with the native cell population; these neurites would act as 'tube electrodes', releasing either electrical charge or a neurotransmitter that would activate the target cells. An alternative approach would be an electrode structure that stimulates nearby retinal cells to form new neurites, spurred on by favorable growth conditions and a coating on the implant. Such coatings promoting cell growth, formed by microcontact printing, are under investigation [76].

The distance between implant and target cells could also be reduced by enticing native retinal cells to migrate toward the electrode surface, adding a new twist to the cell migration seen in naturally occurring retinal repatterning [46]. Such approaches are not as farfetched as they may sound. Several groups are experimenting with surface modifications on semiconductor chips that will allow cells to adhere to these surfaces and be activated by neurotransmitters [77], while researchers at Stanford University have reported the tendency of retinal neurons to migrate around pillar-shaped electrodes forming an array placed under the photoreceptor layer (Fig. 69.6) [78]. Combinations of such technologies may lead to improved implant-tissue interfaces.

A fundamentally different approach to improving (sub)retinal prostheses may be the use of high-yield photoconversion systems. Such high-yield conversions are known to be performed by Photosystem I (PSI), a macromolecule present in the membrane of thylakoids, which can be found inside the chloroplasts that give the green color to plant leaves and algae. Early experiments with thylakoids [79] showed that it is possible to anchor these structures onto a metal surface and use them as miniature photovoltaic elements. Lee et al. [80] also demonstrated that it is possible to chemically modify thylakoid surface membranes to create charge displacement in a specific direction. Kuritz et al. [81] demonstrated the ability

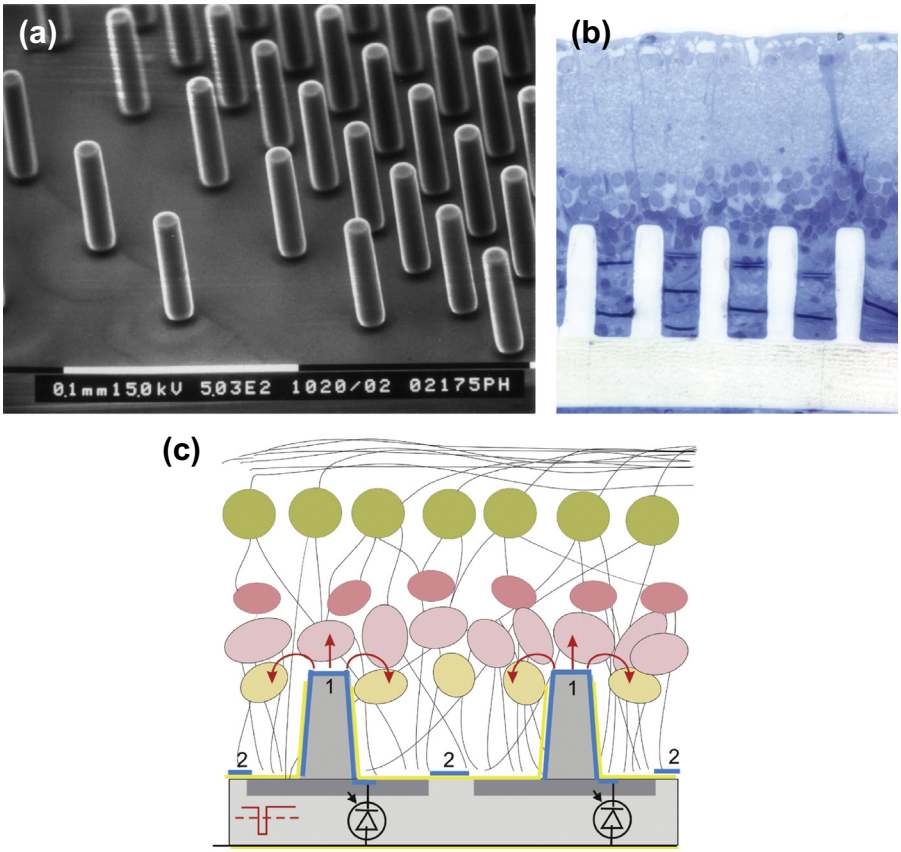

FIGURE 69.6
Pillar electrodes penetrating into the middle of the inner nuclear layer of degenerated retina. (a) Electron micrograph of an array having pillars of 10μm in diameter and 70μm in height. (b) Histology of the RCS rat retina with a pillar array six weeks after implantation. (c) Schematic of a retinal prosthetic: retinal cells in the inner nuclear layer stimulated by pulsed electric fields emanating from the exposed tops of the pillars. Images provided by Daniel Palanker. Ph.D., Stanford University.

of PSI to impart photosensitive polarization and Ca^{2+} ion movement to retinoblastoma cells in tissue culture, as a first example of cellular engineering that may eventually lead to artificial photoreceptors. The engineering successes of thylakoids and PSI open the opportunity to create cells that assume a dipole charge distribution or, with the help of some form of intracellular electronics, produce a biphasic pulse between the 'poles'; in a subsequent stage of development, automatic gain control could be incorporated, as a limited form of light/dark adaptation. If these cells can be made to attach to the outer retinal layers or to migrate into the inner nuclear layer, one could achieve microscopic local current sources with sufficient conversion efficiency to ensure vision at a broad range of (day)light levels. To achieve true night vision, an external device, such as a night vision scope, could be used.

Note that in this idealized situation, the synthetic 'photoreceptors' might be small and sensitive enough to provide good vision without external image preprocessing (e.g., magnification and contrast/edge enhancement). It is to be expected that preliminary forms of such light-sensing units will be neither small nor sensitive enough to provide the dynamic range and resolution required; at this intermediate solution level, external image processing with an advanced portable low vision visor would be a necessary complement to this intraocular light conversion array.

Optogenetics

Since 2006, research into sight restoration has been exploring the prospects of conveying artifical phototransduction to the cell membrane of retinal neurons [82,83]. These optogenetic approaches, as termed by Deisseroth et al. [84], introduce DNA to the target cells which codes for proteins reacting to certain wavelengths of light by depolarizing or hyperpolarizing the cell. The two primary proteins used in this research are channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR), although melanopsin [85] is also considered a candidate for gene therapy.

Neuron depolarization is achieved by stimulating ChR2. This protein is a microbial-type rhodopsin from the green alga, *Chlamydomonas reinhardtii*. Nagel et al. [86] found ChR2 to be a plasma membrane channel that passively conducts both mono- and divalent cations upon capture of a photon by its attached chromophore. Similar to melanopsin ($\lambda_{max} = 480$ nm), ChR2 has an absorption peak at approximately 460 nm [86].

Conversely, neuron hyperpolarization can be achieved by stimulating NpHR. Originally thought to be an outward-directed cation pump, Schobert et al. [87] identified halorhodopsin as an inward-directed Cl^- pump. Although these experiments were performed on halorhodopsin from *Halobacterium halobium*, a variant (NpHR) from *Natronomonas pharaonis* is used for current experimentation because of its higher affinity for extracellular Cl^- [88,89]. Both forms of halorhodopsin absorb light at longer wavelengths than ChR2, with λ_{max} at approximately 580 nm. The two labs simultaneously reported the use of NpHR to optically hyperpolarize neurons.

These tools can be delivered to retinal cells by a variety of methods. The most widely used delivery system is that of adeno-associated virus type 2 (AAV2) vectors. These vectors were first reported for human preclinical gene therapy trials by Flotte et al. [90], applied to cystic fibrosis. Since then, the use of AAV2 for human gene therapy has expanded to cover a number of conditions [91]. Encouragingly, Simonelli et al. [92] reported the use of AAV2-based treatment for LCA with maintained single-dose efficacy and no serious adverse events as of 1.5 years post-treatment.

Bi et al. [82] injected AAV2, loaded with ChR2/GFP coding attached to a strong ubiquitous promoter, into the intra- and subvitreal spaces of mice. The authors found expression of the ChR2/GFP chimera in ganglion, amacrine, horizontal, and to a lesser extent, bipolar cells of the retina. Light responses were confirmed by whole-cell patch clamping in retina slices and

visually evoked potential (VEP) recordings from the visual cortex of otherwise blind mice. These authors further demonstrated that even non-specific transduction of ChR2 in degenerated retinae can produce light responses in the visual cortex. The quality of such information reaching cortex, however, may not be particularly meaningful. Specifically, the simultaneous activation of pathways that normally exhibit direction selectivity, lateral inhibition, and ON, OFF, or ON-OFF responses would likely generate a highly corrupted image of the original signal.

One way to avoid such signal corruption would be to limit the classes of cells that receive photic input. Busskamp et al. [93] reported transducing surviving cone cell bodies with eNpHR [94], a variant of NpHR with improved cell membrane localization. The target cones were in mouse models of RP and in human *ex vivo* retina explants. In mouse models, the authors demonstrated that such cone transduction could create light responses in the retinal network that display lateral inhibition, direction selectivity, and ON and OFF responses. Live mice with transduced cones also displayed visually guided behavior. In human retina explants, the authors showed that their AAV2 delivery method successfully transduces human cones, and results in the generation of photocurrents indicative of eNpHR activity. Although cone transduction would not be a viable treatment for patients with too few remaining cones, the authors identified an existing population of RP patients that had some remaining cone cell bodies, despite the apparent loss of their outer segments.

Another set of targets for cell-specific transduction in the retina are ON bipolar cells. While there is no promoter that is known to specify OFF bipolar cells [93], ON bipolar cells can be targeted using the promoter for the gene encoding the metabotropic glutamate receptor, mGluR6 [95,100]. Although not as advantageous as stimulating the retinal network from the level of the cones, specifying ON bipolar cells should allow for signals far less corrupted than one would expect from indiscriminate stimulation. Doroudchi et al. [95] used AAV2 to transduce ON bipolar cells in mouse models of blindness with ChR2. The authors observed ON-type retinal ganglion cell responses and visually guided behavior in the test animals. Unfortunately, Fradot et al. [96] performed a similar experiment in human retina explants, in which only a very small portion of ON bipolar cells was transduced. More efficient promoters and/or delivery methods will need to be explored before useful, selective transduction of ON bipolar cells can be realized [83,96].

While AAV2 delivery has been shown to be very productive, a number of researchers are doubtful of its practical use in the clinic. Specifically, the use viral vectors may introduce its own risk of immune responses, and it imposes limits on the size of DNA packages to be delivered [97]. Parallel to research using AAV2 vectors are experiments that generate pores in target cells through which desired genetic material can be absorbed. These pores can be generated using electric fields [98] or a femtosecond-pulsed laser beam [102].

In 2008, Lagali et al. [100] used electroporation to transfect ON bipolar cells of mouse models of retinal degeneration. Actually predating the analogous AAV2 experiments, these authors also observed expected ON ganglion cell responses and optomotor behavior, as well as center-surround organization and cortical responses. There is no indication, however, whether electroporation would have any more success than AAV2 at transfecting human ON bipolar cells. More recently, Gu et al. [101] also transfected ganglion cells with ChR2 in mouse models of retinal degeneration, generating visually guided behaviors.

Electroporation, by its nature, acts over a relatively wide area and does not offer more spatial specificity than AAV2 vector delivery. Should the treatment be aimed at specific parts of the retina, as in RP patients with remaining central vision, a more focal method of transfection may be desirable. By applying a 10^{12} W/cm 800 nm laser beam using femtosecond pulses, researchers can create transient perforations in specific cells through which DNA can enter [99].

Gu et al. [101] used this optoporation to transfect RGCs of goldfish with ChR2. Using multi-photon targeting, the authors were able to specify the 3D location of each pore, which had

radii of approximately 400 nm, and were laid out in 3 μm intervals over the targeted area. In a follow-up study, Villalobos et al. [102] transfected retinae of the same species using both optoporation and photothermal poration with carbon nanoparticles. Stimulation through ChR2 was observed by intracellular calcium imaging.

Separate from these experiments on targets and delivery methods, Nirenberg and Pandarinath [103] recently addressed the issue of optogenetic signal fidelity using a computational approach. Unlike other groups discussed here, these authors bypassed the typical issue of transfection by generating mouse lines that both were models of retinal degeneration and possessed ChR2-expressing RGCs. To determine proper stimulation parameters for these ganglion cells, the authors observed the spiking patterns of different classes of ganglion cells from normal retinae in response to movies with both natural and artificial scenes. Using these data, they constructed encoders, specific for each type of ganglion cell, which combined to model the signal processing performed by the normal retina. The models were data-driven, in that parameters for each model were chosen so the output for any given input would have the highest probability of matching actual retinal spike trains, as tested by cross-validation.

Nirenberg and Pandarinath stimulated the retinae of blind mice using either patterns generated by their encoder or the unprocessed spatial patterns of the stimuli. The authors demonstrated that cells stimulated by the encoder's patterns more closely matched the firing patterns of normal retinal neurons. Further, the authors used a drifting grating on an LCD screen to elicit optomotor tracking, which was only possible when the stimulus was presented though the stimulation encoder. No tracking was observed with the unprocessed stimulus or in control animals. These data imply that, although cerebral cortex is plastic, meaningful perception of visual information may depend on the preservation of the neural code normally exhibited by the retina.

For clinical purposes, Nirenberg and Pandarinath acknowledge that it may not be practical to target multiple classes of ganglion cells, each receiving its own coded version of a given stimulus. They do argue, however, that targeting a single ganglion cell class, with proper encoding, may be sufficient to provide more faithful percepts than those generated by conventional stimulation patterns. Their experiment inducing optomotor tracking, in fact, only utilized transient ON cell encoders.

Once applied to human subjects, optogenetic approaches to vision restoration may outpace less target-specific electrical retinal processes. Reactivation of cone remnants using NpHR could provide many benefits related to intrinsic retinal processing. For patients in whom such treatment would not be viable, stimulation of bipolar cells, or of ganglion cells in the fashion described by Nirenberg and Pandarinath, could provide similar benefits. To introduce light-sensitive properties into the target cells, electro- and optoporation may offer new avenues of treatment, should cases arise in which AAV2 vectors are insufficiently effective or specific to use as a delivery system.

Outer retinal cell transplantation

Despite almost two decades of careful investigation, reliable and widespread formation of synapses between transplanted photoreceptor precursors and native inner retinal cells — and thus full integration of the transplanted cells into the host retina — has remained elusive. Also, the effects of the host immune response on graft survival — especially of transplanted RPE — cannot be ignored, even if it appears to be mild, and this host reaction will have to be effectively controlled without long-term systemic immune suppression. Both areas can profit from tissue-engineering approaches. A complex set of conditions — cells in the proper stage of development, properly matched to the host retina, and spurred on by a proper combination of neuroprotective and neurotrophic modifications to the host environment — needs to be met for successful graft-host integration. Recent reports indicate that even

widely held beliefs regarding the proper developmental stage of the transplanted cells may need to be revisited. In a mouse model, early postnatal photoreceptors showed widespread synapse formation with native retinal cells [104], whereas most previous research has been directed at the use of postmitotic photoreceptor precursors in a much earlier stage of development.

Even if the cell population affected by the primary degeneration process is successfully replaced, secondary degeneration stages — of RPE cells in photoreceptor degenerative disease and of photoreceptors in RPE degeneration — may still prevent restoration of visual function. This problem can be addressed adequately only by performing a combined graft of RPE and photoreceptors, presumably prepared in a tissue culture environment and stimulated toward integration through a carefully tuned combination of neuroprotective and neurotrophic factors. Culturing stem cells or differentiated cell lines might allow the creation of heterogeneous structures such as RPE/photoreceptor double-sheets. Culture conditions and the growth stage of these sheets should be modulated to prepare the cells for integration with the host retina. In order to provide sufficient structural support to these fragile sheets during growth and transplantation, a resorbable polymer layer could be used as a substrate.

Cell matrices supporting axonal regrowth

Cell culture conditions and retinal integration alone will not be sufficient in the case of ganglion cell transplantation with the objective of repopulating the optic nerve with axons carrying visual information to the brain. As discussed earlier, this will require the (re)-creation of conditions favorable for axonal growth over distances of many centimeters and the ability of sprouting axons to contact target cells with the correct retinotopic mapping. This feat is accomplished effortlessly by RGC axons in the developing embryo, but to recreate the conditions for this to occur in an adult organism will be a considerable challenge.

As was noted earlier, an RGC axon damaged by glaucoma, optic nerve disease, or injury can only form new neurites under optimal environmental conditions, which essentially mimic those in a developing organism. At the same time, neuroprotective factors are required to sustain the RGC long enough for the axon to assume this support function. If the glaucoma diagnosis is made early and safe and effective neuroprotective agents become available, it may be possible to save most of the threatened axons and thus spare most RGCs and the patient's vision. In practice, however, many axons may have been lost by the time the diagnosis is made, and protection of the cell somata and stimulation of axon regrowth become the treatment objectives [105]. The experimental conditions employed thus far, using peripheral nerve sheaths to create a substrate for axonal regrowth, are less than ideal, because they do not provide an integrated environment in which regrowing axons combine with intact remaining axons and in which protection of the threatened RGCs is built into the environment. This is a very active field of research, offering great potential for tissue-engineering approaches, and one may expect such approaches to play a major role in achieving these objectives. Understanding the necessary conditions to create an integrated environment for axonal regrowth and creating novel synthetic materials to provide these conditions should provide wonderful challenges to tissue engineers.

One approach to such an integrated solution might be that engineered cells could be grown *in situ*, as a loose skeleton of supporting tissue, to follow the course of the optic nerve to the chiasm and optic tract; these cells would be programmed to exude the necessary factors promoting axon growth and RGC protection. Alternatively, it might be possible to modify the normal environment of the optic nerve (temporarily) to allow or even stimulate axonal growth. Assuming that it would be possible to guide outgrowing axons through the optic

chiasm toward the appropriate structures and retinotopic projections in the midbrain, RGCs may restore their connections to the target cells and resume functional visual processing.

Repopulating ischemic or diabetic retina

As mentioned earlier, new capillaries — formed under the influence of an angiogenic tissue response — tend to be poorly organized and fragile, causing leakage and thus a great deal of damage to the already stressed retinal tissue. Therefore, the prospects of restoring vision in such retinal areas are, at the present time, poor.

This may change, however, if cell populations can be grown *in vitro* and introduced into the retina under physiological conditions mimicking those during embryonal development. In that case the formation of new blood vessels could follow a more orderly pattern, and implanted cells would have a much better chance of forming functional connections. Whether and when it will be possible to recreate embryonal conditions and grow such integrated retinal tissue as is required to restore vision to an ischemic portion of the retina, from RPE to RGC axons, is difficult to predict. It is a challenge whose magnitude exceeds that of RPE/photoreceptor transplants, functional stimulation of inner retinal cells, and RGC protection/axonal regrowth combined.

Assessing the functional outcomes of novel retinal therapies

Electrophysiologic assessment of the functionality of a therapeutic intervention is considered a gold standard by many researchers and clinicians, but recording the responses in eyes that have undergone experimental treatments may not always be feasible. Retinal implants are in the unique position that they allow clinical functional evaluation directly from the implant provided the necessary signal collection hardware is available. This is exemplified by developments in cochlear implants. Cochlear implants have been clinically approved in the US since 1984 [106], and over the years advanced built-in electronics based on reverse telemetry [107] have been developed to record electrically evoked auditory nerve responses (electrically evoked compound action potentials, or eCAPs). eCAPs are recorded by stimulating the auditory nerve through a subset of electrodes in the implant array and recording the elicited neural activity at a more distant electrode. The recorded data is sent back through the same wireless radiofrequency link that is used to stimulate the implant. eCAP recordings are nowadays routinely applied intra-operatively [108], enabling the surgical team to assess functionality of the implanted device while the patient is still under general anesthesia. In addition, eCAPs are being used for rehabilitation purposes such as automated device fitting [109,110] and fitting of cochlear implants in young children [111,112].

The visual equivalent of the eCAP is the electrically elicited electroretinogram (eERG), which could fulfill similar purposes to the eCAP. However, none of the present retinal implant systems, such as the Argus® II device, has reverse telemetry capabilities sufficient for recording the eERG. Recording the eERG with standard Electroretinogram (ERG) methods such as corneal electrodes is difficult, due to the small signal amplitudes and the presence of electrical as well as physiological artifacts in the eERG signal [116]. Electrical artifacts generated by the implant can be effectively reduced by using filtering techniques such as stationary wavelet transformation [113–116]. Physiological artifacts are more difficult to deal with, because filtering techniques reducing these contaminations will inevitably also reduce the retinal responses of interest. In a recent conference presentation reporting on recordings from monocularly implanted Argus II recipients [116], it was demonstrated that a physiological artifact attributable to pupil innervation responses in both eyes can be largely eliminated through subtraction of the contralaterally recorded pupil response from the combined retinal+pupil response recorded at the implanted eye (Fig. 69.7). Although the decontaminated corneal responses have the predicted negative polarity, and show increasing amplitude with increasing stimulus level, their retinal origin remains to be definitively confirmed. It is likely

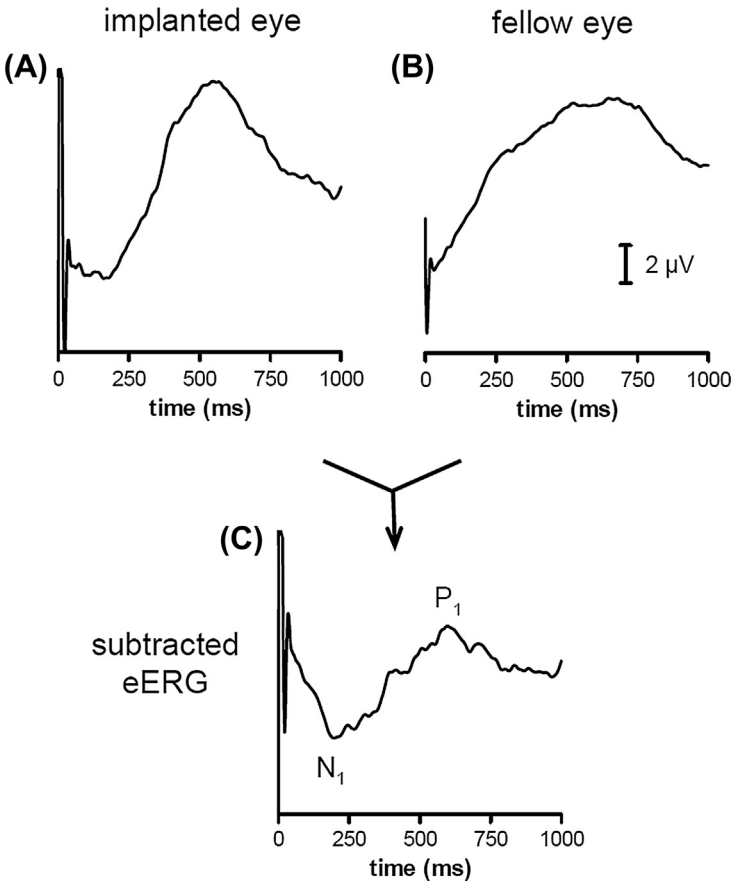

FIGURE 69.7
Electrically elicited electroretinograms (eERGs) evoked by unilateral stimulation of the implanted eye and recording the eERG bilaterally (a, b). The decontaminated eERG (c) was obtained by mathematically subtracting the physiological artifact recorded from the fellow eye.

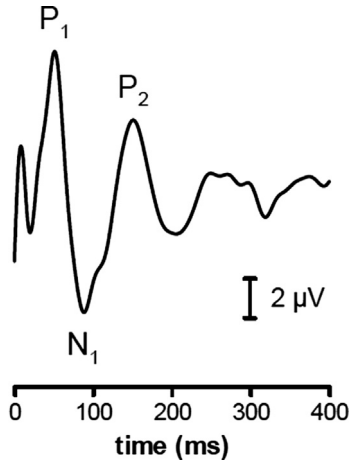

FIGURE 69.8
Cortical response of retinal implant stimulation: the electrically elicited visually evoked potential (eVEP).

that this confirmation will have to await direct recording, with high temporal and dynamic resolution, from implant electrodes through improved telemetry.

In the interim, cortical responses, i.e., electrically elicited VEPs (eVEPs) recorded from the scalp, can provide a promising alternative [117,117a] to retinal responses. Cortical [118] and brainstem [119] responses have been used to assess cochlear implant function, but eCAPs are applied more widely, due in no small measure to the availability of high-quality reverse

telemetry systems [120]. Nevertheless, advantages of cortical over peripheral potentials include the fact that cortical responses represent central nervous system activity (V1 in case of eVEPs), and may provide a more accurate indication of perception than eERGs. In addition, eVEPs can also be applied in case of visual prostheses implanted in the optic nerve [121] or central nervous system; are relatively easy to record; show the expected dependence on stimulus level; and accurately reflect subjective threshold [117a]. Unfortunately a single eERG or eVEP recording can take up to 15 minutes to complete, using current technology, thus limiting the usefulness of this approach at the present time. For the eERG and eVEP to be of clinical value, recording times will have to be substantially reduced; once this is accomplished, these recordings can be used in a similar fashion as is currently the case for eCAPs in cochlear implants.

CONCLUSION: TOWARD 2020 VISION

The potential applications of tissue engineering sketched in the previous sections pose enormous challenges, well exceeding the competency of any single group or institution. Concerted research efforts by multidisciplinary groups may allow the implementation of the complex systems required to restore and enhance vision. As researchers improve, on the one hand, their fundamental understanding of processes such as photoconversion, graft integration, immune regulation, and neuroprotection and, on the other, the engineering ability to control tissue properties and neurite growth, both *in vitro* and *in situ*, crude but functional vision restoration at the RPE/photoreceptor level and at the RGC level may advance to the level of experimental and even clinical therapy. Integration of all these areas to recreate the full range of retinal processing is a much more distant goal, for which the header of this section may well be too ambitious.

To accomplish any of these forms of vision restoration, however, funding mechanisms for multidisciplinary research and interest from the corporate sector will have to rise well beyond their current levels. While the number of severely visually impaired individuals and the economic impact of vision restoration alone may not justify that these approaches receive priority over treatment of life-threatening conditions, the investment required is relatively modest, and the improvement in quality of life for (nearly) blind patients can be very significant.

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PART 17

Oral/Dental Applications

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CHAPTER 70

Biological Tooth Replacement and Repair

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INTRODUCTION

Teeth might not at first seem an obvious organ for which to develop biological methods for replacement or repair. Teeth are non-essential organs, and modern dental treatments enable most dental problems to be treated using traditional non-biological approaches. Teeth do, however, offer unique opportunities to develop tissue-engineering-based approaches on an organ that is not only easily accessible and non-life-threatening but one where there are very large patient numbers and a significant clinical problem. The challenges of developing methods needed to restore and repair complex organs are immense, but the biggest challenge will be the testing of any such organs on patients. Patients requiring repair or replacement of major internal organs (heart, liver, lungs, pancreas, etc.) are by definition likely to have a serious illness. Access to these organs requires major surgery; should the treatment fail, the consequences will be severe and life threatening. Teeth do not present any such problems and thus provide an opportunity for the proof of concept to be tested safely, with little chance of danger to the patients.

Although there are an increasing number of alternative treatments in dentistry, most are non-biological and many are based on techniques that have been practiced for thousands of years. Dental implants, for example, involve the replacement of missing teeth with metal rods that are screwed into holes drilled into the jawbone. The practice of replacing missing teeth with metal implants can be traced back to the ancient Romans and Egyptians [1,2]. The number of dental implants installed is increasing each year, and thus there is a need to develop new biologically based approaches. Similarly, many tooth fillings still use mercury-based amalgams, and the possibility that repair of dental hard tissues (dentine and enamel) might involve the use of cells to remineralize the damage to teeth naturally is an exciting prospect. This chapter explores the current status of research directed toward whole tooth replacement and repair of dental disease.

TOOTH DEVELOPMENT

Teeth are ectodermal appendages (hair, sweat glands, salivary glands, etc.) that develop from an increasingly well-understood series of reciprocal epithelial mesenchymal interactions (Figs. 70.1 and 70.2). In mammals, these interactions take place in the developing oral cavity between the oral ectoderm (epithelium) and cranial neural crest-derived mesenchyme (ectomesenchyme). The cellular origins of the signals that initiate tooth development have

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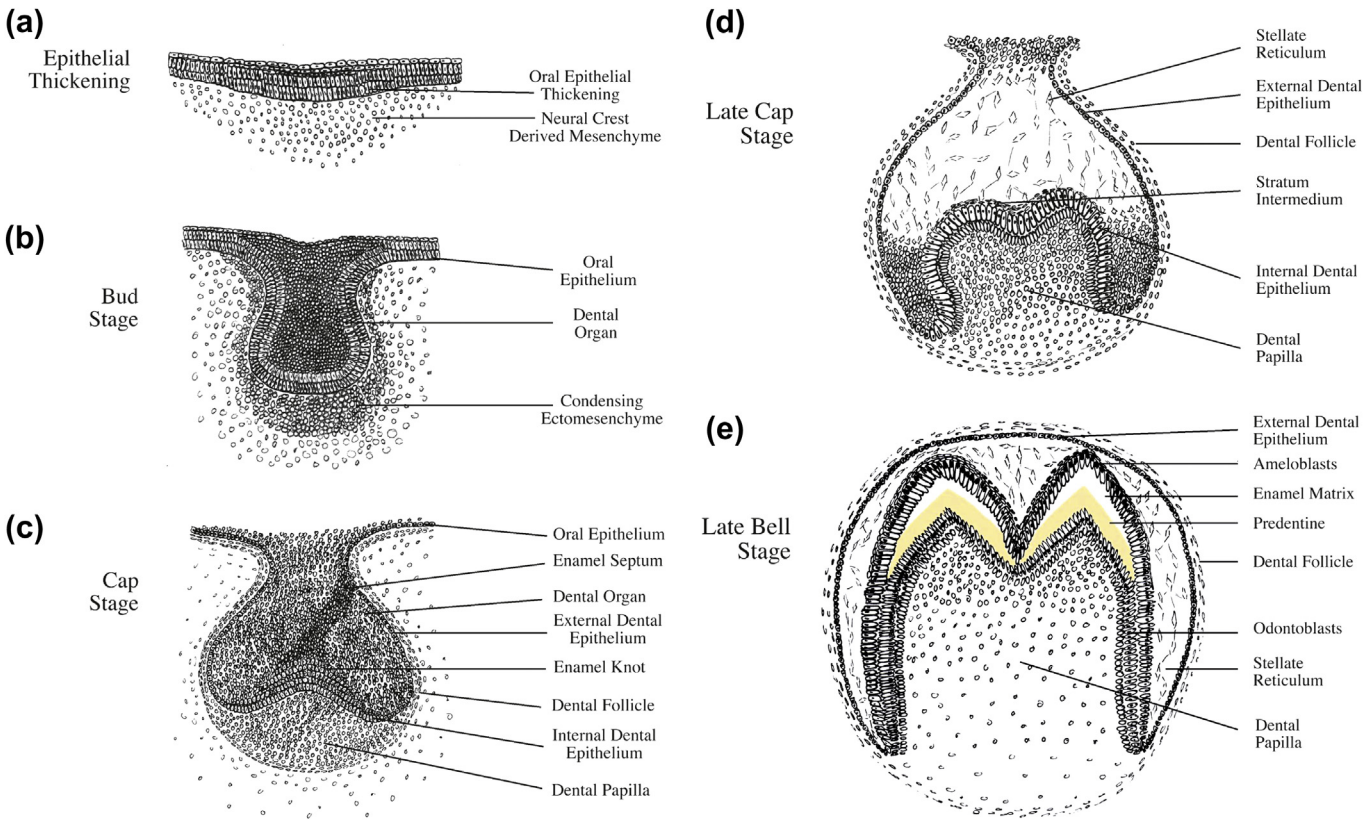

FIGURE 70.1
Drawings of histological sections of mammalian first-molar-tooth development.

been the subject of much controversy over the years, but modern molecular and animal techniques have confirmed that the oral (predental) epithelium is the source of the signals that initiate odontogenesis (reviewed in references [3,4]).

Recombination experiments between dental cells and non-dental cells have identified temporal changes in the direction of inductive signals. Following the initial epithelial-to-mesenchymal inductive signals, a long series of temporally and spatially controlled exchange of signals governs each step in the increasingly complex development of a tooth. With the exception of the nerve supply, all the cells of a mature tooth originate from the oral epithelium and ectomesenchyme. The epithelial cells form only one functional cell type, the ameloblasts which are responsible for enamel formation. All of the other cell types, including the odontoblasts that are responsible for dentine formation, periodontal ligament cells, the pulp cells, etc., are derived from ectomesenchyme. The ability of ectomes-enchyme to differentiate into these different cell types illustrates the stem cell-like properties of these cells. Classic recombination experiments in the 1980s established that non-neural crest cell populations of mesenchymal cells cannot respond to tooth-inductive signals, and, more recently, the requirement for dental mesenchymal cells to have stem cell properties has been established (see later). An understanding of the properties of predental epithelium, mesenchymal cells, and the interactions and molecules involved is the key to developing biological approaches for replacement and repair.

WHOLE TOOTH-TISSUE ENGINEERING

Stem cell-based tissue engineering of teeth

The aim of this approach is to reproduce an embryonic tooth primordium from cultured epithelium and mesenchymal cells. Since it is established that embryonic tooth primordia are

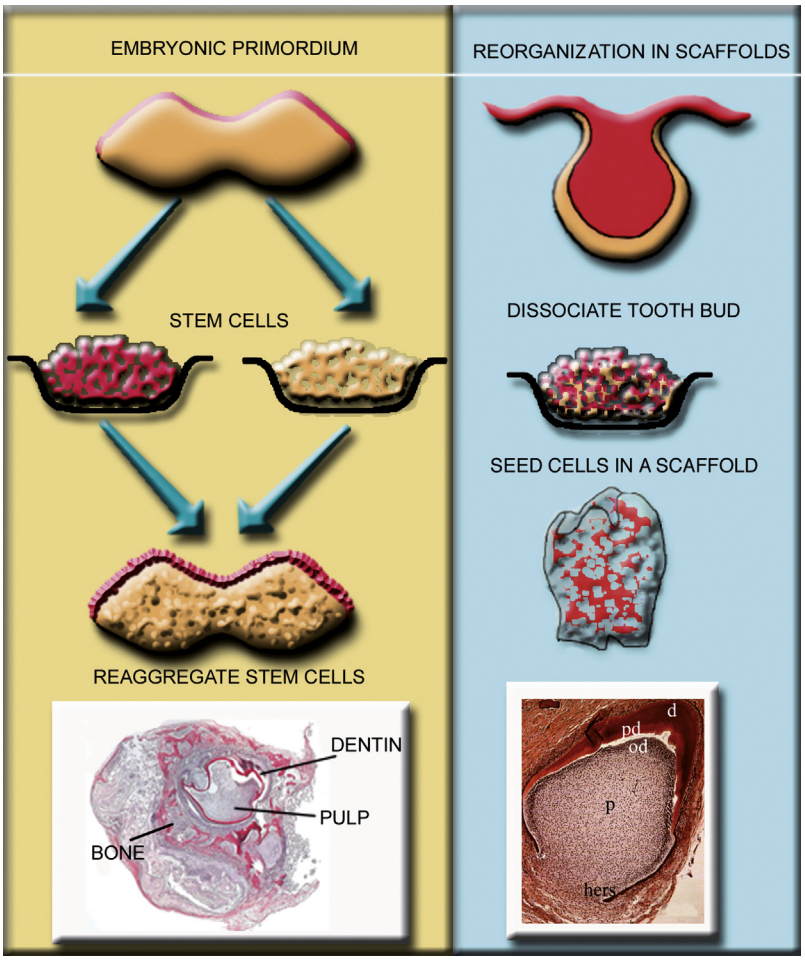

FIGURE 70.2
Diagrammatic representation of two methods currently being explored for producing biological tooth replacement. (*Figure kindly drawn by Rachel Sartaj.*)

able to continue their embryonic development in ectopic adult sites such as the kidney, the expectation is that the artificial primordia will develop into teeth following implantation as tooth rudiments into the adult mouth. Thus, rather than a metal dental implant, a cell-based biological implant will be transplanted. Since the ectomesenchymal cells of the tooth primordium form the majority of tooth cells and regulate tooth shape, it is finding replacements for these cells that has been the main initial focus of research. The early inductive interactions that take place *in vivo* show that the mesenchymal cells must have stem cell-like properties. Cultured populations of stem cells have thus been used to replace these ectomesenchymal cells in recombinant tooth explants. Cultures of mouse embryonic stem (ES) and neural and adult bone marrow cells were aggregated to form a semi-solid mass, on which embryonic oral epithelium was placed [5]. When cultured for two to three days, the initiation of an odontogeneic response in the stem cell 'mesenchyme' cells could be visualized with molecular markers. Transfer of the explants in the kidney capsules of adult mice was then used to assay for tooth formation. 'Bioteeth' explants made with mesenchyme derived from ES and neural stem cells proved difficult to transfer intact into kidneys. Explants made from adult bone marrow stromal cells, however, were substantially more robust and survived transfer. Following incubation of explants in kidneys for 10–14 days, clearly identifiable tooth crowns were formed, surrounded by bone. By using cells from genetically distinct transgenic mouse lines (GFP, LacZ), the stem cell origins of dental mesenchyme cells and bone cells could be confirmed. Cells derived from adult bone marrow stromal were thus capable of contributing to tooth formation is a way identical to ectomesencyme cells. Moreover, development of the tooth crowns appeared to follow the normal pathway of embryogenesis, with the formation of new bone completely surrounding the

tooth. New bone formation is an essential (but often overlooked) component of biotooth formation. A biotooth has to be able to anchor itself to the jawbone with roots and a periodontal ligament. Concomitant bone formation must therefore occur during tooth development.

The growth of tooth primordia following surgical transplantation into the adult jaw appears remarkably straightforward. Transplantation into ectopic sites or into tooth sockets following extraction provides a suitable environment to support continued development, postnatal growth, root formation and eruption [5–7].

Bioteeth from cell-seeded scaffolds

In the early 1950s, Shirley Glasstone-Hughes demonstrated that when early-stage embryonic tooth primordia are physically divided into two halves, each half develops into a normal-sized tooth [8]. This pioneering experiment demonstrated the developmental plasticity and regenerative capacity of embryonic tooth germs. This regenerative capacity has been exploited to devise a simple, biodegradable scaffold-based approach to biotooth generation. The early pioneering work of Vacanti and coworkers on the use of biodegradable scaffolds to act as supports for guided tissue regeneration has provided the basis for using the reorganizational properties of dental primordia cells to reform teeth *in vitro* [9]. The basic principle is to create scaffolds in the shape of the tooth required. These are then seeded with cells isolated following dissociation of third-molar-tooth germs. Third molars, or wisdom teeth, erupt late in human development and thus in young adults are present as dormant primordia. Both pig and rat third-molar primordia have been used with essentially the same procedure, utilizing biodegradable polymer scaffolds [10–12]. Cells from third molars at the late bud stage of development were dissociated by incubation with collagenase and dispase, and the cells were then either seeded directly into scaffolds or cultured for up to six days in Dulbeccos modified eagle medium (DMEM) plus 10% fetal bovine serum (FBS) before seeding. The scaffolds were composed of polyglycolate/ poly-L-lactate and poly-L-lactate-co-glycolide prepared using polyvinyl/siloxane and molded into the shape of human incisors and molars. For pig cells, scaffolds of $1 \times 0.5 \times 0.5$ cm were used; whereas for rat cells, rectangular scaffolds of $1 \times 5 \times 5$ mm were used. Scaffolds containing seeded cells were surgically implanted into the omentum of rats (athymic rats for pig cells) and left for 12–30 weeks. Histological sectioning of the explants revealed the formation of tiny tooth-like structures. The structures were between 1 and 2mm in size and showed many of the features of molar-tooth crowns, including differentiation of ameloblasts and odontoblasts. Experiments using pig cells detected the tooth-like structures after 20 weeks, whereas with rat cells these formed after 12 weeks. In all cases, the shapes of the toothlets formed were independent of the shape of the scaffold, and, unlike natural tooth formation, no bone was formed in association with the teeth. Based on the previous results of Glasstone-Hughes, the most likely explanation for these results is one of reorganization rather than *de novo* formation. The small size of the teeth indicates that small numbers of dental epithelium and mesenchyme cells reassociated to form tooth germs. The fact that cells could be cultured for up to six days indicates that either the epithelial and/or mesenchymal cells are able to retain their odontogenic properties, or the possibility exists that stem cells were present in the cell populations, but this remains to be demonstrated. A functional biotooth must be able to form roots; in order to do this, new bone must form at the same time as the tooth. In the scaffold approach, no new bone is formed. In order to address this, scaffold tooth-like structures have been generated together with bone implants produced from osteoblasts induced from bone marrow progenitor cells seeded onto polyglycolide-co-lactide-fused wafer scaffolds [13]. The codevelopment of bone and tooth-like structures permitted the early formation of roots and thus demonstrated the possibility of utilizing a form of hybrid tooth/bone-tissue-engineering approach.

The phenomenon of reorganization of dental primordia cells into teeth has been investigated in detail by Lesot and colleagues. Using cap-stage-tooth germs (E14 – mouse) (Fig. 70.1), they showed that following complete dissociation of both the epithelium and mesenchymal cell derivatives, teeth could be produced when the cells were reaggregated and reassociated [14,15]. Thus, as observed with third-molar-tooth primordial, the cells retained their odontogenic capacity following dissociation. This property was utilized as a method of investigating the potential of non-dental cells to participate in tooth development. The experiments of Ohazama et al. [16] showed that bone marrow stromal cells were able to replace all dental mesenchyme cells. Bone marrow cells were mixed with dissociated dental epithelial cells and reassociated with dental mesenchyme [17]. The bone marrow cells were found to contribute to ameloblast formation. In order to determine which cells within the crude marrow population might give rise to epithelial ameloblasts, cell sorting with c-kit was used to separate hematopoietic progenitor cells. When mixed with dental epithelial cells, these cells were observed to form both ameloblasts and odontoblasts. The ability of bone marrow mesenchymal progenitors (stem cells) to contribute to ameloblast formation was not assessed in these experiments. However, the principle, as originally proposed by Ohazama et al. [16], that bone marrow cells can be a potential source of cells for tissue-engineering bioteeth is further supported by these reaggregation experiments. In all the experiments reported by Lesot and colleagues, the ability of non-dental cells to contribute to tooth formation occurs only when the cells are mixed with dental cells from cap-stage tooth germs. The experiments reported by Ohazama et al. [5] did not require a mixed population. It remains to be seen therefore whether any commercial biotooth procedure could feature the use of dissociated embryonic third-molar dental cells. Since bone marrow can be used totally to replace dental mesenchyme in the absence of any dental mesenchyme cells, the prospect exists that if a source of epithelial cells can be identified that can replace dental epithelial cells in the absence of any epithelial cells, then bioteeth could be formed entirely from adult cells. Dental epithelial cells produce the signals that initiate the whole process of tooth formation, and, thus, a non-dental source of these cells must in part be capable of reproducing these signals.

One signal that has been identified is BMP4, which is specifically expressed in early predental epithelium before bud formation. When exogenous BMP4 is added to non-dental embryonic explants, odontoblast and ameloblast differentiation can be detected [16]. Although these cells are organized together in cell layers as they appear *in vivo*, they are not present in a recognizable tooth structure. Nevertheless, the fact that one molecule applied to cells early can stimulate the differentiation of non-dental cells into both epithelium and mesenchymal dental cells offers the possibility of engineering a dental epithelium from non-dental epithelial cells.

Root formation

In order to be functional, bioteeth must develop roots. Root formation is a complex and little-understood process. However, although little is known of the molecules that stimulate and coordinate root formation, the absolute requirement for bone is established. If teeth form in the absence of bone, they cannot form roots, as observed in the seeded scaffold tooth-like structures. When these scaffold teeth develop alongside new bone, root formation is stimulated [13]. Tooth formation is most often studied experimentally by transplantation of tooth primordia to ectopic sites in adult rodents. The anterior chamber of the eye, the kidney capsule, the omentum, and the ear have all been used as sites that permit tooth formation. Root formation can occur at all of these sites if accompanied by bone development [17]. A prerequisite of any method of biotooth formation therefore is that tooth primordia are able to form teeth and roots integrated into jaw bone in the mouth. The mouth is, however, not routinely used as an experimental ectopic site for tooth development because of the difficulty of the surgery involved. In order to determine whether the adult mouth can support tooth formation, embryonic tooth primordia have been surgically transplanted into the mouths

of adult mice. Not only do these develop into teeth in the mouth, but they also produce functional roots and erupt [5].

Cell sources

The biggest hurdle to a successful clinically-translatable protocol for whole tooth-tissue engineering is the issue of the source(s) of cells to be used. Both epithelial and mesenchymal cell sources need to be identified and with the exception of the use of bone marrow stromal cells, all other reports of whole tooth 'bioengineering' have employed cells dissociated from tooth primordia. A feature of the successful experiments using dissociated tooth germ cells is that the cells are not expanded *in vitro* before use. In order to obtain sufficient cells to generate one tooth, many tooth primoria need to be dissociated to produce at least 5 x 10^4 cells [17a]). For any viable clinical approach, the cells, whatever their source, will have to be expanded *in vitro*. However, when tooth primordia cells are expanded *in vitro* they rapidly lose their ability to re-participate in tooth formation [17b,18]. A major challenge therefore is to develop ways of maintaining tooth forming capacity following *in vitro* cell expansion.

Since tooth formation requires both epithelial and mesenchymal cells and these cells exhibit reciprocal signaling interactions, one of the cell types used must be able to induce odontogenic capacity in the other. In the bone marrow stromal cell experiments, embryonic tooth epithelium was used to induce this capacity in the bone marrow-derived cells [5]. Cells isolated from adult oral mucosa are capable of responding to odontogenic-inducing signals from embryonic tooth mesenchymal cells and forming tooth-like structures [19,20]. Potential adult cell sources are thus available but none have inherent odontogenic-inducing properties.

DENTAL-TISSUE REGENERATION

An important long-term goal for dental-tissue engineering is developing strategies for biotooth formation, thereby addressing many of the clinical problems arising from developmental anomalies and dental disease. However, in the shorter term there are many opportunities to be exploited for partial reconstruction of the tooth organ by tissue regeneration. Such approaches may be more readily achieved and still provide the potential for very significant impact on delivery of oral health care. Natural tissue regeneration, i.e., wound healing in the dental environment, is well recognized but represents a rather serendipitous event, and the approaches used to encourage such processes are somewhat empirical. A key factor in encouraging natural tissue regeneration is facilitating a conducive tissue environment in which the regenerative processes can take place, including moderation of chronic inflammatory events, control of bacterial infection, and minimizing tissue injury during any restorative surgical intervention. A good understanding of how to control these influences on tissue regeneration is fundamental to clinical success. There are also significant opportunities for the application of agents, whether directly through bioactive molecules or indirectly through agents that can release tissue-sequestered pools of these molecules locally, to promote regenerative processes and tip the balance between tissue degeneration and regeneration.

Natural tissue regeneration

Natural tissue regeneration implies a cellular basis to regeneration, and the non-vital nature of mature dental enamel provides major hurdles to its regeneration by any but physicochemical remineralization processes. However, the vitality of the dentin-pulp complex provides significant opportunities for regeneration. Regeneration in the dentin-pulp complex will only take place, however, if there is a conducive tissue environment. Early studies highlighted the causal link between bacteria and inflammatory events in the dental pulp, and the presence of pulpal inflammation provides an effective barrier to initiation of regeneration therein [21]. Thus, if natural tissue regeneration is to be facilitated, it is essential that bacterial infection and the consequent inflammation in the tooth are controlled. The inhibitory action of

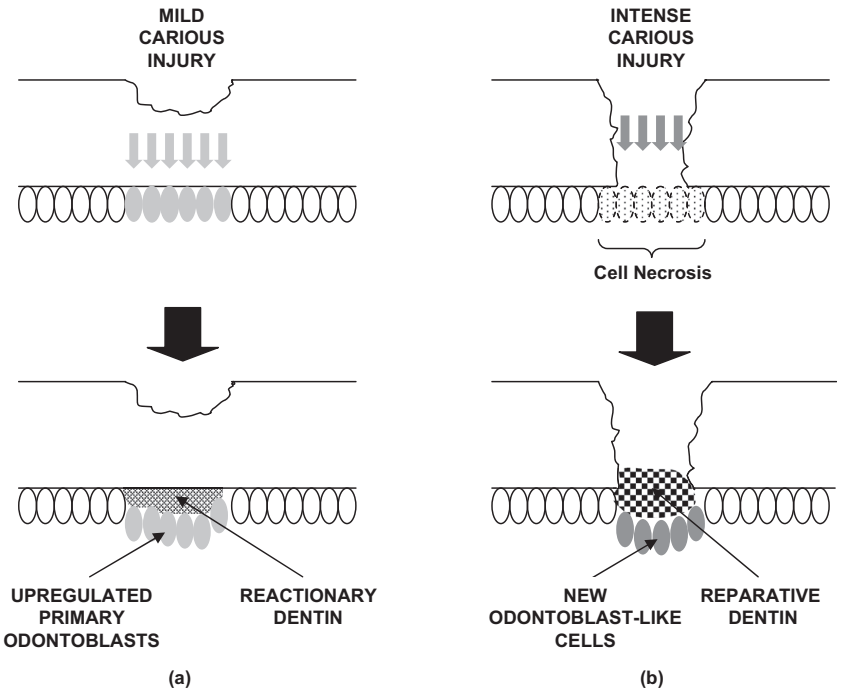

FIGURE 70.3
Schematic diagrams of reactionary (a) and reparative (b) dentinogenesis. (a) Mild carious or other injury signals upregulation of the primary odontoblasts underlying the injury site, leading to secretion of reactionary dentin. (b) More intense injury causes necrosis of the odontoblasts underlying the injury site, and progenitor cells are recruited from the pulp to differentiate into a new generation of odontoblast-like cells, which secrete reparative dentin. Dentin bridge formation at sites of pulpal exposure arises from reparative dentinogenesis.

inflammation on regeneration may represent an effect more on signaling events than stem/progenitor cell survival, since inflamed pulp tissue can allow isolation of cells with some stem cell properties [22,23]. The close interplay that is emerging between inflammation and tissue regeneration [24], however, emphasizes the complexity of events in the post-injury tissue environment where intervention should be carefully targeted. Traditionally, bacterial control has often been achieved through extensive surgical removal of infected dental hard tissues, although the trend toward minimal intervention therapy during tooth restoration potentially places the tooth at risk through incomplete bacterial control. Sealing or 'entombing' the bacteria within the restoration may help to compromise their viability, as may some of the chemical agents used in the placement of dental materials. Nevertheless, control of pulpal inflammation may be desirable in the context of tissue regeneration, and an improved understanding of the inflammatory mediators involved [25,26] may allow specific targeting with novel anti-inflammatory molecules.

The concept of tissue regeneration in the dentin-pulp complex has been recognized since the first report of tertiary dentinogenesis in response to injury from caries by Hunter in the 18th century, and dentistry has long been a pioneer in regenerative medicine through the use of calcium hydroxide to stimulate reparative dentinogenesis to bridge pulpal exposures in the dentin [26a]. Tertiary dentinogenesis (reactionary and reparative dentinogenesis are subvariants) represents the upregulation of the dentin-secreting cells, the odontoblasts, in teeth after completion of tooth formation to initiate tissue regeneration in response to injury. With mild injury, the odontoblasts underlying the injury survive and respond by upregulation of their secretory activity to form reactionary dentin, while with injury of greater intensity a number of these odontoblasts undergo necrosis and may be replaced by a new generation of odontoblast-like cells secreting a reparative dentin matrix [27] (Fig. 70.3).

Importance of the injury-regeneration balance

Dental caries is one of the most widespread infectious diseases globally and continues to be a major health care problem. Initially, colonization of bacteria on the tooth surface within the dental plaque biofilm leads to demineralization and proteolytic degradation of the dental

hard tissues following diffusion of bacterial acids and metabolites. As the disease progresses, both the hard and soft tissues of the tooth become infected, and a sustained bacterial challenge ensues, with consequent host inflammatory responses. The dynamics of the disease process determine the opportunities for tissue regeneration through tertiary dentinogenesis – with a rapidly progressing lesion, little tertiary dentinogenesis or regeneration is seen, but in a more slowly progressing lesion, induction of tertiary dentin secretion occurs immediately beneath the lesion [28]. Classically, this has been assumed to reflect the intensity of microbial challenge, but more recent data indicate that the response is in part due to local release of dentin matrix components. During intense carious injury, appreciable levels of dentin matrix components are released, and these compromise survival of the odontoblasts [29]. It is possible that growth factors contained within the dentin matrix, particularly those from the TGF-β family, contribute to this loss of odontoblast survival, since levels of TGF-β similar to those released from the dentin matrix have a comparable effect on odontoblast survival *in vitro* [30]. At lower doses, however, these dentin matrix components and the cocktail of growth factors contained therein can stimulate regenerative events.

It has long been recognized that particles of dentin displaced into the pulp during surgery can act as a nidus for regeneration. Implantation of solubilized dentin matrix components in the base of either unexposed [27] or exposed cavities [31] induces a regenerative response of tertiary dentinogenesis. Such responses mirror those seen beneath more slowly progressing carious lesions [28] and suggest that the natural regeneration seen during carious and other dental tissue injury is induced by local release of bioactive tissue matrix components. If such processes are to be successfully mimicked for development of novel regenerative therapies, it is important that we understand the nature of the signaling molecules involved.

Signaling events in dental regeneration

It seems probable that the signaling events during tertiary dentinogenesis and regeneration in the dentin-pulp complex recapitulate many of those occurring during embryonic development. During tooth development, the cells of the inner enamel epithelium of the enamel organ induce the ectomesenchymal cells at the periphery of the dental papilla to differentiate into odontoblasts through the mediation of the dental basement membrane, which may function in the immobilization and presentation of the signaling molecules [32]. Growth factors, especially those of the TGF-β family, may be key signaling molecules during induction of odontoblast differentiation [33,34]. While an epithelial source of these molecules is not available in the mature tooth for signaling regeneration, sequestration of these molecules in the dentin matrix [35,36] following secretion by the odontoblasts may provide such a source. Their release during carious demineralization [29] by lactic and other bacterial acid metabolites would allow their diffusion through the dentinal tubules to the odontoblasts and pulp cells, which express receptors for these growth factors. Application of recombinant members of the TGF-β family, either *in vitro* [37–39] or *in vivo* [40–42], has been demonstrated to induce a regenerative response of tertiary dentinogenesis.

However, while this mirrors some of the signaling events of embryonic development, there is likely to be much reduced control over the signaling, since the rate of release of these molecular signals from the dentin matrix will be of variable intensity and the spectrum of cells with which they can interact will differ from embryogenesis.

Control of specificity of dental-tissue regeneration

During wound healing in the dentin-pulp complex, a broad spectrum of tissue responses may be observed, ranging from regeneration of dentin tissue virtually indistinguishable from primary physiological dentin in terms of its tubularity and structure to secretion of atubular tissues with many of the structural features of bone. While terms such as tertiary dentin, reparative dentin, and irritant dentin have been used to encompass all of these responses, the

dentinogenic specificity of some of the responses remains to be demonstrated. It is unclear, though, as to what the determinants are of the specificity of the response during natural tissue regeneration. Is it control of the molecular signaling processes, is it heterogeneity in the types of cells participating in the regeneration, or is it some other factor? Answering these questions will be fundamental to future strategies for exploiting tissue regeneration in the tooth in a controlled manner. The ability to determine whether tissue regeneration gives rise to tubular or atubular dentin matrix could be of great value in developing designer regenerative clinical approaches. For example, there may be significant benefit in directing secretion of a tubular dentin matrix in the tooth crown during regeneration to restore the normal physiological tissue architecture and function. However, it could be advantageous in possible exploitation of tissue regeneration for endodontic applications, such as root canal therapy, to generate an atubular dentin matrix, thereby providing an effective seal to the periapical region of the tooth.

Dental postnatal stem cells

The cells of the dental pulp have traditionally been considered to be neural crest-derived ectomesenchymal cells, although it is clear that during embryogenesis the migrating neural crest cells will intermingle with mesenchyme in the first branchial arch [43]. Thus, cells of the pulp may not all share the same lineage, although those at the periphery, including the odontoblasts, appear to be of neural crest origin. In reparative situations, there are potentially a variety of possible progenitors in the pulp for differentiation of a new generation of odontoblast-like cells, including undifferentiated mesenchymal cells in the cell-rich layer of Höhl adjacent to the odontoblasts, perivascular cells, undifferentiated mesenchymal cells, and fibroblasts. This highlights the diversity in reparative response, which may occur after injury to the tooth. While the tissues that regenerate in such situations are all referred to as tertiary dentin (reactionary or reparative variants), in reality they represent a spectrum of tissue responses, dependent on the origin of the formative cells.

During normal tooth development, it has been suggested that cells destined to differentiate into odontoblasts have to achieve a level of competence before they can respond to an inductive signal for terminal differentiation [32]. If this is the case, then it may be a very restricted population of cells able specifically to give rise to odontoblasts during regeneration in the mature tooth. Just prior to terminal differentiation during tooth development, the pre-odontoblasts align perpendicular to the dental basement membrane, and after the final cell division, one daughter receives the inductive signal to differentiate into an odontoblast, while the other daughter shares a similar developmental history, with the exception of this final inductive step. It has been presumed that this latter group of cells resides in the cell-rich layer of Höhl just beneath the odontoblast layer in the mature tooth and as such, these cells would be prime candidates as progenitors for odontoblast-like cells during regeneration. Certainly, there is a decline in the numbers of cells in this subodontoblastic site with age [44], which mirrors anecdotal clinical reports of impaired tissue regeneration with age. Interestingly, many of the cells in the Höhl layer express the stem cell marker Thy-1 and those cells showing high expression of Thy-1 demonstrated enhanced potential to differentiate into hard tissue-forming cells [45]. Undifferentiated mesenchymal cells are also found within the central core of the pulp, which could contribute to regenerative processes. A specific population of postnatal dental pulp stem cells (DPSCs) has also been described [46], which show many characteristics of postnatal stem cells, although it is possible that this population of cells may be heterogeneous in its phenotype. Transplantation of these cells subcutaneously into immunocompromised mice in association with hydroxyapatite/tricalcium phosphate powder generated ectopic deposits of reparative dentin with expression of dentin sialoprotein [47]. These DPSCs appear to be distinct from the stem cells from human exfoliated deciduous teeth (SHED) [48] and stem cells from the apical part of the papilla (SCAPs), the latter of which have been isolated from apical areas of the tooth [49]. Despite this focus of looking for specific cell populations in pulp, it is unclear

whether these various populations simply represent mesenchymal stem cells (MSCs) recruited through the circulation from sites outside the tooth and if their exposure to the niche environment within the pulp provides their phenotypic characteristics. Genetic lineage tracing provides evidence to support such an origin for some cells involved in odontoblast-like cell differentiation [50], which has significant implications for regenerative strategies in dentin-pulp. Issues with phenotypic change of pulp cells during expansion culture [51] also highlight the opportunities to source stem/progenitor cells from non-pulp tissue, such as bone marrow and adipose tissue [52,53] for dentin-pulp regeneration.

As interest in dental regeneration increases, it is important that detailed characterization be performed of the progenitor cells involved. Primary cultures of dental pulp cells give rise to many cells with myofibroblastic characteristics, and similar results have been observed during extended serial expansion of such pulp cultures [29]. Whether this represents asymmetric growth of these cells or the myofibroblast phenotype represents a default phenotype is unclear, but it is probable that such cells are not true postnatal stem cells, and any attempt to label them as such should be resisted in the absence of fuller characterization. Clearly, there is potential for a spectrum of derivations for the cells potentially involved in regeneration of the dentin-pulp complex, and the consequences of this may be a variety of phenotypic responses. It seems probable that only some of these responses may involve true postnatal stem cells. However, the diversity of cellular responses highlights the potential benefits of achieving control over these regenerative processes and the opportunities for developing strategies for directed tissue regeneration.

Directed tissue regeneration

The concept of directing tissue regeneration will be successful only if it builds on the foundation of our knowledge of natural tissue regeneration, which in turn exploits our appreciation of the molecular cellular signaling events during normal tooth development. Focus on both cell- and signaling-based approaches has provided many interesting initial avenues for directed tissue regeneration in the tooth, but it is probable that the combination of these two approaches will prove the most effective in providing us with novel solutions for regenerating tooth structures. For example, seeding of SHED cells in a poly-lactic acid scaffold within a tooth slice has allowed regeneration of pulp tissue resembling that seen physiologically [54] and the acidic conditions created by the scaffold were hypothesized to be responsible for release of signaling molecules from the dentin matrix.

Signaling-based strategies

Signaling-based strategies have generally aimed to mimic those signaling events responsible for cell differentiation and secretion during embryonic tooth development and natural tissue regeneration. Thus, there has been a strong focus on application of growth factors, as well as various matrix-derived molecules, which also appear to stimulate regenerative events. Growth factor application has also been investigated for chemotaxis-induced cell homing in pulp regeneration [55], which lends support to the concept of MSC recruitment from non-dental locations [50]. Approaches have been used to upregulate secretion by existing cells (reactionary dentinogenesis) as well as to induce the differentiation of new odontoblast-like cells (reparative dentinogenesis) for tissue regeneration, although the former has been constrained by our lack of understanding about the physiological control of odontoblast secretion during primary dentinogenesis.

Both *in vitro* [37–39] and *in vivo* [40–42] application of growth factors, particularly of the TGF-β family, to the exposed pulp in pulp-capping situations induces regeneration, although the tissue formed has shown a range of appearances, from osteodentin to tubular dentin-like. There have also been several reports of the application of matrix molecules [56–59] promoting dentin regeneration, and a Phase II clinical trial with a synthetic peptide derived from

matrix extracellular phosphoglycoprotein (MEPE) is presently in progress. While all of these approaches provide the foundation for a new era of biologically based regenerative therapies for the teeth, there are a number of limitations. The half-life of free growth factors is generally short; for instance, that of free TGF-β1 is estimated to be of the order of 2−3min. In fact, when the growth factors are sequestered within the dentin matrix, they are remarkably well protected from degradation by association with extracellular matrix components. This protective mechanism can allow the growth factors to remain 'fossilized' for the life of the tooth, thereby providing an exquisite life-long potential for natural regeneration. Also, the consequences of short half-life of these molecules and the ubiquitous presence of proteolytic enzymes in the tissue milieu at sites of injury require high concentrations to be applied for regenerative signaling. As for all tissue regenerative and engineering strategies, the mode of delivery of signaling molecules is key to their effective action. To date, most signaling molecules have been simply applied as lyophilized powders or aqueous solutions, but considerable potential exists for development of novel delivery systems. An alginate hydrogel has shown encouraging results for delivery of TGF-βs for induction of *de novo* dentinogenesis on cut surfaces of pulp tissue [60], and innovative approaches are needed to deliver such molecules through the dentin matrix via the dentinal tubules. While passive diffusion through the tubules will have some effect, histomorphometry of restored human teeth suggests that natural regeneration occurs optimally when there is only a very limited residual dentin thickness, and a challenge will be to develop systems for delivery across greater distances in the dentin matrix. Gene therapy (see later) offers interesting opportunities for targeting signaling molecules to the regenerative site, although safe and suitable delivery systems are still required.

Calcium hydroxide has long been used to stimulate dentin bridge formation for dental regeneration, although its mechanism of action has remained largely elusive. Recent studies, however, have indicated that both calcium hydroxide, and the closely related material mineral trioxide aggregate (MTA), probably acts by release of stores of endogenous bioactive molecules and growth factors sequestered within the dentin matrix, which are responsible for the cellular signaling [61,62]. The limited control of growth factor dissolution from the dentin matrix by calcium hydroxide may in part help to account for its rather variable activity during regeneration. However, its action does point to an interesting approach for regeneration, whereby agents target release of endogenous signaling molecules from the tissue, thereby obviating some of the issues associated with the application of exogenous sources of these molecules. Such agents might also include some of the cavity etchants and irrigants commonly used in restorative dentistry [63,64].

Cell- and gene-based strategies

Cell- and gene-based strategies offer exciting opportunities for dentin-pulp regeneration, although many hurdles have to be overcome before these can become a clinical reality. Using electroporation, Gdf11 gene transfer to an exposed pulp *in vivo* failed to provide effective regeneration [65], although use of ultrasound for transfer of the Gdf11 plasmid provided an osteodentinogenic regenerative response [66]. *In vivo* gene transfer of a recombinant adenovirus containing a full-length cDNA encoding mouse bone morphogenetic protein (BMP)-7 failed to induce reparative dentinogenesis in inflamed ferret dental pulps, while *ex vivo* transduced dermal fibroblasts induced regeneration in the same model, although the dentinogenic specificity of this response requires further characterization [67]. These studies highlight the negative impact on regeneration of a non-conducive tissue environment, due to inflammation and, also, the limited numbers of stem/progenitor cells in the pulp for recruitment to participate in regeneration. The latter issue might be overcome by transplantation of suitable stem/progenitor cells for regeneration at sites of injury, especially if transduced with suitable signaling molecules. *Ex vivo* gene therapy by transplantation of Gdf11 [68] or Bmp2 − electrotransfected pulp stem/progenitor cells [69] − shows promise for induction of regeneration, although the tissue formed was osteodentin-like in appearance. It is

unclear whether the lack of dentinogenic specificity in these responses can be ascribed to the nature of the cells transplanted or to the inductive signaling molecules. However, the results highlight the importance of considering not only the cells and signaling molecules involved, but also the delivery mechanisms for effective therapies to be developed. Novel approaches may also include epigenetic modification of pulp cells with histone deacetylase inhibitors (HDACi) to stimulate regenerative events [70,71].

The anticipated low numbers of stem/progenitor cells for regeneration in the dental pulp indicate the need to optimize recruitment strategies for these cells, whether locally recruited from within the tissue or transplanted from without. The former approach avoids possible issues of immune reaction to non-autologous cellular material. However, local recruitment requires selection of those cells of appropriate lineage and phenotype. Cell markers for potential selection stem cells from embryonic first branchial arch tissue have been investigated [72], and low-affinity nerve growth factor (NGF) receptor is being targeted for selection of odontoblast-like cell progenitors from the mature dental pulp [29]. A combination of c-kit, CD34, and STR-1 has been reported for selection of stromal stem cells from human deciduous dental pulps, which can be induced to differentiate into osteoblasts for bone regeneration [73], while c-kit has been used for selection of odontoblast progenitor cells from bone marrow [17]. CD105 has been reported to allow for selection of pulp cells capable of regenerating pulp tissue including nerves and vasculature followed by new dentin formation [74]. Use of such cell selection approaches may overcome problems associated with recruitment of stem/progenitor cells for odontoblast differentiation from a relatively small niche within the pulp during dental regeneration. Future strategies might include *ex vivo* selection of these cells for transplantation or immobilization of antibodies or other capture molecules for selection at regenerative sites for recruitment of the cells. A combination of these approaches with novel delivery of signaling molecules may prove effective in optimizing the regenerative response.

CONCLUSIONS

Tissue-regenerative strategies offer exciting possibilities for development of novel clinical solutions for treatment of dental developmental anomalies and disease. The exquisite natural tissue-regenerative capacity of the dentin-pulp complex in the tooth provides an invaluable foundation on which to develop novel biologically based regenerative therapies. Particular attention will need to be focused on both the control and the specificity of the regenerative processes, and a combination of natural and directed tissue regeneration offers many exciting opportunities for the future.

Progress in the identification, isolation, and understanding of the differentiation of embryonic and adult stem cells, together with a continuing understanding of the control of tooth development, will undoubtedly aid the production and refinement of approaches for biotooth formation. Although there remain many potential problems and pitfalls, biological tooth replacement is now a realistic possibility.

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CHAPTER 71

Tissue Engineering in Oral and Maxillofacial Surgery

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INTRODUCTION

The oral and maxillofacial region is defined as the mouth and surrounding structures, bounded superiorly by the cranial base and inferiorly by the lower jaw. It is a highly complex area that includes morphologically intricate skeletal elements (Fig. 71.1), organs responsible for the special senses, lining and covering tissue (i.e., skin and subcutaneous fat), a rich neural and vascular network and teeth. The prominent position adopted by this area makes it particularly vulnerable to injury, while the complicated interplay of events during embryogenesis of the craniofacial skeleton increases the possibility of developmental aberrations. Regular exposure to various disease-inducing agents including complex carbohydrates (e.g., sugar), ultraviolet rays, thermal insults, and carcinogens (e.g., tobacco products) produces a variety of common pathological conditions such as dental caries, burns and malignant neoplasms. The loss of tissue integrity and continuity resulting from trauma, developmental deformities, and pathology imposes upon both physicians and dentists considerable

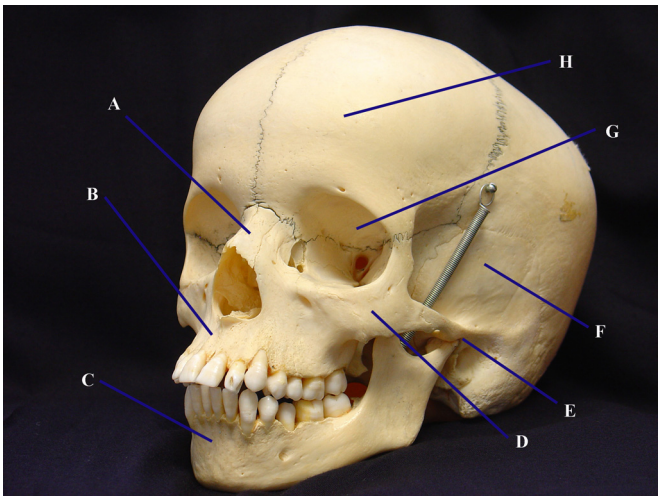

FIGURE 71.1
The human skull is a complex region composed of many bones. Several key structures include the: (A) nasal bone; (B) maxilla; (C) mandible; (D) zygomatic bone; (E) temporomandibular joint; (F) temporal bone; (G) orbital cavity; and (H) frontal bone.

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reconstructive challenges. Meeting these requirements in the twenty-first century will include strategies based upon tissue-engineering principles. While the discipline itself is several decades old, clinical applications for the reconstruction of the oral and maxillofacial region are currently absent. The purpose of this chapter is therefore to describe the special challenges posed by oral and maxillofacial reconstruction, outline existing reconstructive techniques, and review the available literature on tissue-engineering protocols that may be relevant. Providing tissue engineers with a description of current reconstructive modalities and in particular, their shortcomings, creates a reasonable starting point on which to base the development of revolutionary, new methods. We have specifically excluded descriptions of tissue engineering of teeth and special sensory organs, because these subjects have been covered elsewhere in this textbook.

SPECIAL CHALLENGES IN ORAL AND MAXILLOFACIAL RECONSTRUCTION

Virtually all tissue types of ectodermal, mesodermal and endodermal origin are candidates for tissue-engineering strategies and are present in the oral and maxillofacial region. However, certain structures are more commonly impacted by disease, trauma, and developmental failures and constitute the focus of our discussion, though the reconstructive methods described can be applied to more rare conditions. Common *pathological* entities include both benign and malignant cystic and neoplastic processes affecting the upper (maxilla) and lower (mandible) jaws, as well as degenerative conditions involving the mandibular articulation (temporomandibular joints). These diseases, or the subsequent removal of pathologically involved tissue, can produce continuity defects of the jaws requiring the replacement of bone, cartilage and lining epithelium (Fig. 71.2). Since many of these conditions are frequently silent and the dimensions of the structures involved relatively small, their initial presentation is usually associated with significant tissue involvement. In addition to disease, non-physiological loading of bone can also produce loss of skeletal tissue affecting the jaws and joints. *Edentulous bone loss* involves the resorption of the alveolar processes of the jaws (i.e., that portion of the jaw bone surrounding the tooth roots) following tooth removal (Fig. 71.3). This phenomenon is believed to be the result of direct loading of bone during mastication and the loss of physiological maintenance forces transmitted by the teeth. Over time, the loss of bone produces severely atrophied alveolar ridges, posing significant challenges to prosthetic reconstruction of the dentition and a predisposition to pathological fracture of the mandible [1].

Degenerative diseases of the temporomandibular joints (TMJs) are commonly the result of non-physiological mechanical forces produced by excessive ranges of motion of the joints or

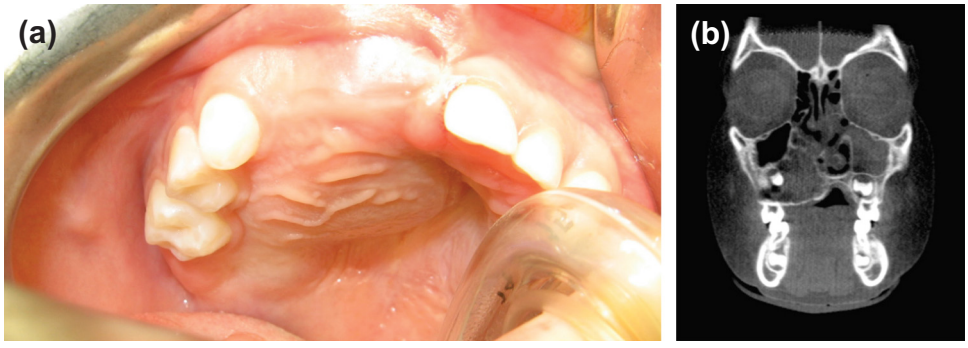

FIGURE 71.2
(a) Intra-oral photo of a patient with a giant cell tumor of the maxilla, which presents as a swelling of the palate and erosion of the supporting bone resulting in the loss of adjacent teeth. (b) Coronal computed tomography (CT) scan of the same patient with a giant cell lesion of the right maxilla which has produced marked bone destruction and displacement of developing teeth. Sinusitis of the left maxillary sinus is an incidental finding.

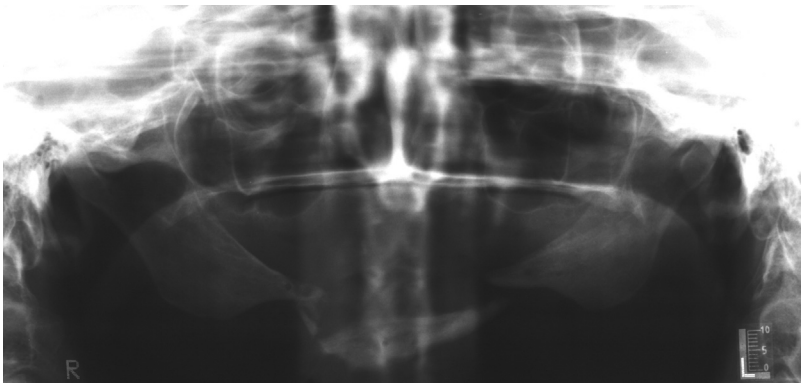

FIGURE 71.3
Panoramic radiograph of a patient with a fractured atrophic mandible. *(courtesy of Kamal Busaidy, D.D.S.)*

chronic micro-trauma from various parafunctional habits [2]. The habits in question include jaw clenching or tooth grinding (nocturnal bruxism) and repetitive motion habits such as nail biting and gum chewing. Injury to the TMJs commonly affects both the articulating surfaces of the condylar head and glenoid fossa as well as the interpositional disk, producing a spectrum of disease from chondromalacia to severe osteoarthritis. As a synovial joint, the TMJs can also fall victim to various immune-mediated disease processes, such as rheumatoid or psoriatic arthritis. The inflammatory component is responsible for progressive structural tissue loss leading to changes in skeletal relationships and malocclusion [3]. In advanced forms of disease, replacement of both cartilage and bone as a total joint reconstruction may be necessary to restore function or skeletal support to the mandible.

Maxillofacial trauma constitutes another group of conditions providing opportunities for tissue engineering reconstruction. Whereas most forms of blunt trauma result in fractures where tissue loss is minimal, penetrating injuries produced by high velocity missiles and projectiles often create significant loss of bone and overlying soft tissue (Fig. 71.4). Finally, consideration should be given to the various forms of congenital facial clefts that commonly affect the oral and maxillofacial region. In a limited form, failure of the maxillary processes to fuse unilaterally or bilaterally produces alveolar clefts (Fig. 71.5). When the upper lip, maxilla and palate are involved, a constellation of deformities associated with unilateral or bilateral cleft lip and palate patients is present.

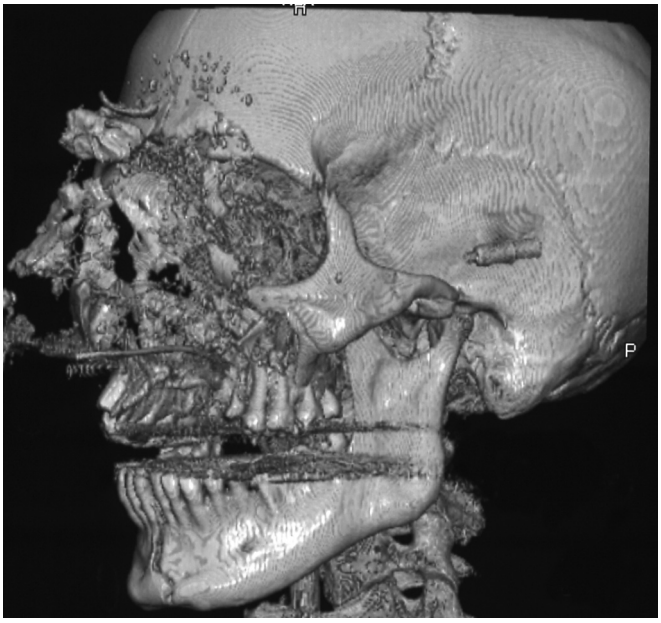

FIGURE 71.4
3D reconstructed radiograph of a patient with a self-inflicted gunshot wound demonstrating the significant disruption and loss of maxillofacial skeletal structures.

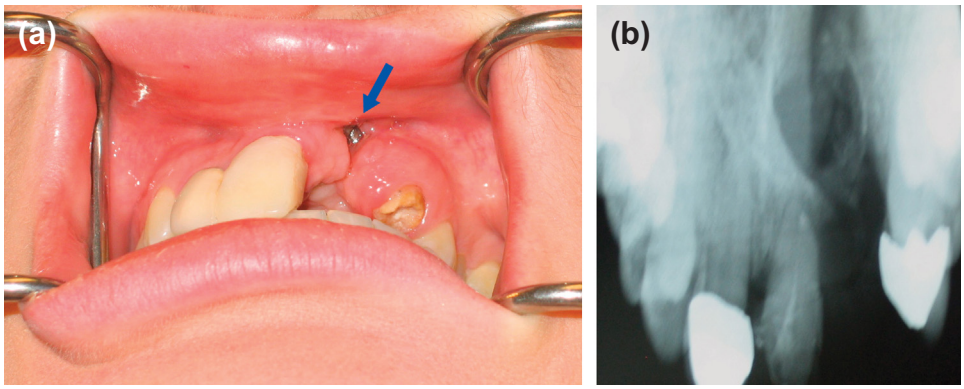

FIGURE 71.5
(a) Intra-oral view of a left maxillary alveolar cleft. An oro-nasal fistula is present at the superior-most aspect of the cleft (see arrow). (b) Radiograph of a cleft of the maxillary alveolar bone.

In the reconstruction of anatomical defects, the causative events must be taken into account to ensure long-term success. Defects produced by traumatic, developmental and pathological conditions are associated with a defined end-point. Assuming that pathology has been completely eradicated or further traumatic insults do not occur, defects produced by these mechanisms can be fully characterized with respect to size and missing tissue types. In contrast, tissue loss as a result of parafunctional habits, non-physiological loading patterns and immunologically mediated degeneration often continue following reconstruction. This set of circumstances will adversely affect any biological constructs produced by tissue-engineering techniques and impose an important limitation on the clinical application of their usage. Before biological, rather than alloplastic materials can be employed, correction of the underlying etiology is of paramount importance.

A special concern in oral and maxillofacial reconstruction is the potential exposure of grafted tissue to the external environment. Constructs used to restore defects involving the jaws, orbits, nose and ears are potentially in direct contact with the mouth, sinuses (maxillary, ethmoidal and frontal), nasal passages and external environment. These areas are characterized by high moisture content, significant bacterial populations, and functional loads imposed by physiological activities such as chewing. If biological (i.e., tissue engineered) constructs are to survive under these conditions, modifications to account for the dilutional effects of moisture, presence of infective organisms, and mechanical loads must be provided by the engineered tissue. For example, when *in vivo* polymerization of materials is intended, the presence of fluid must be considered. Alternatively, pre-formed constructs can be used. Colonization of constructs with a mixed population of aerobic and anaerobic bacteria is expected with reconstructions involving oral, nasal and sinus-related structures. Porous constructs, capable of harboring potentially pathological organisms might be modified to reduce either bacterial attachment or replication until lining tissue develops over the implant, forming a barrier to the external environment. In addition to contaminated wound sites, tissue constructs may be exposed to complicated mechanical loads before anisotropy is restored with the regeneration of biological tissue. Both the mandible and TMJs are subject to a combination of compressive, shear and tensile loads depending on the type and degree of function [4,5].

Another special feature of the maxillofacial region is the number of tissue types within a relatively small region. As a result of this proximity, traumatic, pathological, and developmental events often lead to the creation of composite defects requiring reconstruction of multiple tissue types. This results in a special challenge not only to engineer composite tissues, but also to attach the various constructs to each other in their normal anatomical relationship.

Facial symmetry is an important consideration in oral and maxillofacial reconstruction. Since most structures are paired or contiguous (e.g., the orbits, zygomas, left and right maxillae and mandible), accurate reproduction of the external form is an important aspect to preserve facial esthetics. The paucity of overlying soft tissue as camouflage contributes to the exacting

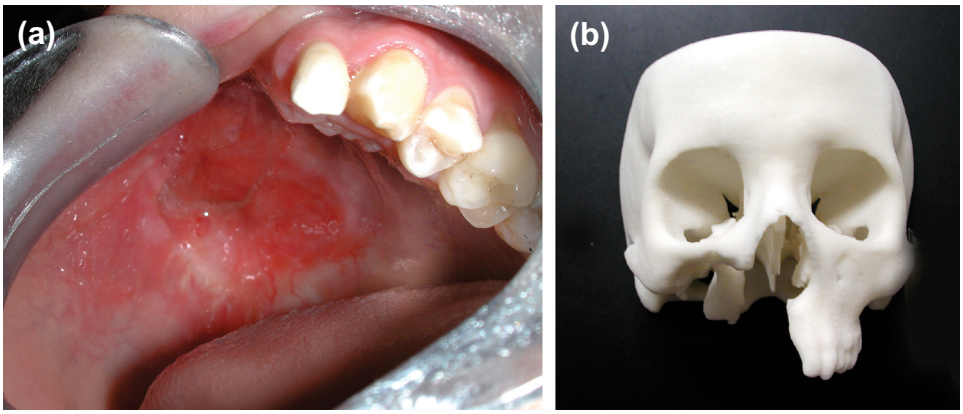

FIGURE 71.6
(a) Patient following a right maxillectomy for removal of a benign odontogenic neoplasm. Defect has filled in with fibrous tissue stimulated by grafting the site with an allogeneic dermal matrix.
(b) Stereolithographic model of the same patient demonstrating the extent of the maxillary hard tissue defect.

nature of oral and maxillofacial reconstruction, and these requirements impose upon tissue engineering methods the ability to compose and maintain accurate morphology. The advent of new three-dimensional imaging techniques with the capacity to produce stereolithographic skeletal models that mirror both the normal anatomy and defect is a valuable adjunctive tool (Fig. 71.6). These models assist in the fabrication of scaffolds to support the reconstruction of missing tissue.

CURRENT METHODS OF ORAL AND MAXILLOFACIAL RECONSTRUCTION

There are several methods used for oral and maxillofacial reconstruction, and the selection of a particular modality takes into account a number of important issues. Major factors that guide this process include the presence (or absence) of associated soft tissue, the vascularity and vascular pattern present, a multi-dimensional characterization of the defect size, the types of missing tissue, availability of tissue for transfer, and both patient and surgeon preference. Most reconstructive techniques can be categorized into four categories:

1) Soft tissue pedicled flaps
2) Non-vascularized soft and hard tissue grafts (the graft establishes in a delayed fashion, a vascular network following implantation, relying upon tissue diffusion to preserve the viability of the transplant)
3) Soft and hard tissue vascularized grafts (the graft is immediately perfused through an existing arterial-venous system)
4) Alloplastic reconstructions with prosthetic appliances

On occasion, composite techniques can be used, such as the staged reconstruction of a defect where soft tissue is first added to a defect site followed by bone at a later time.

Mandibular defects

Reconstruction of the lower jaw is indicated following removal of tissue during surgical excision of a pathological lesion or following loss of tissue due to a traumatic injury. When malignant disease is present, not only is more radical removal of tissue required, but post-operative radiation therapy produces lasting compromise to both the cellularity and vascularity of the remaining tissue. Blast effects from missile injuries can also produce significant composite injuries with loss of bone soft tissue, and diminished vascularity of the tissue bed. Two techniques are commonly employed for the reconstruction of mandibular defects. Vascularized grafts are indicated when the vascularity of the tissue bed is compromised by radiation or excessive scarring. They are also valuable when there is a requirement to replace both hard and soft tissue at the same time [6]. Hard and soft tissue defects can also be reconstructed using non-vascularized grafts, but their success relies

upon an adequately vascularized tissue bed, which can be compromised in irradiated or traumatically injured tissue, to support the survival of transplanted cells before a new supply is established [7].

As composite structures, vascularized grafts contain either soft tissue alone (muscle, subcutaneous tissue with or without epithelium) or include hard and soft tissue components (bone and soft tissue). Since the vascular supply to bone is contained within a peri-osseous cuff of muscle and fibrous tissue, it is not possible to transplant only bone. The additional tissue transferred into the site of a bony defect often produces a bulky graft. While this can be easily excised once a new vascular network is established, a second procedure performed several months after the initial transplantation is required. Another potential limitation to the use of vascularized bone grafts for the reconstruction of mandibular defects is the amount of bone available, since the dimensions of the graft are determined by the morphology of the donor site and not the size of the defect. Special techniques such as osteotomizing the graft and folding it upon itself have been described, but this can compromise the blood supply to the graft. Vascularized grafts are harvested from a limited number of anatomical sites characterized by a dominant arterial supply or venous drainage system. In addition, the *en bloc* harvesting of the graft must not compromise either the function of the donor site or the vascular and neural supply of structures distal to the harvest. Commonly used donor sites that meet these requirements include the fibula, ilium, scapula, and distal radius. Vascularized grafts transplanted to mandibular defects are anastomosed to patent vessels adjacent to the mandible, such as the facial, lingual or superior thyroid arteries and veins. This reconstructive approach is highly technique sensitive, and, while experienced microvascular surgeons achieve successful outcomes in over 90% of cases, less experienced surgeons or patients with underlying vascular disease (e.g., diabetes) enjoy less success.

Mandibular defects can also be reconstructed using non-vascularized transplantations of autologous bone from various sites. Successful bone grafts rely upon adequate cellularity and a sufficiently cellular and vascular recipient bed. When the soft tissue bed is deficient or lacks a decent blood supply, addition of well-vascularized soft tissue is achieved by the rotation of a muscle flap (with or without skin) into the mandibular defect. The pectoralis major, latissimus dorsi and deltopectoral flaps have all been described for this purpose. The bony reconstruction is delayed for a period of three to six months until the soft tissue flap has healed. In patients whose soft tissue is adequate, but avascular as a result of radiation therapy, hyperbaric oxygen therapy can improve the quality of the vascular supply in a course of treatments lasting between four to six weeks where repeated exposures to pressurized room air promote tissue angiogenesis. This process adds both time and considerable expense to the reconstructive process, but has been shown to be effective in improving the quality of the recipient bed. Once the soft tissue in a mandibular defect has been optimized with respect to quantity, cellularity and vascularity, autologous bone is transferred from a donor site and molded to fit the dimensions of the defect. The bone graft can be retained with screws fixed to a rigid bone plate, or held in position with the aid of cribs fashioned either from processed allogeneic bone or alloplastic materials. Depending upon the size of the defect, bone can be harvested from the anterior ilium (suitable for defects up to 4 cm in length), the posterior ilium (defects up to 8 cm in length), and the tibia or mandibular symphysis and rami (defects of less than 2 cm in length). Non-vascularized grafts, especially those combined with allogeneic bone are susceptible to infection especially following exposure to the intra-oral environment, with a frequency of 43% in one case series [8]. When a non-vascularized graft is colonized by organisms, infection often ensues and the graft fails to survive or integrate with the host bone. Aside from the potential for infection, non-vascularized grafts are less technique sensitive, allow 'complete' reconstruction of a defect by customizing the volume of bone harvested, and are associated with less donor site morbidity [9].

Maxillary defects

Defects of the upper jaw pose difficult reconstructive challenges from several perspectives, but must be undertaken to preserve speech, prevent the escape of food and fluids during eating, and maintain esthetics. Unlike the mandible, which is related to the oral cavity alone, the maxilla is bounded both inferiorly by the mouth and superiorly by the nasal cavity and maxillary sinuses. Even when present, the thin lining epithelium does not provide a sufficiently cellular or vascular bed to support the transplantation of sufficient quantities of non-vascularized bone, and the potential for exposure of the graft to oral and nasal environments is high. Post-operative or post-traumatic scarring reduces the tissue envelope even more, further complicating reconstructive efforts. Staged reconstructions have been described involving the initial transfer of vascularized soft tissue with a pedicled flap, such as the temporalis muscle or temporo-parietal flap, followed by the addition of bone several months later [9]. As an alternative, vascularized flaps have been used because of their ability to transfer both hard and soft tissue at the same time [10]. However, the accompanying soft tissue and vascular pedicle may not be accommodated by the smaller dimensions of a maxillary defect and this has limited their use to hemi- or total maxillary reconstructions. The simultaneous transfer of a large bulk of overlying soft tissue also results in a post-operative recovery period of several months where the flap can prevent mouth closure and compromise eating.

As a result of these challenges, prosthetic appliances have become the most commonly used method to reconstruct maxillary defects. These devices incorporate teeth and a fitted base to separate the mouth from the superior defect [11]. Excellent restoration of both esthetics and function can be achieved, but the fact that they are not permanently fixed in place and in fact, require daily removal and cleaning, reduces their acceptability by patients. In addition, adjustments are required periodically to account for remodeling of the underlying tissue bed.

The reconstruction of maxillary alveolar clefts is one exception to the use of prosthetic appliances as a primary reconstruction technique, even though they can be used very effectively to restore missing teeth in the cleft site or obturate an oral-nasal communication. When teeth are present in a cleft site, provision of bone is essential for eruption and support [12]. Alveolar grafting is therefore timed according to the presence and stage of development of adjacent teeth and is usually performed between the ages of eight and eleven years. The procedure involves the development of soft tissue flaps to isolate the mouth from the nasal cavity and placing autogenous bone between the cleft segments to restore maxillary continuity. Loss of the graft from infection, insufficient vascularity or lack of functional stimulus is not an infrequent occurrence and opportunities for tissue-engineering alternatives exist. This would be especially true if new interventions minimized the extent of surgery, since post-surgical scarring has been associated with restricted growth and development of the maxilla.

RELEVANT STRATEGIES IN ORAL AND MAXILLOFACIAL TISSUE ENGINEERING

Driven by the limited supply and inherent shortcomings of various autogenous, allogeneic, and prosthetic materials currently used for the reconstruction of oral and maxillofacial tissues, the potential for tissue-engineered biomaterials as alternatives is under serious investigation with the hope that significantly improved therapies will result.

While a diverse number of strategies are presently under development, the fundamental tenets of tissue engineering (TE) remain the same. These include consideration of the biological and mechanical properties of the scaffold material and its interactions with relevant bioactive molecules and cell populations.

Although multiple tissue types exist in the oral and maxillofacial region, TE research in this field has focused primarily on the regeneration of single tissues: the bony craniofacial skeleton,

lining epithelium, the cartilages of the temporomandibular joint, auricle, and nose, and the teeth and surrounding periodontal tissue (please refer to Chapters 74 and 76 for teeth and periodontal regenerative initiatives, respectively).

Bone applications

An ideal biodegradable TE bone construct should combine the biocompatibility and osteoinductive potential of autologous bone, with the availability and structural characteristics of allogeneic bone. Additional scaffold design considerations include porosity, pore interconnectivity, surface chemistry, and the ability to reproduce complex three-dimensional defects.

Scaffolds are responsible for a construct's initial mechanical integrity and provide surface area for cell attachment. Several biocompatible scaffold materials are currently used in oral and maxillofacial surgery, including naturally derived materials like collagen, gelatin, or hyaluronic acid, synthetic polymers like poly(lactic acid) and poly(glycolic acid) and ceramics like calcium phosphate granules, blocks and cements [13]. In addition to those materials clinically used, several materials have been tested using *in vivo* models for craniofacial bone, including alginate, chitosan, poly(propylene fumarate), oligo(poly(ethylene glycol)-co-fumarate), poly(caprolactone), and polyurethanes [13]. These materials are typically processed as porous structures or hydrogels that guide the morphology of regenerated tissue, allow for tissue ingrowth, and control the release of bioactive molecules such as growth factors or nucleic acids.

Other approaches to tissue engineering use novel biomaterials capable of implantation through minimally invasive surgery. This can be achieved via *in situ* crosslinking or polymerization via chemical reactions initiated by mixing chemicals immediately before injection, transcutaneous photopolymerization or thermogelation [14–17]. Delivery of osteogenic factors or cells has been demonstrated with these techniques; however, parameters such as cell viability must be weighed against gel stiffness with such materials.

Growth factors act as mediators of cellular growth and differentiation during tissue regeneration and play an important role in extracellular matrix synthesis. Utilized as recombinant proteins in TE strategies, growth factors require a local population of target cells capable of effecting the desired response [18]. This constituency of cells may be naturally present at the wound site or added to the scaffold at the time of fabrication [19] prior to implantation.

Factors that have been used for the regeneration of *in vivo* TE craniofacial bone include the bone morphogenetic proteins (BMPs) [19,20], transforming growth factor-beta (TGF-β) [21], fibroblast growth factors (FGFs) [22], insulin-like growth factors (IGFs) [23], and platelet-derived growth factor (PDGF) [24]. The bulk of experience concerning the use of growth factors for bone repair has involved BMPs [25] and this popularity has been extended into clinical investigations using recombinant human BMP-2 (rhBMP-2) for alveolar ridge augmentation [26], maxillary sinus floor augmentation [27], mandibular reconstruction following tumor resection [28], and distraction-assisted alveolar cleft repair [29]. A review by Herford et al., covers several cases of BMPs used in mandibular reconstruction secondary to tumor excision, trauma, and infection, highlighting the versatility of growth factor mediated bone regeneration [30]. However, the review notes a relatively small number, 37, of documented clinical cases in the literature with a significant failure rate of 13.5%. This highlights the need for further investigation of these growth factor-based technologies in the craniofacial complex.

BMP-2 has found some definitive success in other aspects of oral and maxillofacial surgery, such as dental implants and sinus floor augmentation. Jung et al. [26] examined the effect of combining recombinant human BMP-2 with a xenogeneic bone substitute in order to improve membrane-guided bone regeneration therapy of osseous defects in areas of dental implant placement. Although there was not a statistically significant difference in percentage of newly

formed bone at the rhBMP-2 treated site versus the control site at six months, a larger fraction of mature lamellar bone (76% vs. 56%) was present in the experimental sites, as well as increased graft to bone contact (57% vs. 29.5%). In addition, Boyne et al. [27] completed a Phase II randomized controlled study investigating the safety and efficacy of rhBMP-2 combined with an absorbable collagen sponge (ACS) versus bone graft for staged maxillary sinus floor augmentation. It was concluded that rhBMP-2 had a similar safety profile to bone graft with the added benefit of lacking donor site morbidity. In addition, the rhBMP-2/ACS treatment induced similar amounts of bone to the bone graft group, allowing for the placement and long-term functional loading of dental implants in approximately 75–80% of the patients treated.

The clinical use of rhBMP-2 to regenerate much larger bone defects has also been reported in the literature. Carstens et al. [29] described the use of 'distraction-assisted *in situ* osteogenesis' (DISO) to treat a severe facial cleft, in which rhBMP-2/ACS implantation was combined with distraction osteogenesis to create the patient's ramus and condyle as part of the surgical reconstruction. A similarly spectacular application of rhBMP-7 has been described by Warnke et al. [31] for the reconstruction of a 7 cm mandibular continuity defect in a patient who had received ablative tumor surgery and subsequent radiation treatment. A bone-muscle-flap prefabrication technique was utilized, in which computed tomography and computer aided design techniques were used to fabricate a custom titanium mesh cage replicating the contours of the missing mandible. Within this cage, a combination of xenogeneic bone mineral blocks coated with rhBMP-7 and autologous bone marrow were placed, prior to implantation of the entire construct within the latissimus dorsi muscle of the patient. Following seven weeks of implantation within this '*in vivo* bioreactor', the viable mandibular replacement was harvested from the patient along with part of the muscle containing a major artery and vein which were subsequently anastomosed with vessels at the recipient site using microsurgical techniques. Four weeks after this transplantation surgery, the patient was able to undertake a small amount of mastication and enjoy more solid foods. In a similar case of prevascularization, Mesimaki et al. implanted a titanium mesh containing β-TCP granules seeded with adipose derived stem cells (ASCs) cultured in rhBMP-2 into the rectus abdominus muscle of a patient who had undergone hemimaxillectomy [32]. The mesh was harvested with a vascular pedicle eight months later and anastomosed to vasculature in the face replacing the resected bone. This patient went on to receive dental implants for complete dental rehabilitation.

A significant drawback to growth factor strategies in tissue engineering is the shortage of naturally derived factors isolated from biological tissue. This deficiency has been addressed with the development of techniques to produce biologically active proteins using recombinant engineering techniques. However, the use of recombinant proteins is not without concern [25]. Compared to animal models, bone regeneration in humans does not appear to be as robust. In order to overcome this species recalcitrance, administration of factors in excess of naturally occurring concentrations appears to be necessary. The augmented administration of exogenous factors may potentially stimulate harmful biological effects, such as malignant transformation of cells, and could also prove to be too expensive when compared to alternative techniques for tissue regeneration. In an effort to mitigate cost, potential for harmful stimulation or disease transmission, autologous supplies of growth factors have been investigated, primarily through the use of platelet rich plasma (PRP). As a natural source of growth factors, PRP has been applied to craniofacial bone tissue-engineering scaffolds, but with limited success, illustrating the inherent difference in the effect of therapies across species [33,34].

Attempts to address the shortcomings of recombinant protein-based strategies have spurred investigation into the use of gene delivery for tissue engineering. By delivering the gene for the expression of a protein with specific effects on a target cell population, successfully transfected cells will elaborate the protein constitutively. This results in higher and more constant levels

of protein production [35]. However, while both viral [36,37] and non-viral [38] gene delivery vectors have been utilized for bone regeneration in cranial defect animal models, compromises must be made with each. Adenoviral constructs have commonly been used as viral vectors to transfect craniofacial tissues and have the advantage of efficiently transfecting both replicating and quiescent cells [39]. In addition, adenoviruses are easily manipulated, can be produced in high titers, and large amounts of genetic information can be inserted into them. However, concerns related to viral vectors include *in vivo* homologous recombination and the possibility of an immune response from the expression of viral antigens on the surfaces of transfected cells. These concerns have led to the development of non-viral vector agents [35].

While numerous non-viral gene delivery systems exist, a common problem is their low *in vivo* transfer efficiency [39]. Nonetheless, such systems are able to deliver much larger genes with minimal immunogenicity. One promising modality of non-viral gene delivery for craniofacial applications is the use of cationic liposomes which have been used to regenerate cranial bone defects in rabbits by delivering BMP-2 plasmid cDNA [40]. The low transfection efficiency of uncondensed, naked plasmid DNA has also been addressed by the use of the cationic macromer poly(ethylene imine), which has been used to condense BMP-4 plasmid DNA and deliver it in a sustained and localized manner from poly(lactic-co-glycolic acid) scaffolds within critical size cranial defects [38].

Gene transfection can take place directly within the defect site by releasing the delivery vector *in vivo* from the TE scaffold [36,37]. Indirect delivery methods have also been described utilizing a target cell population harvested from the patient, performing *in vitro* transfection of the cells, and then re-implanting the transfected cells into the defect along with the TE scaffold material [41]. While the direct technique may be simpler, it has a lower transfection efficiency and targets cells in a non-specific manner [25]. The indirect *ex vivo* approach on the other hand, requires additional harvesting and culturing procedures, but avoids the risks associated with placing viral vectors directly into the patient and disturbing the host genome. *Ex vivo* transfected cells are not immunologically privileged and may still express viral antigens on their surface which can lead to a host response following implantation.

As a corollary to gold standard approaches where bone grafts and flaps include the donor site cells, some TE approaches to craniofacial reconstruction employ cell-seeded scaffolds as implants. These have potential benefits for regenerating tissues in large defects or those with compromised healing capacity, such as those affected by radiation therapy [7]. The majority of cell-seeded scaffolds have investigated mesenchymal stem cells (MSCs) or ASCs. Reviews have covered some of the work in these areas looking at various stem cell sources, delivery and other parameters such as *in vitro* expansion and differentiation [7,42]. To highlight a few studies, MSCs were applied to ceramic and polymer scaffolds with and without PRP in both cranial and alveolar defects of rats and minipigs, respectively [33,34]. In both studies, the addition of MSCs enhanced bone regeneration over all other groups irrespective of PRP presence. In another study, autologous, culture expanded MSCs were utilized in combination with alginate hydrogels for the treatment of large cranial bone defects in sheep [43]. Finally, pre-differentiated ASCs were applied to rabbit cranial defects on gelatin scaffolds showing enhanced bone regeneration within the defect [44]. However, in another rabbit cranial defect study, rhBMP-2 on collagen regenerated greater amounts of bone compared to ASCs or pre-differentiated ASCs, indicating that although cell delivery for bone regeneration in the craniofacial complex shows promise, issues of cell sourcing, purification and processing need to be investigated further [45].

Aside from the biological components of tissue-engineering constructs, scaffold properties are also extremely important to the overall success of any particular strategy. A common misconception is that bone TE scaffolds for craniofacial applications do not require substantial strength since the craniofacial skeleton is not subjected to heavy loading. However, *in vivo* studies demonstrate that many craniofacial bones undergo levels of strain similar to that

experienced by the appendicular skeleton [46], substantiating the need for mechanical strength of potential bone TE scaffolds. Ideal scaffold design must therefore reconcile the need for high porosity and interconnectivity which promotes tissue in-growth and scaffold degradability, with a requirement for mechanical strength. Computational methods for designing and fabricating scaffold architectures to optimize both pore interconnectivity and load bearing characteristics have been performed [47], and a proof of concept study has illustrated the effectiveness of scaffold design in fabricating a mandibular condyle for a minipig [48].

The surface characteristics of bone tissue-engineering scaffolds also determine their ability to regenerate tissue in the wound healing environment. Surface chemistry has a significant effect on the interactions between the cell populations present in the defect and the biomaterial. Hydrophilic synthetic polymers such as oligo(poly(ethylene glycol) fumarate) (OPF) have been shown to impede bone healing in extraction sockets as compared to the hydrophobic polymer poly(propylene fumarate), based on the OPF macromer's prevention of protein adsorption and hence cell adhesion [49]. Interestingly, the resistance of OPF hydrogels to generalized cell adhesion has been used to advantage in the fabrication of biomimetic scaffolds, which are able to selectively encourage the migration of osteoblasts *in vitro* through the addition of specific binding peptides to their surfaces such as osteopontin-derived peptide [50]. In addition to surface chemistry, surface topography can impact healing. In a series of studies, porous materials in the mandibles of New Zealand White rabbits were shown to enhance the healing of the overlying mucosal surface in a composite tissue defect model [51–53]. However, contrary to traditional tissue-engineering materials with high porosity led to an increased inflammatory response not seen with lower porosity materials [51]. Two of these studies, investigated porous poly(methyl methacrylate) (PMMA), a material currently part of many Food and Drug Administration (FDA) cleared products. By creating porous PMMA and enhancing soft tissue interaction with the implant, these materials show promise for rapid translation to clinical use in a staged approach, whereby the implants serves to temporarily maintain the bone space during soft tissue regeneration and is removed for the definitive therapy.

Cartilage applications

While there has been extensive research in bone tissue engineering in the craniofacial complex, there has been less research in cartilage tissue engineering. However, cartilage tissue engineering in the craniofacial complex remains a significant challenge due to the inherent lack of remodeling in cartilage due to decreased cellularity and vascularity and due to the various cartilaginous tissue types in the craniofacial complex. As in musculoskeletal tissue engineering, articulating cartilage is part of the TMJ on the mandibular condyle, while the TMJ disk has different morphology. Additionally, there is structural cartilage in the nose and ears, which supports the skin to create openings critical for the senses.

The earliest tissue-engineering study directed at reconstruction of the TMJ disk utilized a porous collagen scaffold seeded with articular cartilage cells. After two weeks, the construct appeared similar to a disk with regards to gross morphology and cell shape [54]. Later efforts tested fibers of poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) and concluded that both materials were able to support cell attachment, matrix production, and exhibited acceptable mechanical properties after 12 weeks [55]. Another study comparing PGA, polyamide filaments, expanded polytetrafluoroethylene (ePTFE) filaments, and bone blocks [56] demonstrated cell attachment and limited collagen production, but neochondrogenesis was not observed after four and eight weeks. While PGA is an acceptable scaffold substrate, the material degrades exceedingly rapidly, leaving constructs with limited mechanical integrity after only a few weeks. As an alternative, PLA non-woven mesh has been tried and initial results show promise with retention of tensile and compressive integrity over a similar timescale [57].

However, replacement of fibrocartilage to restore the articulative surfaces or interpositional disk of the TMJs constitutes a special challenge including identifying a suitable source of healthy fibrochondrocytes. In a review by Wang et al., cell sources for TMJ tissue engineering are discussed [58]. Primary cells such as chondrocytes from articular cartilage or the TMJ disk have enhanced phenotypic characteristics for tissue engineering, but require expansion which commonly results in dedifferentiation and loss of this phenotype [59,60]. Stem cells on the other hand proliferate extensively and can be induced into a chondrocyte-like cell through growth factors and other parameters. In a study by Bailey et al., condylar chondrocytes were compared to human umbilical cord cells on PGA scaffolds and were not only able to proliferate more but also produced more extracellular matrix with components similar to that of native articulating cartilage [61]. Beyond differences in cell type, chondrocytes from various anatomical regions have shown varied ability to proliferate or synthesize extracellular matrix components *in vitro*. Specifically, costal and ankle chondrocytes have shown greater synthesis of collagen and glycosaminoglycans (GAGs) compared to chondrocytes harvested from the TMJ disk [62,63]. Other studies have investigated paracrine and cell contact signaling by coculturing differentiated cells together or with undifferentiated cells [64,65]. In these studies, coculture of fibrochondrocytes with either chondrocytes or with embryonic stem cells resulted in greater extracellular matrix production.

In addition to cell types, growth factors have been utilized to enhance the regeneration of cartilage tissue for the craniofacial complex. This potential was first observed in an experiment comparing the effects of transforming growth factor-β_1 (TGF-β_1) with prostaglandin E_2 (PGE_2) on bovine TMJ disk cells in monolayer. TGF-β_1 increased cell proliferation 2.5-fold, while PGE_2 had no significant effect [66]. The effects of PDGF, IGF and basic fibroblast growth factor (bFGF) have also been assayed using monolayer cultures of porcine TMJ disk cells. The results of these studies suggest that lower concentrations favor biosynthesis, while higher concentrations favor proliferation [67]. The most beneficial growth factors appear to be IGF-I and bFGF, both of which produce significant increases in collagen synthesis and cell proliferation [63,67]. Since native tissue is exposed to a variety of growth factors, combination strategies are likely to prove more beneficial than single factor therapy. To explore this hypothesis, IGF-I and bFGF in low concentration were combined with bFGF and TGF-β_1. This cocktail successfully demonstrated increased collagen production when applied to porcine TMJ disk cells seeded on PGA scaffolds [68]. However, while constructs exposed to growth factor combinations improved structural integrity and overall cellularity, a statistically significant improvement in biochemical or mechanical properties was not demonstrated [69]. Finally, other combination strategies employed coculture with growth factor delivery, which were shown to increase matrix production and structural integrity of a gel construct over the individual strategies alone [64,65].

The TMJ is not the only cartilaginous tissue of the craniofacial complex, and research into cartilage tissue engineering of the ear has also been investigated. *In vitro* expansion and seeding of harvested chondrocytes into degradable polymer scaffolds is capable of producing cartilage following implantation in immunocompromised and immunocompetent animal models [70], but the clinical experience has been somewhat disappointing with resorption of tissue-engineered auricular cartilage after several months [71]. However, to enhance the structural aspects of engineered cartilage and to increase the viability and maintenance of overlying tissue, Lee et al. seeded porous polyethylene implants with chondrocytes in fibrin gels [72]. These implants showed dimensional stability unlike previous auricular tissue-engineered constructs.

Although growth factors have received the most attention, positive biochemical stimulation is also likely to come from culture conditions and cellular interactions as well. An ascorbic acid concentration of 25 μg/mL has been shown to produce constructs with higher total collagen content and higher aggregate modulus relative to concentrations of 0 μg/mL or

50 μg/mL [73]. This was likely associated with improved seeding observed for the constructs cultured in 25 μg/mL of ascorbic acid. Additionally, a concentration of 100 μg/mL of glucosamine sulfate was found to enhance condylar cell proliferation and matrix production as a media supplement [63]. Metabolic supplements have also been shown to impact cell proliferation and matrix synthesis. L-glutamine, sodium pyruvate and insulin increased cell proliferation, while L-proline at high concentrations decreased matrix production [74]. Initial cell seeding is another important consideration in any tissue-engineering construct due to cell-to-cell interactions and signaling. It has been shown that PGA scaffolds seeded at saturation increased cellularity and extracellular matrix (ECM) content relative to scaffolds seeded below saturation [75].

The native TMJ disk undergoes significant compression, tension, and shear [76]. While cells proliferate and produce ECM in static culture, mechanical stimuli may be required to produce an optimal tissue-engineered construct. A variety of mechanical stimuli may be beneficial including compression, tension, hydrostatic pressure, and fluid shear stress. An extensive review of the mechanical bioreactors that have been used in engineering cartilaginous tissues has been published on the subject [77].

Three recent studies have investigated the effects of mechanical stimulation on TMJ disk constructs. A low-shear fluid environment by means of a rotating wall bioreactor created constructs with dense matrix and cell composition [78]; however, when the biochemical content of these constructs was compared to those grown in static culture, no clear benefit of the bioreactor was observed. When disk cells were exposed to hydrostatic pressure in monolayer or PGA scaffolds, constant hydrostatic pressure at 10 MPa increased collagen production compared to static culture [79]. In contrast, intermittent hydrostatic pressure from 0 to 10 MPa at 1 Hz frequency was detrimental to the constructs, producing less collagen and GAGs than unloaded controls. These results were consistent in both two- and three-dimensional culture. In another recent study, dynamic tensile strain significantly reduced interleukin-1β induced up- regulation of matrix metalloproteinase [80]. This may have implications on future tissue-engineering studies since matrix metalloproteinases (MMPs) play an important role in ECM degradation and remodeling.

Oral mucosa applications

Oral mucosa regeneration has followed many of the strategies of skin tissue engineering, in that cultured epithelial sheets showed inadequate results due to fragility, contractility and failure due to lack of underlying supporting tissue. Thus many approaches have considered the use of thick cultured grafts containing single or multiple cell types. An extensive review by Moharamzadeh et al. covers scaffold materials, cell sources and culture medium [81]. Many of the same materials have been investigated for oral mucosal engineering as in bone and cartilage such as collagen, fibrin, gelatin, poly(lactic-co-glycolic acid (PLGA), and polycaprolactone (PCL). Additionally, various cell sources have been used, primarily keratinocytes and fibroblasts either from oral or skin origins. Finally, growth factors like epidermal growth factor have been employed to promote proliferation. In one study, fibroblasts and keratinocytes were cultured on a collagen composite scaffold [82]. The culture led not only to cell specific markers for a full thickness mucosa but structural components including a basement membrane and extracellular matrix. A similar study used gingival fibroblasts and keratinocytes harvested from patients on a clinically available collagen matrix, resulting in cell markers and tissue structures similar to mucosal tissue [83]. *In vivo*, mucosal grafts of collagen precultured with gingival fibroblasts and keratinocytes have maintained their phenotype as mucosal tissue after 60 days of implantation [84]. Finally, one group has shown translation of tissue-engineered mucosal graft. Creating a mucosal graft *in vitro* using canine cells cultured on AlloDerm, the group translated this technique by first using human cells *in vitro* [85,86]. Subsequently, in a 30 patient study comparing AlloDerm alone to AlloDerm precultured with autologous cells,

the precultured grafts show enhanced wound healing with earlier vascularization and maturation of the submucosal layer at 28 days after grafting [87].

In addition to oral mucosa, composite grafts for muco-cutaneous junctions such as the lips. Peramo et al. presents a novel approach to creating these junctions, in which oral mucosal cells and skin cells are cultured on AlloDerm *in vitro* with a separation barrier [88]. This barrier is lifted and the cells are allowed to migrate into the junction space and interact. The cell construct was then lifted to air-liquid interface for maturation of the construct and characterized for morphology and immunohistochemical staining for keratin content. Biochemical markers were consistent with spatial distribution in mucocutaneous tissues, illustrating the possibility of this strategy to be employed for tissue-engineering lips.

Composite tissue applications

Success with the regeneration of single tissue types such as bone and cartilage has encouraged investigators to attempt the reconstruction of structures composed of multiple tissue types. Such anatomic structures may exist as composites of hard and soft tissues, which differ in their cellular composition and mechanical properties, yet perform as a single functional unit [89].

The temporomandibular joint condyle serves as an example of a maxillofacial composite structure consisting of articular cartilage and subchondral bone and provides an excellent opportunity for composite osteochondral tissue engineering. A study performed by Alhadlaq et al. [90] used adult bone marrow MSCs, expanded in culture and induced to differentiate into separate osteogenic and chondrogenic lineages *in vitro*. The resultant cells were then encapsulated in poly(ethylene glycol) based hydrogels and the cell-polymer solutions cross-linked in a mold which provided the correct stratified organization of the osteogenic and chondrogenic layers. Finally, the osteochondral constructs were implanted into the dorsum of immunodeficient mice for up to eight weeks. Histological and immunohistological analysis revealed both structural and immunohistochemical differences between the osteogenic and chondrogenic layers, which served as a primitive proof of concept of the potential for composite tissue-engineered constructs in the craniofacial region. In a similar study trying to regenerate tissue within an osteochondral defect, a gradient scaffold releasing BMP-2 on the osteogenic side of the scaffold and TGF-β1 on the chondrogenic side was fabricated [91]. This construct showed increased osteo- and chondrogenesis on the respective sides.

Animal models

As tissue-engineering strategies become more sophisticated, the complex interactions between multiple cell populations, growth factors, and scaffold biomaterials require testing within clinically-relevant *in vivo* healing environments. While computational models and *in vitro* testing provide proof of concept verification of a particular tissue-engineering strategy, more challenging test beds utilizing animal models are ultimately required, to allow discrimination of the healing potential of different biomaterial constructs [92].

The ideal model for testing the validity of tissue-engineered bone constructs is the *critical size defect* (CSD): namely, an intraosseous defect which will not heal by bone formation during the lifetime of the animal [93]. Several CSD models have been described in the literature including the calvarial defect [21,41] and long bone segmental defect [94,95]. These models do not accurately describe the oral and maxillofacial environment, because they do not simulate the unique masticatory stresses and cell populations seen in mandibular or maxillary wound healing. Mandibular defects have been described in rats, but they have the disadvantages of poor surgical access and a tendency for implanted materials to fall into the fascial spaces [93]. Conversely, larger animals such as dogs [96], minipigs [97], goats [98], and non-human primates [99] offer the advantages of easy surgical access and the ability to create large defects, but are expensive to maintain. As such, a critical size mandibular defect in a rabbit was developed. This 10 mm, cylindrical, bicortical defect in the New Zealand White rabbit has been shown to

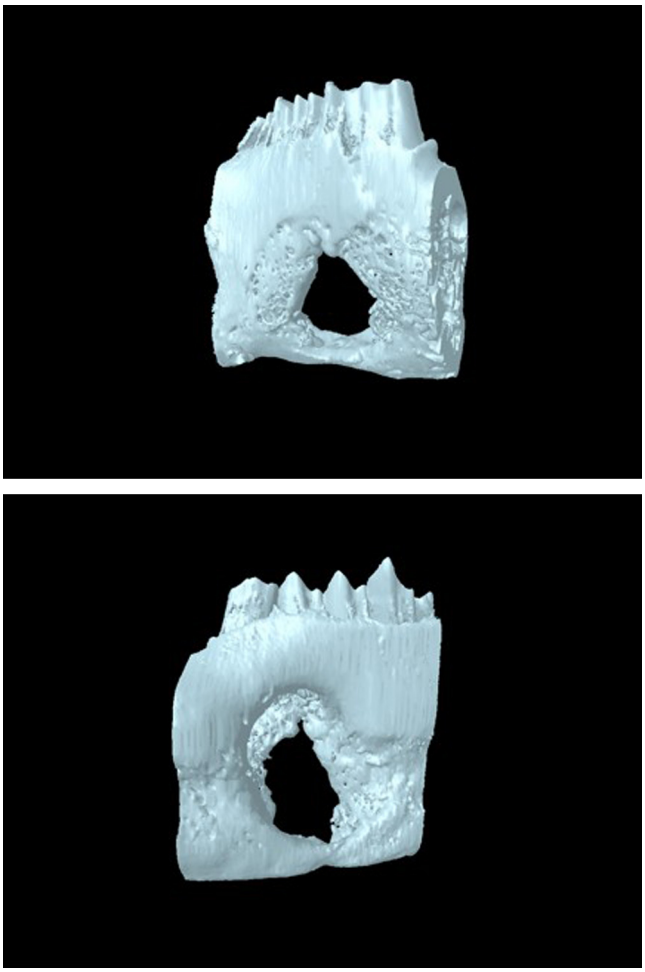

FIGURE 71.7
Micro-CT generated representative three-dimensional reconstructions. Note the buccal aspect of the specimen is shown on the top, with the lingual aspect on the bottom. The critical size defect at 16 weeks shows a 'through-and-though' defect and a lack of bony bridging across its center. *Reprinted from Young S, Bashoura AG, Borden T, Baggett LS, Jansen JA, Wong M, et al. Development and characterization of a rabbit alveolar bone non-healing defect model. J Biomed Mater Res A. 2008 Jul;86(1):182–94 with permission.*

be non-healing after 16 weeks (Fig. 71.7) [100]. Additionally, this model was expanded into a composite tissue defect model, whereby an overlying mucosal defect is created (Fig. 71.8) [51–53]. Composite tissue defect models represent an important direction for evaluation of tissue-engineered constructs. Defects resulting from trauma and resection of pathology frequently involve multiple tissue types and the combination of successful therapies for each individual may not suffice when both tissues need regeneration.

THE FUTURE OF ORAL AND MAXILLOFACIAL TISSUE ENGINEERING

While significant progress has been made towards our ability to fabricate tissue in the laboratory for the reconstruction of defects in the oral and maxillofacial skeleton, considerable challenges remain before these techniques can be embraced as a feasible clinical modality. Some of the issues pertain to tissue engineering itself, such as the ability to identify and harvest a suitable population of cells capable of fulfilling the functions of the desired tissue, supporting cellular differentiation and reproduction through physiological levels of growth and attachment factors, promotion of vasculogenesis, and the development of matrices that fulfill the physical requirements of a skeletal site. Since many of the structures in the oral and maxillofacial region are composed of multiple tissue types, the ability to engineer composite structures is also important, as is consideration of the unique environment that tissue-engineered constructs are exposed to. Even as these demands are met, other factors must be considered before TE tissue can be adopted as part of a reconstructive surgeon's armamentarium. Existing reconstructive techniques were originally based on macroscopic concerns for

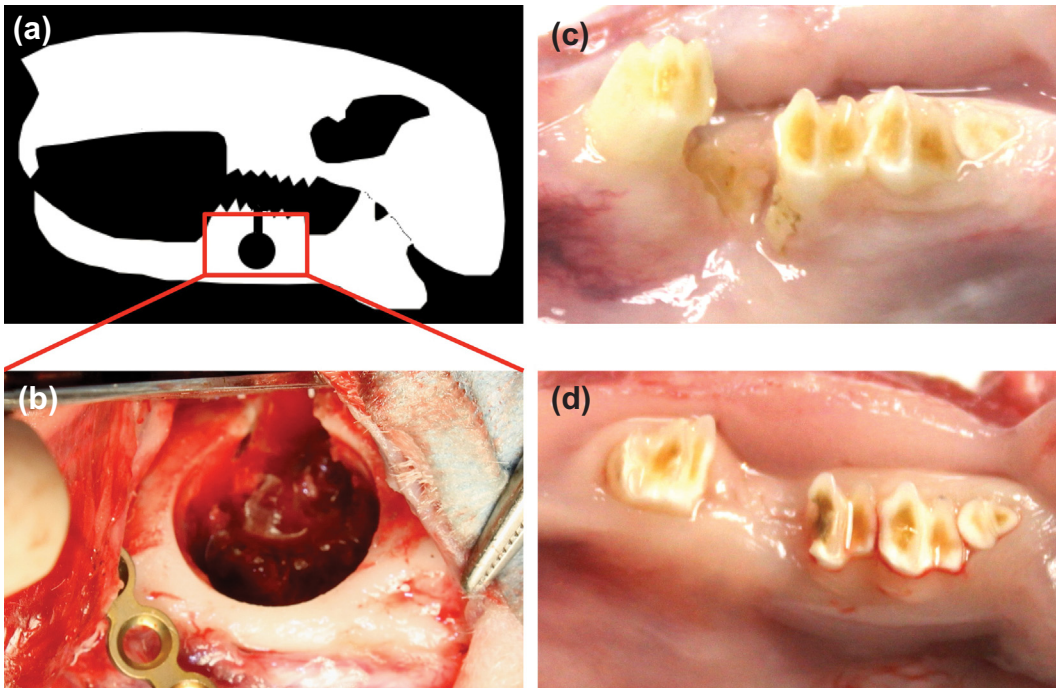

FIGURE 71.8
Schematic (a) and intraoperative photo (b) illustrating the composite tissue defect model of the rabbit mandible with a critical size bone defect and overlying mucosal defect. A healed (c) and non-healed (d) mucosal defect are seen after 12 weeks of biomaterial implantation. *Reprinted from Spicer PP, Kretlow JD, Henslee AM, Shi M, Young S, Demian N, et al.* In situ *formation of porous space maintainers in a composite tissue defect. J Biomed Mater Res A. 2012 Apr 15;100(4):827–33 with permission.*

restoring the shape and size of a missing structure. Since TE is essentially based upon cells, a microscopic appreciation of the function of a particular tissue type must first be derived. At this time, characterization of the function and pathological degradation of many of the structures in the oral and maxillofacial region is unfortunately deficient. In other words, before the replacement for a structure can be appropriately engineered, a better understanding of the function and local environment must first be achieved. This is particularly true of defects produced by on-going pathological processes where correction of the condition must precede replacement with yet another biological substrate. The future of oral and maxillofacial TE therefore lies in the hands of close collaborations between engineers and clinicians together.

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CHAPTER 72

Periodontal Tissue Engineering

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INTRODUCTION

Periodontitis is an infectious disease caused by pathogenic microorganisms in the subgingival biofilm, in combination with other risk factors, such as aging and cigarette smoking. Untreated periodontitis results in irreversible destruction of the tooth-supporting tissues, including periodontal ligament (PDL), alveolar bone and cementum, and may lead to early tooth loss [1]. It has also been linked to many systemic disorders, such as diabetes, rheumatoid arthritis and cardiovascular complications, and has been associated with adverse pregnancy outcomes [2]. Consequently, periodontal disease significantly impacts a patient's quality of life and financially impacts both patients and the public healthcare system. Blocking the progression of periodontitis has been achieved by mechanically removing bacterial biofilm with scaling and root planning procedures. Although the elimination of periodontal pathogens may improve clinical attachment levels of a tooth, reconstruction of the support and function of the affected tooth attachment apparatus represents an important therapeutic endpoint that has been an ongoing challenge in clinical periodontitis treatment [3]. Conventional treatment is generally unable to promote the regeneration of the damaged periodontal structures. Although numerous periodontal regenerative treatments have been established, such as techniques that use bone grafting, guided tissue/bone regeneration (GTR/GBR), enamel matrix derivative (EMD) and platelet-rich plasma (PRP), a positive outcome is difficult to predict, and in principle, these existing therapeutic paradigms fall short of reaching a consistent and complete regeneration of the periodontium, particularly in cases where the disease has caused large defects in the periodontal tissue [4].

The recognition that periodontal tissues possess the capacity, albeit in a very limited manner, to repair/regenerate has led to substantial efforts focused on understanding the biological basis for this activity and employing the accumulated knowledge to devise tissue-engineering technology that predictably promotes functional periodontal regeneration, i.e., the restoration of all components of the periodontium and the re-establishment of their appropriate connections [5]. Tissue engineering, which is a term often used interchangeably with regenerative medicine, merges the fields of life sciences and engineering biotechnologies and aims to orchestrate body regeneration by specifically controlling the biological environment or developing biological substitutes to restore, maintain or improve tissue function. The 'engineering' of irreversibly damaged tissues is progressing toward reality by improvements in our understanding of wound repair and recent advancements in mysenchymal stem cell

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(MSC) biology and biomaterials science. Such advancements help to target molecules and pathways in an effort to restore the regenerative capacity of tissue, and the periodontium could be considered a prime candidate for such endeavors [6]. Applied to periodontal therapy, tissue engineering offers the opportunity to improve periodontal regeneration in a more predictable and qualitative, but possibly less invasive manner than currently available regenerative procedures [3,5].

It is an inherent belief in the current concept of periodontal tissue engineering that naturally occurring tissue regeneration can be reproduced *in vitro*. Clearly, a tissue-engineering approach to periodontal regeneration will need to utilize the regenerative capacity of stem cells residing within the periodontium (or other related tissues), and would involve the isolation of such cells and their subsequent proliferation/differentiation within a three-dimensional (3D) framework followed by implantation into the defect [6]. The *in vitro* design of such therapeutics primarily considers the delivery of appropriate cells in sufficient quantity, the sustained release of chemical and mechanical signals that are often popularly termed growth factors (GFs) in a near-physiological level, and the engineering of extracellular matrix (ECM)-mimicking biomaterials with such desirable properties as macrostructures and microstructures, biodegradability and biocompatibility [5]. The scaffold not only acts as a delivery vehicle for the cells to the site of regeneration, but it also plays an essential role in cell attachment, space retention, determination of morphological features and recruitment of oxygen and nutrients [7]. After *in vivo* transplantation, blood supply, mechanical loading and pathogen control will become the critical factors which influence the predictability of final outcome. New vascular networks promoted by angiogenic signals provide the nutritional base for periodontal tissue growth and homeostasis, while appropriate mechanical loading would be essential for the development of highly organized, functional PDL fibers [8]. Finally, because of the microbial load at the periodontal lesion, strategies to control infection and host response are required to optimize periodontal regeneration [3].

We must be aware that periodontal tissue engineering is now still in its early stage, with no current paradigm that takes all the aforementioned factors into proper consideration. If periodontal regeneration relies on the *in vitro* conditioning of a cell-seeded construct for implantation, then the transplants should be designed to incorporate biochemical and biophysical signals that recreate important *in vivo* stimuli, albeit in simplified form, to create a tissue functionally equivalent to native periodontal tissue in terms of composition, biomechanical properties and physiological performance [9,10]. Although *in vitro* periodontal tissue engineering has greatly increased our understanding of cellular behavior and cell-material interactions, this methodology is often unable to recreate tissue with the hierarchical organization and vascularization found within the native periodontium. Thus, most, if not all, efforts in this field are focusing on *in vivo* tissue-engineering strategies, in which the traditional triad (cells, regulatory signals and scaffolds), or a combination thereof, is directly implanted at the damaged tissue site, or within ectopic sites capable of supporting neotissue formation, instead of attempting to recreate tissue replacements/constructs *ex vivo* [6]. In the broader scope of tissue engineering, cell-free methods can also be considered; nonetheless, it is widely accepted that most of the developing and established strategies rely on the use of cells of different origin. *In vivo* tissue engineering via cell transplantation or biomaterials design may offer a preferential route for regeneration of periodontal tissue with distinct advantages over *in vitro* methods that are based on the specific location of endogenous cultivation, recruitment of autologous cells and patient-specific regenerated tissues [11]. In the future, these exciting developments are likely to help reconcile the clinical and commercial pressures on tissue engineering. However, even if *in vivo* tissue-engineering strategies are to be used, many issues still that need to be addressed before such strategies become routine in periodontal practice. In particular, the events following cell transplantation are poorly understood. Furthermore, the efficacy and safety of prospective tissue-engineering-based therapies have not been fully evaluated, and the risks have already been underscored by several clinicians and researchers [12].

Approaches to the regeneration of periodontal tissue have made some progress recently, and these paradigms provides useful experimental models for the evaluation of future strategies for treating other connective tissue diseases. This chapter explores the potential cell types, signaling molecules, biomaterials and various technologies based on the concept of tissue engineering for periodontal regeneration. In addition, possible new directions that need to be exploited to make periodontal tissue engineering a clinical success are highlighted.

STEM CELLS FOR PERIODONTAL BIOENGINEERING

Stem cells are the foundational cells for every organ and tissue, and they have a remarkable self-renewal ability, in that they can divide to replenish any other cell type, thus functioning as a part of a repaired tissue. The healthy PDL harbors stem cell niches throughout adulthood and exhibits a limited regenerative capacity. However, in a diseased periodontal environment, the lack of robust stem cells renders the use of *ex vivo* expanded/manipulated stem cells a necessity [5,13]. In this context, periodontal cell therapy involves the treatment of periodontitis by transferring new cells into a defect site with the goal of improving the regeneration process [14]. The transplanted cells may participate in the repair of damaged periodontium, serving as building blocks by differentiating into multiple cell types, or regulate repair via secretion of growth or cellular signals, instead of, or in addition to, directly participating in regeneration of the tissue [15]. Both intraoral and extraoral MSCs have been evaluated for the treatment of periodontal disease and for the regeneration of damaged periodontium [3,12].

Intraoral MSCs

Of note, several cell populations with stem cell properties have now been harvested from different tissues in and around a tooth and subjected to enrichment and expansion techniques (Fig. 72.1). These populations generally include dental pulp stem cells (DPSCs) [16], stem cells from exfoliated deciduous teeth (SHED) [17], PDL stem cells (PDLSCs) [18], stem cells from apical papilla (SCAP) [19,20], dental follicle stem cells (DFSCs) [21,22] and MSCs

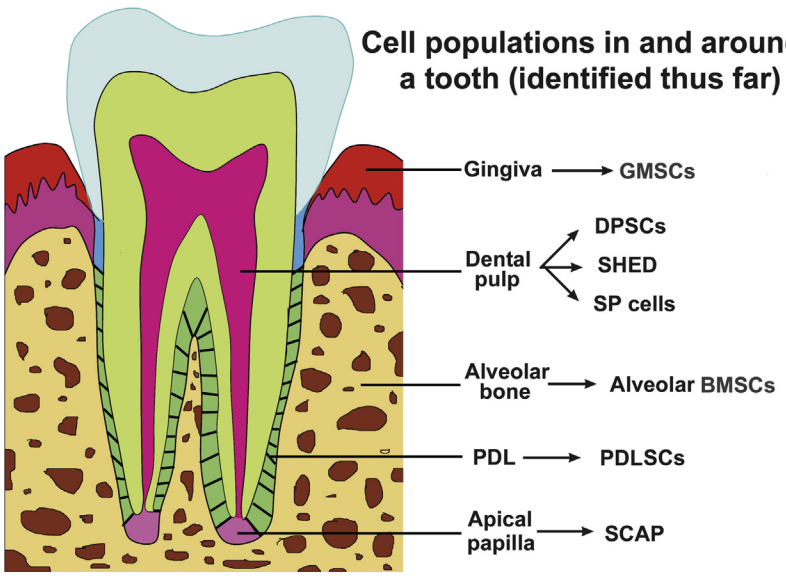

FIGURE 72.1

Selected stem/progenitor cells identified thus far in and around a tooth. PDLSCs: periodontal ligament stem cells [18]; DPSCs: dental pulp stem cells from pulp of permanent teeth [16]; SP cells: side population cells isolated from dental pulp tissue [61]; SHED: stem cells from human exfoliated deciduous teeth [17]; SCAP: stem/progenitor cells from apical papilla [19,20]; GMSCs: mesenchymal stem cells from gingiva [23,24,52]; alveolar BMSCs: bone marrow stromal cells isolated from alveolar bone marrow [25,26].

from gingival tissues [23,24] and alveolar bone [25,26]. These stem/progenitor cells can be obtained with ease from medical waste, such as teeth extracted for orthodontic, impaction or irreversible periodontitis reasons, exfoliated deciduous teeth or even gingiva that was removed for hyperplastic or aesthetic reasons. The ease of access makes them an important source of autologous stem cells for use in the regeneration of PDL and bone lost in periodontal disease. These stem cell populations have an advantage over other stem cells, such as bone marrow-derived MSCs (BMMSCs), because they can be obtained from patients in the dental clinic rather than requiring an invasive bone marrow aspiration procedure at a secondary clinic [7]. Because of their particular characteristics of proliferation, differentiation and plasticity, dental stem cells have also enabled significant progress towards clinical orthopedics and oral maxillofacial bone reconstruction [27–29]. Thus, while not largely exploited, it can be seen that the humble tooth has an important role to play in the development of future regenerative therapies [30].

Periodontal tissue-derived stem cells

When considering application to periodontal regeneration, stem cells derived from the tissues surrounding the teeth, i.e., the periodontium should be considered the first choice [12]. The PDL is a fibrous connective tissue that contains specialized cells located between the bone-like cementum and the inner wall of the alveolar bone socket. Just as PDL is essential for osteogenesis and cementogenesis during development and remodeling, cells derived from this tissue are supportive for the healing response to injury [31]. Early observations indicated that the PDL has a regenerative capacity and that a population of multipotent progenitor cells may exist within this tissue [32]. Transplantation of PDL-derived cells has shown the potential to regenerate bone and periodontal attachment apparatus *in vivo* [33–35]. PDLSCs were first isolated in 2004 and have been shown to give rise to adherent clonogenic clusters resembling fibroblasts [18]. They are positive for the stem cell markers STRO-1 and CD146, and they are capable of developing into adipocytes, osteoblast-like cells and cementoblast-like cells *in vitro* as well as producing cementum-like and PDL-like tissues *in vivo* [18,36]. In recent years, sources of stem cells from human [37], swine [38,39] and canine PDL tissues [40,41] are reported to be the most potent for periodontal tissue regeneration. Furthermore, *in vitro* studies have also found that PDLSCs were able to differentiate into mesodermal (i.e., adipocytes, osteoblasts, chondrocytes), ectodermal (i.e., neurons) and endodermal (i.e., hepatocytes) lineages [42]. Meanwhile, PDLSCs have also shown the capacity to differentiate into vascular cells, forming blood vessel-like structures in rats [43]. These findings suggest that the PDL constitutes an important stem cell source, not only for the regeneration of periodontal tissues, but also for the regeneration of other tissues and organs, making them highly amenable for use in periodontal regeneration. The PDL is under constant strain from the forces of mastication, and thus PDLSCs are likely to play an endogenous role in maintaining PDL cell numbers. This might explain why they are better than other dental stem cell populations at regenerating the complex structures of the periodontium [36,40,41]. Despite the considerable promise that the PDL holds for periodontal regenerative medicine, a need still exists for further characterization and validation of PDLSCs for clinical use [44].

The alveolar bone is a main part of the tooth-supporting apparatus, functioning as an anchorage of the tooth root to the alveoli and resorbing the forces generated by the function of mastication. Progenitor cells responsible for alveolar bone formation lay in the periosteal region, the PDL or around the blood vessels. Alveolar bone marrow is considered to be a useful and easily accessible source of bone-related progenitor cells; alveolar MSCs have an osteogenic potential that is similar to, or even more responsive than, cells derived from the iliac crest, although alveolar MSCs show poor chondrogenic or adipogenic potential [25]. Recently, putative stem cells from human PDL on the alveolar bone surface were isolated, characterized, and provisionally named as 'alveolar PDLSCs'. Interestingly, these 'alveolar PDLSCs' exhibited a strong proliferative capability, expressed high percentages of MSC markers, and were found

to have a higher osteogenic/adipogenic differentiation potential than those from the PDL of the root surface [26]. However, further studies need to be carried out to demonstrate that the reported 'alveolar PDLSCs' are PDL-derived.

The periosteum is also considered to be a suitable cell source for periodontal regeneration [45]. Mizuno et al. [46] grafted autologous cultured cell membrane that was derived from periosteum peeled from the mandibular body into a mechanically created class III furcation defect in dogs. Proper regeneration of multiple periodontal tissues was observed three months after the surgery. In addition, a treatment for human periodontal disease using periosteum-derived cells has already been conducted. It was shown that cultured periosteum combined with PRP and porous hydroxyapatite (HA) granules induced significantly more favorable clinical improvement in infrabony periodontal defects, when compared with the control group (PRP with HA) [47].

The cementum is a specialized calcified substance with ultrastructural similarity to bone that covers the root surface. It is excreted by cementoblast cells within the root of the tooth and is thickest at the root apex. These cementoblasts develop from undifferentiated mesenchymal cells in the connective tissue of the dental follicle at the interface between the dentinal tissue and the PDL, and they contribute to periodontal repair/regeneration after damage. The organic ECM of cementum contains proteins that selectively enhance the attachment and proliferation of cell populations residing within the PDL space [48]. In 1998, human cementum-derived cells were isolated, cloned and characterized *in vitro* and *in vivo*. These cells are capable of differentiating and forming mineralized tissue that exhibits several features identical to cementum when transplanted into immunodeficient mice, although different from the mineralized matrix produced by human BMMSCs [49]. In a proof-of-concept investigation, cementoblasts showed a marked ability to induce mineralization in periodontal wounds in a rodent periodontal fenestration model when delivered to the defects via biodegradable polymer sponges. However, the implanted dental follicle cells seemed to inhibit periodontal healing [50]. These results confirm the selective behaviors of different cell types *in vivo* and support the role of cementoblasts as a tool to better understand periodontal regeneration and cementogenesis.

The human gingiva is the oral mucosal tissue that surrounds the teeth and forms a mucoperiosteum covering the alveolar bone. Since the epithelial layer shows a unique fetal-like scarless healing process after wounding and has the capacity for continuous renewal, it is anticipated that this tissue could also be a source of stem cells. Recently, cells with MSC properties (GMSCs) have been isolated from gingival tissues and characterized [24,51,52]. GMSCs are easy to isolate and homogeneous, and they proliferate more quickly than BMMSCs in the absence of GFs. GMSCs display a stable morphology and do not lose MSC characteristics at higher passages. In addition, they maintain normal karyotype and telomerase activity in long-term cultures and are not tumorigenic, suggesting that they are superior to BMMSCs for cell therapy in regenerative medicine [23]. Very recently, the *in vivo* efficacy of utilizing GMSCs in bone regeneration was demonstrated in a mandibular defect as well as a critical-sized calvarial defect model in rats [53]. Furthermore, these cells gave rise to high-quality iPS cells, which suggests that gingival tissue is also a promising cell source for investigating the basis of cellular reprogramming and pluripotency for future clinical applications [54].

Stem cells from apical papilla

The apical papilla tissue is only present during root development before the tooth erupts into the oral cavity [55]. A unique population of dental stem cells known as SCAP is located at the tips of growing tooth roots. These cells form adherent clonogenic clusters and, similar to other MSC populations, they are capable of differentiating into adipocytes and odontoblasts/osteoblasts *in vitro* [20]. By co-transplanting SCAP cells, to form a root, and PDLSC, to form a PDL, into tooth sockets of mini pigs, dentine and PDL was formed [19]. These findings

provide support for the use of combined MSC populations in root and periodontal regeneration. Most human tissues from early in their development are not clinically available for stem cell isolation; however, because roots develop postnatally, the root apical papilla is accessible in dental clinical practice from extracted wisdom teeth. Thus, a very active source of stem cells with embryonic-like properties can be readily obtained. Further experiments on the properties of these cells obtained from human teeth following expansion in culture are needed.

Dental follicle stem cells

The dental follicle is a loose mesenchymal tissue surrounding the developing tooth germ which participates in the formation of periodontal progenitor cells. It is believed that this tissue contains stem cells and lineage-committed progenitor cells or precursor cells for cementoblasts, PDL cells and osteoblasts [56]. The presence of stem cells in the dental follicle (DFSCs) of human third molar teeth was demonstrated in 2005 [22], suggesting an alternative population of dental stem cells capable of differentiating into cells of the periodontium [57]. These fibroblast-like, colony-forming and plastic-adherent cells express stem cell markers (STRO-1 and nestin) and can be maintained in culture for at least 15 passages. STRO-1-positive dental follicular progenitors have been shown to differentiate into cementoblasts *in vitro* [57] and to form cementum *in vivo* [21]. Current evidence has shown that immortalized DFSCs were able to recreate PDL-like tissues that expressed periostin, Scx and type XII collagen, as well as the fibrillar assembly of type I collagen when transplanted into immunodeficient mice [58], while EMD [59] or dentin non-collagenous proteins (dNCPs) extracted from dentin [60] could stimulate DFSCs to differentiate into cementoblast lineages. Similar to SCAP, DFSCs represent cells from a developing tissue and might therefore exhibit a greater plasticity than other dental stem cells. However, further research is needed to explore the properties and potential uses of both SCAP and DFSCs.

Stem cells from dental pulp or exfoliated deciduous teeth (SHED)

The first human dental stem cells were isolated from dental pulp tissue of extracted third molar teeth (DPSCs) and were characterized relative to BMMSCs [16]. DPSCs were found to be highly proliferative, clonogenic cells capable of differentiating into odontoblast-like cells and forming dentin/pulp-like complex when implanted into immunocompromised mice. Subsequently, human MSCs were isolated from exfoliated deciduous teeth and were observed to induce bone and dentin formation *in vivo*. Furthermore, compared with DPSCs or BMMSCs, SHED grow and proliferate more rapidly and have a higher number of population doublings [17]. Further data demonstrate that pulp side population (SP) cells maintain self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis and neurogenesis [61]. Although there is no evidence that supports the use of DPSCs to regenerate PDL, they showed the highest osteogenic potential among BMMSCs, PDLSCs and periosteal cells, indicating that they may form a useful cell source for bone reconstruction around teeth and dental implants [62,63]. In 2009, the clinical trial of alveolar bone reconstruction using DPSCs was successfully carried out, and the data suggest that a DPSC/collagen sponge biocomplex can completely restore human mandible bone defects [27]. Taken together, these results suggest a potential role for DPSCs in bone and dentin regeneration; however, significant further work is required in this area.

Extraoral MSCs

Extraoral MSCs, i.e., non-dental stem cells, such as BMMSCs and adipose-derived stem cells (ASCs), have also been investigated as alternative cell sources for periodontal regeneration and bioengineering [64–67].

Bone marrow-derived MSCs

BMMSCs are the most widely investigated MSCs in tissue engineering and regenerative medicine because they are easily accessible, their isolation is straightforward, they can be biopreserved with minimal loss of potency, and they have shown no adverse reactions to allogenic versus autologous MSCs transplants. These cells are clonogenic and have demonstrated the ability to form bone and cartilage *in vivo*. Because of their differentiation potential, BMMSCs have been used in various phases of clinical application, particularly in orthopedics [68]. Indeed, BMMSCs can efficiently regenerate not only bone tissue, but also periodontal tissue in various animal models [69]. Recent data have shown that bone marrow progenitor cells can communicate with dental tissues and become tissue-specific mesenchymal progenitor cells to maintain tissue homeostasis [70]. Animal experiments have demonstrated that autotransplantation of BMMSCs induced periodontal regeneration in experimental class III furcation defects, and that the defects were regenerated with cementum, PDL and alveolar bone in the MSC-atelocollagen groups. Less periodontal tissue regeneration was observed in the control group than in the MSC-atelocollagen groups [64]. In addition, transplanted BMMSCs labeled with green fluorescent protein (GFP) were detectable by immunohistochemical analysis four weeks after transplantation. The periodontal defects were almost regenerated with periodontal tissue. Cementoblasts, osteoblasts, osteocytes and fibroblasts of the regenerated periodontal tissue were positive for GFP, which suggested that transplanted BMMSCs could survive and differentiate into periodontal tissue cells, resulting in an enhancement of periodontal tissue regeneration [71]. Moreover, clinical evidence indicated that periodontal tissue could be successfully regenerated using autologous BMMSCs combined with PRP [28]. Significantly, when evaluated in periodontal defects in dogs, cryopreserved MSCs showed no altered regenerative capacity compared with freshly isolated MSCs when applied to periodontal regeneration [72]. Notably, a few clinical cases showed the successful treatment of challenging periodontal defects with a novel cellular allograft that contains native MSCs and osteoprogenitor cells [73]. These data suggest that bone marrow is an excellent candidate source of MSCs for periodontal regeneration. Nonetheless, the established shortcomings in relation to the isolation of BMMSCs, i.e., pain and morbidity associated with bone marrow harvest, complicated procedures, as well as low harvested cell number, all mean that the use of this cell population in periodontal regeneration in the clinical setting will be a significant challenge in the future. Consequently, researchers have shifted their efforts on assessing alternative sources of MSCs for periodontal therapies [7].

Adipose-derived stem cells

Adipose tissue is an abundant source of MSCs. ASCs can be readily harvested in large quantities and can be obtained with relative ease with low donor site morbidity. ASCs have adipogenic, myogenic, osteogenic and chondrogenic potential, and they are very angiogenic in nature. These multipotent cells can be utilized in regenerating diseased or damaged tissue throughout the body, including the periodontium. More importantly, the safety and efficacy of ASCs have been demonstrated in numerous preclinical studies, supporting the use of these cells in curing human disease. Although ASCs originate from mesodermal lineages, recent preclinical studies have demonstrated that the use of ASCs in regenerative medicine is not limited to mesodermal tissue, but can also extend to both exodermal and endodermal tissues and organs [66]. Recently, ASCs were investigated for periodontal regeneration. For example, eight weeks after the transplantation of ASCs mixed with PRP into rat periodontal defects, new PDL-like and alveolar bone-like structures were formed, which implies that ASCs could promote periodontal regeneration *in situ* [67]. In a canine model of periodontal tissue regeneration, ASCs were transplanted with PRP into dental root bifurcation defects. ASC and PRP transplantation were found to prevent epithelial invasion into the defect area after four weeks, while newly induced bone was observed at the site of implantation after eight weeks [66]. Recently, freshly isolated, uncultured stromal vascular fraction of adipose tissue has been suggested as

a source of autologous progenitor/stem cells [74], offering a practical, promising candidate for future periodontal tissue engineering or cell-based periodontal therapy.

Selection of cell types

Although both dental and non-dental MSCs for periodontal regeneration are promising (Table 72.1), we must be aware that mesenchymal cell populations from different tissues display distinct biological properties [12]. Even if they carry common genetic markers, they are likely to be conditioned by their specific microenvironment and to be committed towards a specific differentiation pathway. Dental stem cells are isolated from specialized tissue with potent capacities to differentiate into odontogenic cells. However, they also have the ability to give rise to other cell lineages similar to, but different in potency from, that of BMMSCs [55]. When comparing different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model, PDLSCs resulted in more newly formed cementum and well-oriented PDL fibers than other cells (BMMSCs and alveolar periosteal cells). In addition, nerve filament was observed in the regenerated PDL tissue only in the periodontal ligament cell (PDLC) group. The amount of alveolar bone regeneration was highest in the PDLC group, although it did not reach statistical significance among the groups. These results indicate that PDLSCs combined with a β-tricalcium phosphate (β-TCP)/collagen scaffold serve as a promising tool for periodontal regeneration [41]. In another dog model with advanced periodontitis, PDLSCs were also demonstrated to be the most favorable candidate for cell therapy when compared to DPSCs and DFSCs. Autologous PDLSCs showed the best regenerative capacity of PDL, alveolar bone and cementum as well as peripheral nerve and blood vessel [40]. Except for PDLSCs, it remains to be determined which source of MSCs will be most suitable for regenerative periodontal therapy. The use of dental follicular cells is likely to be limited by the availability of tooth germs, and non-dental stem cells may require the transfer of genes to enhance their odontogenic potential. Although the best stem cell source is yet to be identified, the prospect of using these cells for regeneration represents a step forward in the development of more predictable biologically based therapies for the periodontium [69].

SIGNALING MOLECULES

GFs mediate crosstalk between cells and their microenvironment via their autocrine and paracrine effects [75]. They initiate their action by binding to specific receptors on the surface of target cells and their chemical identity, concentration, duration and context contain information that dictates cell fate for safe and effective regeneration of functional tissue. Hence, the importance of GFs in tissue engineering is unsurprising, considering their importance for tissue regeneration [76]. Accordingly, stimulation of osteogenesis, cementogenesis and connective tissue formation via presentation of various signaling molecules is necessary for periodontal tissue engineering. A number of recombinant human GFs, biological agents (e.g., EMD), and a patient's own biologically active products (e.g., PRP) are now used clinically, or are currently under extensive investigation and some of them have been examined by controlled clinical studies or randomized controlled clinical trials.

Types of signals

BONE MORPHOGENETIC PROTEINS

The bone healing process initiated by a single molecular species of bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-beta superfamily such as BMP-2 or BMP-7, sets in motion a cascade of cellular events resulting in differentiation of progenitor cells into phenotypes involved in periodontal regeneration [77]. Current data suggest that BMP-2, BMP-4, BMP-7 (i.e., osteogenic protein-1, OP-1) and BMP-12 are potent inducers of bone formation during mandibular reconstruction, with OP-1 (occasionally BMP-2) inducing substantial cementogenesis. In this context, BMP-12 supports the

TABLE 72.1 Potential cell types for periodontal tissue engineering

Cell type	Cell association	Therapeutic application	References
PDLSCs[a]	Xenogenous	Rat / ectopic model	Seo et al. [18]
	Autologous	Swine / periodontitis model	Liu et al. [38]
	Allogenous	Swine / periodontitis model	Ding et al. [39]
	Autologous	Dog / 1-wall defect model	Tsumanuma et al. [41]
	Autologous	Dog / Apical involvement defect	Park et al. [40]
	Autologous	Human / periodontitis	Feng et al. [37]
SCAP[b] & PDLSCs	Xenogenous / Autologous	Rat / ectopic model; Minipig / *in situ* (lower incisor extraction socket)	Sonoyama et al. [19]
DFSCs[c]	Allogenous	Rat / ectopic model	Yokoi et al. [58]
	Autologous	Rat / ectopic model	Wu et al. [60]
DPSCs[d]	Autologous	Dog / periodontitis model	Park et al., 2011 [40]
BMMSCs[e]	Autologous	Dog / Class III defects	Kawaguchi et al., 2004; [64]
	Not defined	Dog / Class III defects	Hasegawa et al. [71]
	Autologous	Dog / Periodontal fenestration defects	Li et al. [72]
	Autologous	Dog / Class III defects	Wei et al. [174]
	Allogenous	Rat / periodontal defect	Yang et al. [86]
	Autologous	Dog / 1-wall defect model	Tsumanuma et al. [41]
	Autologous	Dog / Apical involvement defect	Park et al. [40]
	Allogenous	Patient / periodontal defect	McAllister [73]
ASCs[f]	Autologous	Rat / periodontal palatal defects	Tobita et al. [64]

[a] *periodontal ligament stem cells;*
[b] *stem cells from apical papilla;*
[c] *stem cells in dental follicle;*
[d] *dental pulp stem cells;*
[e] *bone marrow mesenchymal stem cells;*
[f] *adipose-derived stem cells.*

reestablishment of the PDL, including regeneration of cementum and functionally oriented fibers, and prevents ankylosis and root resorption following replantation of teeth (reviewed in [77, 78]). There is mounting evidence that recombinant human BMPs stimulate periodontal regeneration if applied with suitable carriers [79,80]. However, the optimal effects are modulated by a range of factors that need careful evaluation in clinical studies. Furthermore, we must be aware that severe side effects, such as ankylosis and root resorption, have also been reported [81], although these were not always in accordance with outcomes obtained by other groups [82]. Considering the results of studies indicating that the application of recombinant human BMP-2 around a periodontal defect induces bone formation but not cementum formation [83], the combined use of BMP-2 and BMP-7 might be suitable for regenerating two hard tissues: bone and cementum [84]. In addition, BMP-2 has also been used in combination with other GFs, such as insulin-like growth factor (IGF)-1, to synergistically enhance differentiation of PDLCs [85,86] or recombinant human β-nerve growth factor [87] to improve the quality and quantity of regenerated bone. Of note, the absorbable collagen sponge (ACS) carrier containing recombinant human BMP-2 is now commercially available as InFuse® Bone Graft (Medtronic, Minneapolis, MN, USA) and has been applied for sinus floor augmentation and for localized alveolar ridge augmentation following tooth extraction in patients.

PLATELET-DERIVED GROWTH FACTORS

Platelet-derived growth factor (PDGF), as a serum GF for fibroblasts, has five isoforms (AA, AB, BB, CC and DD); of these, PDGF-BB was found to be more potent than the other isoforms in

promoting mitogenesis of PDL cells [88]. At concentrations of 10–20 ng/ml, *in vitro* studies suggested that PDGF-BB stimulated the proliferation of fibroblasts and osteoblasts [89,90], whereas a higher concentration (≥50 ng/ml) is required for the adhesion of PDL fibroblasts to diseased roots; concentrations of 5–20 ng/ml were not effective [91]. PDGF has also been shown to be effective when combined with either IGF, as shown by significant bone fill upon re-entry into the defects in animal models [92] and in patients with periodontal disease [93], or with dexamethasone [94], the latter being a well-known osteogenic differentiation factor. PDGF and IGF-1 had additive effects on calvarial DNA synthesis, but PDGF opposed the stimulatory effect of IGF-1 on collagen synthesis, and IGF-1 prevented the PDGF effect on collagen degradation [89]. In further research, IGF-1 alone at a dose of 10 μg did not significantly alter periodontal wound healing, while PDGF-BB alone at the same dose significantly stimulated new attachment, with trends of effect on other parameters. For example, the PDGF-BB/IGF-1 combination resulted in significant increases in new attachment and osseous defect fill above vehicle at both four and 12 weeks [95]. In humans, the use of purified rhPDGF-BB mixed with bone allograft resulted in robust periodontal regeneration in both class II furcations [96] and interproximal intrabony defects [97]. Based on those preclinical and clinical studies, recombinant human PDGF-BB homodimer in β-TCP is approved for the treatment of intrabony and furcation defects, as well as gingival recession in periodontal disease and is commercially available as Gem-21® (Osteohealth Co., Shirley, NY, USA). Furthermore, a large multicenter randomized controlled trial study of PDGF-BB homodimer, together with β-TCP, in the surgical treatment of a 4 mm or greater intrabony periodontal defect demonstrated significant increases in clinical attachment levels (CAL) reduced gingival recession at three months post-surgery, and improved bone fill when compared with those of β-TCP alone at six months [97]. The safety and effectiveness of this product was further demonstrated recently in a double-blind, prospective, parallel, active-controlled, randomized, multicenter clinical trial involving 54 patients with periodontal osseous defects [98]. Further longitudinal clinical data are still needed to confirm the long-term effect of this product [99]. Although far from ideal for meeting the needs of complex periodontal therapy, the road from basic research to clinical applications of PDGF-BB, or BMP-2 suggests a potential use of protein-based therapeutics for stimulating and accelerating periodontal tissue healing and bone regeneration [100].

FIBROBLAST GROWTH FACTOR-2

Fibroblast growth factor (FGF)-2 is a heparin-binding protein that regulates cellular functions including migration, proliferation and modulation of ECM production, in the wound healing cascade [101]. In periodontal regenerative therapies, the *in vivo* effectiveness of FGF-2 has been evaluated in beagle dogs as well as in non-human primates (reviewed in [102]). The results of these studies suggest that FGF-2 induces significant periodontal tissue regeneration with new cementum and new alveolar bone formation. Recently, a double-blind, placebo-controlled clinical trial (Phase II) was conducted in 253 adult patients at 30 Japanese dental facilities. The data obtained demonstrated that FGF-2 was efficacious in regenerating periodontal tissue without clinical safety problems [103]. Further clinical trials (Phase III) are still needed in order to establish the clinical efficiency and value of FGF-2 products [104].

GROWTH/DIFFERENTIATION FACTOR-5

Growth/differentiation factor (GDF)-5 is a member of the TGF-β superfamily, which shows a close structural relationship to BMPs and plays critical roles in skeletal, tendon and ligament morphogenesis [105]. In the periodontal field, it has been reported that cells involved in root- and PDL-forming stages exhibit significantly stronger signals of GDF-5 in comparison to those in more mature, well-established tissues [106]. *In vitro* studies have shown that recombinant human GDF-5 at concentrations of 10–1000 ng/ml inhibits alkaline phosphatase activity in human PDL cells [107]. Recently, the effect of recombinant human GDF-5 on periodontal repair was demonstrated in animal models by using various biomaterials as vehicles for delivery, such

as ACS, β-TCP or an injectable poly-lactide-co-glycolide-acid (PLGA) composite (reviewed in [108]). The combined results suggest that recombinant human GDF-5 appears to safely and effectively support periodontal wound healing/regeneration in intrabony periodontal defects without complications, while in a dose-dependent order. Recently, a Phase IIa randomized controlled clinical and histological pilot study evaluated recombinant human GDF-5/β-TCP for periodontal regeneration in twenty chronic periodontitis patients [109].

PLATELET-RICH PLASMA

The use of PRP, a platelet concentrate from autologous blood, is one of the strategies available for modulating and enhancing periodontal wound healing and regeneration [4]. PRP includes a pool of GFs such as TGF-β, vascular endothelial growth factor (VEGF), PDGF, IGF-1, epidermal growth factor and FGFs. For this reason, it has been suggested that the use of PRP might increase the rate of bone deposition and bone quality in such dental treatments as sinus lifts, placement of autogenous mandibular bone grafts, implants, and periodontal surgery. Both preclinical and clinical studies demonstrate the effectiveness of PRP in bone and periodontal augmentation when used in conjunction with bone graft materials (reviewed in [4]). Unfortunately, the literature on the topic is contradictory and the published data are difficult to sort and interpret. For example, in a series of clinical studies performed by Döri and co-workers on the healing of intrabony defects, the addition of PRP failed to improve the total outcomes in terms of probing depth reductions and CAL gains [110]. Furthermore, PRP may not provide any additional effect when associated with GBR around dental implants [111]. Although data published thus far suggest that PRP does not always exert additional effects, a systematic review of literature does find evidence for beneficial effects of PRP in the treatment of periodontal defects. Nonetheless, evidence for beneficial effects of PRP in sinus elevation appeared to be weak [112]. While PRP has been somewhat beneficial when used in periodontal or implant regeneration, it has fallen out of favor recently because of the lack of controlled clinical trials providing strong evidence of its efficiency [113]. However, the use of a patient's own biologically active proteins, GFs and biomaterial scaffolds for therapeutic purposes has opened a new way of understanding regenerative medicine. These simple and cost-effective procedures may have a potential impact in reducing the economic costs for standard medical treatments, soon achieving a 'golden age' by the development of user-friendly platelet concentrate procedures and the definition of new and efficient concepts and clinical protocols [4].

ENAMEL MATRIX DERIVATIVE

EMD, an extract of porcine immature enamel matrix, is regarded as a candidate protein mixture that is thought to be the induction of proliferation, migration, adhesion, mineralization and differentiation of cells in periodontal tissue [114]. EMD contains over 95% amelogenin with small amounts of enamelin and other proteins. In 2000, a randomized, double-blind, placebo-controlled, split-mouth study was designed to compare the clinical and radiographical effects of EMD treatment with that of placebo-controlled treatment for intrabony defects. The results demonstrated that treatment with flap surgery and EMD, compared to flap surgery with placebo, produced a significantly more favorable clinical improvement in intrabony periodontal defects [115]. A commercial EMD, marketed as Emdogain® (Institut Straumann AG, Basel, Switzerland), received US Food and Drug Administration (FDA) approval and has been available for clinical application for over 10 years, leading to a number of clinical trials performed to evaluate the clinical effects of Emdogain® (reviewed in [4]). In principle, EMD, which seems to be safe, was able to regenerate lost periodontal tissues in previously diseased sites based on clinical parameters and was better than open flap debridement (OFD) alone or GTR. Combined with allograft materials, EMD may be of additional benefit, but it needs to be further investigated [116]. Thus far, however, no evidence indicates that more teeth could be saved, and no evidence points to any

important differences between EMD and GTR [117]. Of interest, a ten-year investigation following treatment of intrabony defects with EMD, GTR, EMD plus GTR or OFD indicated that the three regenerative treatments showed a statistically significant higher CAL gain, compared with that of OFD at both one and 10 years [118]. However, another double-blind randomized clinical evaluation of EMD for the treatment of proximal class II furcation involvements showed that the use of EMD did not promote a superior reduction in probing pocket depths or a gain in clinical and osseous attachment levels, but only resulted in a higher rate of class II to class-I furcation conversion [119]. It seems that the clinical application of EMD for periodontal therapy still requires additional well-controlled clinical trials. EMD is applied not only for periodontal surgery, but also for tooth transplantation [120] and management of peri-implantitis [121]. However, the clinical predictability of such treatments remains to be determined.

Crucial delivery barriers to progress

The biological basis of using GFs in periodontal tissue engineering stems from their inherent property of inducing chemotaxis and/or mitogenesis of mesenchymal and somatic cell populations, initiating a cascade of events that ultimately lead to proliferation and differentiation of these cells. BMP-2/-7, GDF-5, FGF-2 and PDGF have been intensively investigated in preclinical and clinical trials and the results appear promising [77,78,100,102,108]. However, at this point in time, it appears very difficult, if not impossible, to translate knowledge about the functions of GFs in embryonic development, tissue formation/homeostasis and bone healing into clinically applicable solutions that address the loss of periodontal tissue. First, GF therapy is currently hampered by the lack of a safe and efficient delivery system that can provide a sustained therapeutic effect without cytotoxicity or unwanted side effects as a result of using very high doses of GFs [122]. Second, enormous costs are associated with recombinant human GFs in relation to the relatively small and non-life-threatening periodontal defects for which other treatment options (e.g., implants) exist. Of note, the delivery strategies commonly used today in periodontology are bolus injection or physical combination of GFs with prefabricated biomaterials. The rapid degradation, hence, low local availability of GFs suggests that these delivery strategies do not meet the physiological requirements of periodontal tissue repair processes [123]. Thus, in spite of the many recombinant GFs and cytokines now available for research purposes and the testing of some in humans, the clinical experience has, so far, been disappointing. Consequently, sophisticated approaches must be developed to deliver GFs that allow for the controlled, sustained and localized delivery of proteins [76]. New drug delivery systems that regulate the biological presentation of GFs represent an attractive new generation of therapeutic agents for the treatment of a wide variety of diseases. Such devices essentially allow the loaded GF(s) to be released at a desired rate and concentration and to remain at injury sites for a sufficient time to recruit progenitors and stimulate the tissue healing processes [123] (Fig. 72.2). In addition, drug delivery systems, termed cell scaffolds, can be formulated to have particular structures that facilitate cellular infiltration and growth. To improve their properties, many drug delivery systems serve dual purposes. In addition to providing cell support, cutting-edge scaffolds biologically interact with adhering and invading cells and effectively guide cellular growth and development by releasing bioactive proteins. To design controlled release systems for specific applications, it is important to understand the basic principles of protein delivery and the stability of each applied biomolecule [124].

Many biological and engineering challenges in the use of GFs for tissue regeneration remain to be solved. It is certain that these signaling molecules promote periodontal tissue and bone formation, but the ideal concentrations still need to be determined, and safety issues currently remain. In addition, understanding when to manipulate the cell's differentiation pathway with the application of single or multiple doses of GFs at the appropriate concentration is required to optimize the effect of these agents in periodontal wound healing. It also seems that

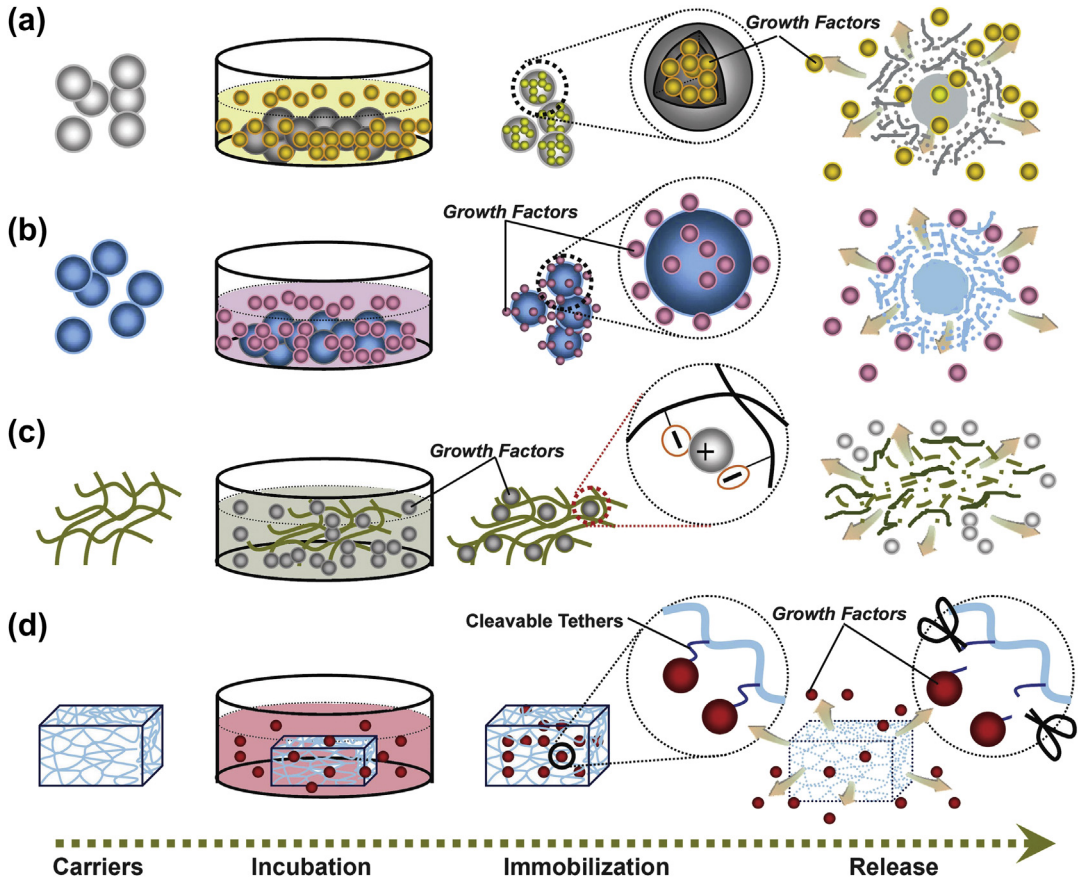

FIGURE 72.2

Schematic illustrations of the frequently investigated methods for immobilization of growth factors (GFs) and their primary release mechanisms (illustration is not to scale). (a-c) Non-covalent immobilization: GFs are directly entraped into (a) or adsorbed onto (b) the carriers, or immobilized via the formation of ionic complexes with polymer matrices (c). (d) Covalent immobilization: GFs are modified and thereafter covalently crosslinked to the hydrogels via crosslinkable and cleavable bonds that can be liberated once the tethers are degraded hydrolytically or enzymatically. *Reproduced from Chen et al. [167] with the permission of Elsevier Inc.*

any treatment aiming to mimic the natural tissue regeneration processes should not be limited to the provision of a single GF, but should deliver multiple agents at an optimized ratio in a specific spatiotemporal pattern [75]. Therefore, different release profiles from the same carrier may be particularly important in tissues with mixed cell populations, such as those found in the periodontium, where similar tissues, like bone and cementum, grow at different rates [124]. Furthermore, treatment of intrabony defects with GFs is likely to require both appropriate temporal release of the agents and a carrier that can serve as a template for new tissue formation, providing space maintenance and supporting the mucoperiosteal flap. Many of these issues have not been adequately addressed, at least from a periodontal standpoint; elucidation of these factors is essential prior to conducting expensive human clinical trials.

Gene delivery as an alternative to GF delivery

Because of the short half-life of topically administered GFs *in vivo*, usually ranging from several hours to several days, researchers have tried to extend protein activity using gene delivery strategies that involve converting cells into protein-producing factories. This is achieved by delivering plasmid DNA encoding the GF(s) of interest into cells/tissues either directly or via gene delivery vehicles or vectors [125]. This therapeutic concept has emerged as a promising

strategy for the modulation of the host response triggered by periodontal microbe and the regeneration of periodontium during disease progression [3].

Using viral vector transduction, cells can be manipulated *in vitro* and when transplanted into patients, these cells might restore normal tissue function. One of the most promising gene therapy approaches for periodontal regeneration, however, is based on the combination of naked DNA with a biodegradable carrier. Using this approach, collagen or other biomaterials are used to engineer a so-called 'gene-activated matrix' (GAM) that can carry and deliver naked DNA that directs an individual's own cells to produce a therapeutic effect [126]. A gene therapy product does not need to carry and deliver manipulated stem/progenitor cells, rather, it is able to signal the available cells to differentiate into a phenotype more favorable to the regenerative process [127].

Viral vector genes can be expressed *in vivo* from weeks to years, thereby having greater sustainability than that of a single protein or compound application. A unique advantage of gene therapy is the possibility of delivering more than one regenerative factor by employing two or more DNAs [128]. This combinatorial approach has shown significant regenerative potential compared to individual gene delivery, offering strong potential in regenerating tissues in three dimensions at the tooth-ligament-bone interface. In addition, gene therapy has also been investigated for the possibility of modulating periodontal disease progression via delivery of either antimicrobial or host modulators, considering that the host response against pathogenic bacteria is a major cause of periodontal tissue destruction [129]. Based on this concept, gene delivery, in combination with tissue engineering, is the only possible strategy that may mimic critical aspects of the natural biological processes occurring in periodontal development and repair [3,9,10,130–132]. The genes of interest in periodontal therapy include, but are not limited to, BMPs, PDGF, FGF-2, IGF-1 and TGF- β (reviewed in [125]). Of interest, the adenovirus encoding the PDGF-B gene delivered in a collagen matrix exhibits acceptable safety profiles for possible use in human clinical studies [133]. Although hundreds of gene therapy clinical trials have taken place in the past 20 years, much work still needs to be done to ensure an ideal safety profile for each gene and delivery method combination. With further efforts in this field, it is expected that gene therapy may augment current protein-based strategies and potentially allow for tissue-specific targeting and delivery of multiple signals in a spatially controlled and bioavailable fashion with the goal of regenerating natural tissue replacement in the craniofacial and periodontal region [127].

SCAFFOLDING AND BIOMATERIALS SCIENCE

The management of periodontal defects began with the introduction of a 'filler' material that aimed to induce bone regeneration. Various types of bone grafts, such as synthetic filler materials (alloplastic materials), autografts, allografts and xenografts, have been investigated for this purpose. Although utilization of such grafting materials for periodontal defects may result in some gain in CAL and radiographic evidence of bone fill, careful histological assessment usually reveals that these materials have little osteoinductive capacity and generally become encased in dense fibrous connective tissue [6]. Frequently, these treatments serve to ease the symptoms, not cure the disease. Tissue engineering offers clear merits over conventional replacement therapies that suffer from a host of drawbacks, such as scarcity of donor source, donor site morbidity, risk of lateral transmission of pathogens and graft-versus-host rejection. A major unmet challenge in tissue engineering today has been the synthesis of complex constructs that are principally comprised of biomaterials and multiple cell types.
This development is also true in periodontal tissue engineering. With the possibility of therapeutic cell cloning becoming a reality, there is an urgency to develop technologies that can precisely control the behavior of stem cells in culture. Central to these technologies would be the probable inclusion of biomaterials as an important component because the scaffold plays a vital role in converting isolated cells into functional tissues. Advances in biomaterial

research will undoubtedly facilitate the transformation of this concept into reality. Factors of great importance in the selection of a suitable scaffold material include such properties as porosity, tissue conductivity, biocompatibility and resorption rate. An appropriate scaffold has several design criteria that ensure not only the precise delivery of therapeutic cells to the site of injury, but also the rigorous control of their fates *in vitro* and *in vivo* toward regeneration.

Requirements of cell scaffolds

The implantation of cells in the afflicted area could be a direct approach in regenerative strategies, but the requirement for a support material to promote regeneration, especially in large sized defects, is a critical consideration. Periodontal tissue and bone are expected to grow into an adjacent defect only when the space is maintained and soft tissue ingrowth is prevented. Thus, the scaffold should act in a manner consistent with the established principles of GTR. To maintain the space for a sufficient time, the scaffold needs to feature such properties as ease of clinical handling and shaping and sufficient rigidity to withstand soft tissue collapse into the defect [6]. Additionally, the scaffold should be biocompatible with no biological hazards, and it should assist tissue functionality by promoting the easy diffusion of nutrients, GFs and cellular waste products. Furthermore, its biodegradability should be adjustable to the time required for tissue regeneration, thus avoiding interference with the physiological healing process. When considering the compatibility of internal structure with cell attachment and colonization and the compatibility of ingrowth tissues with those to be regenerated, the amount of porosity and the pore size of the supporting 3D structure are important features in the design of tissue-engineering scaffolds [134]. ECM components organized in the PDL not only reflect the functional requirements of this matrix, such as mechanical stress and storage of signaling molecules, but also regulate the tissue framework during development and regeneration. This idea is inspired in nature itself because most cells in the body subsist in a 3D world and are anchored onto a network of ECM, which the scaffolding design proposes to recreate. With this in mind, scaffold characteristics should mimic the complex and demanding environment to which cells are exposed and should be able to define a local biochemical and mechanical niche with complex and dynamic regulation that cells may sense, while, at the same time, not inducing an overexpression of inflammatory response [135]. Moreover, biosafety and bioactivity are of great importance. Although no guidelines have yet been established for assessing the safety and efficacy of a biological transplant, clearly the materials should be free from transmittable disease and immunologically inert [6].

Classes of biomaterials

For regeneration, biomaterials are intensively utilized, as a scaffold for cell delivery, a template for cell proliferation and differentiation, or as a capsule/filter to maintain the cell's viability and to deliver the synthesized biomolecules. The resorption, mechanical strength and efficacy of these materials can be manipulated through structural and chemical design parameters. Biomaterials used in periodontal tissue engineering include natural or synthetic polymers, ceramics and composites. Each material offers a unique chemistry, composition and structure, degradation profile and possibility for modification.

NATURALLY DERIVED POLYMERS

Natural biomaterials serve as a cornerstone in the development of matrix-based regenerative therapies that aim to accelerate clinical application due to their excellent biocompatibility, biodegradability, affinity for biomolecules and wound healing activity [136]. Collagen, hyaluronic acid, alginate and chitosan scaffolds have been used in periodontal regenerative research for more than two decades. The natural origin of these materials allows the design and engineering of biomaterial systems that function at the molecular level, often minimizing chronic inflammation. They can also be easily modified, both chemically and physically, to form desired structures, possess optimal properties and perform specific functions for various

applications. The use of natural polymers in the form of hydrogels allows for the incorporation of biological agents by promoting crosslinking when the GF is dispersed in the polymer solution. Because natural polymers are often soluble in water, the creation of hydrogels may occur under mild fabrication conditions that are relatively harmless to the bioactivity of the GFs. Normally, these hydrogels are degraded by enzymes and/or acid hydrolysis at a rate depending on the degree of crosslinking or the molecular weight [136]. In addition, natural polymers can also be modified to become 3D scaffolding materials, such as porous sponges, particles, films and rods, and for the formulation of stimuli-responsive biomaterials.

Purification of the major component of the ECM has been the subject of many potential tissue engineering strategies. Collagen is regarded as one of the most useful biomaterials owing to its excellent biocompatibility and safety associated with its biological characteristics, such as biodegradability and weak antigenicity. While collagen hydrogel fits well with injectable cell delivery, highly porous collagen lattice sponges with crosslinking provide mechanical stability that has been used to support the *in vitro* growth of many types of tissues and to deliver multiple GFs. Following the clinical use of collagen carriers delivering BMPs for tibial shaft fractures [137], spine fusions and long-bone nonunions [138], collagen is currently being evaluated for widespread clinical periodontal regeneration. Particularly, collagen is used clinically as a composite with ceramics [139]. Commercially available collagen composite scaffolds include Formagraft™ (Nuvasive, San Diego, CA, USA) and OssiMend™ (Collagen Matrix, Franklin lakes, NJ, USA). Apart from InFuse® Bone Graft, ACS has been used as a carrier and/or scaffold for periodontal regeneration in animal studies [140] and clinical trials [37].

As a deacetyled derivative of chitin, chitosan is structurally very similar to naturally occurring glycosaminoglycans and is biodegradable in mammals. Chitosan has several merits for use as a cell vehicle material, including its ability to be molded into various geometries (e.g., porous structures) and forms (e.g., gels), ease of chemical modification, high affinity for *in vivo* macromolecules and minimal foreign body reaction [141]. While chitosan can support cell attachment for cell delivery purposes, it is not strongly supportive of tissue regeneration, as demonstrated by its effect on the width of keratinized gingiva in dogs [142]. Accordingly, chitosan needs to be either modified chemically or conjugated with other molecules or peptides to enhance its biocompatibility for cell attachment and capability for tissue regrowth [6]. For example, the addition of HA beads to chitosan gels produced a novel scaffold in which the pore sizes and interconnectivity were preserved. When loaded with FGF-2, this scaffold may provide a suitable 3D environment supporting cellular structure, proliferation and mineralization [143]. Chitosan has also been widely used in combination with collagen [130,144], coral [132], HA [131] and β-TCP [145] to develop new scaffolds for periodontal tissue-engineering applications.

Alginate hydrogels bearing cell adhesion ligands have been used as scaffolds for cell encapsulation and transplantation, and they have yielded promising results in experiments aimed at engineering bone tissue capable of growth from small numbers of implanted precursor cells . An alternative to alginate hydrogels as a cell carrier is the incorporation of cells into beads of alginate to prevent immune cells and soluble complexes from killing the transplanted cells; this property negates the need for immunosuppressant use [146]. As a result of the semipermeable nature of the beads, the soluble factors made by the entrapped cells can be released at the implantation site to guide regenerated tissues [6]. However, these developments have not yet been used in periodontal bioengineering. Of interest, calcium alginate film was found to be more effective for GTR and GBR use than the collagen membrane [147].

SYNTHETIC POLYMERS

Numerous synthetic polymers have been widely used for scaffolding applications. Although synthetic matrices in their native forms lack cellular recognition sites, they have well-controlled and reproducible chemical and physical properties [148]. Synthetic polymers have many

advantages. For example, they offer the ability to provide controllable and reproducible structural properties, they are biocompatible, and their biodegradation rates can be tailored for the intended application through specific chemical manipulation. Synthetic scaffolds are produced by a variety of fabrication techniques, and these materials can be easily manufactured into preformed sizes and shapes according to clinical requirements. Synthetic matrices are typically processed into the form of solid scaffolds, small particles or hydrogels, depending on the mechanical and degradation properties for the particular application [148].

Hydrolytically degradable polymers are widely used materials for scaffold manufacture. The US FDA has approved the use of poly(α-hydroxyester)s, such as poly(glycolic acid) (PGA), poly(L-lactic acid) (PLA) and their copolymers, (PLGA) and polycaprolactone, for a variety of clinical applications. Solid scaffolds are typically porous matrices fabricated by techniques such as solvent casting, gas foaming, particulate leaching, and electrospinning [148]. Normally, these materials do not elicit a permanent foreign body response as they are gradually degraded into natural metabolites and eventually replaced by natural tissue. The physical properties of these polymers can be readily altered by varying the ratio of lactide/glycolide, the molecular weight and the crystallinity, or by combining them with other materials [134]. In some cases, synthetic polymers can be used as additions to natural biomaterials. For example, the rapid degradation of fibrin, a biopolymer critical to hemostasis and wound healing, can be decelerated by modification with poly(ethylene glycol) (PEG), thus creating a hybrid material for cell delivery [149].

PLA is one of the most promising biopolymers, because it is a naturally occurring organic acid, and the monomers may be produced from non-toxic renewable feedstocks [150]. Other degradable polymers include PEG [151], polylactide and polyglycolide [152], and polylactide acetyltributyl citrate and polydioxanon [153], and these polymers have also been explored and tested for guiding periodontal tissue regeneration. Amorphous poly(D,L-actic acid) (PDLLA) is one of the most popular materials considered for scaffold production, and it is used in combination with bioactive glasses because it can be combined with such biomolecules as GFs and antibiotics to establish a locally acting drug delivery system [134]. By this modification, the creation of a macroporous structure within the bioceramic materials is possible. For example, when PLGA microparticles are incorporated into calcium phosphates (CaP) cement, a macroporous CaP cement scaffold is formed after PLGA hydrolysis *in vivo* [154]. Although synthetic scaffolds have excellent mechanical properties and processing ability, their lack of natural biological cues can be a potential weakness. Furthermore, some polymers suffer from shortcomings, such as eliciting persistent inflammatory reactions and eroding or being incapable of integrating with host tissues [11]. These issues need to be addressed prior to clinical application.

CERAMIC-BASED MATERIALS

Ceramic-based materials have been widely used for bone and periodontal regeneration. Commercially available products include CaPs (e.g., HA and TCP), calcium sulfate and bioactive glass [155]. Clinically used forms of HA and β-TCP include injectable formulations that harden at body temperature, such as β-BSM™ (Etex, Cambridge, MA, USA) and BoneSource HA Cement (Stryker, Kalamazoo, MI, USA). These materials exhibit a range of degradation rates that span from weeks to years. Their biocompatibility and high protein-binding affinity make them good vehicles for drug delivery [98]. Recently, several porous ceramic scaffolds have also been examined for their utilization as cell delivery materials [6].

Both synthetic and coral-derived porous HA have been shown to support significant clinical improvements in periodontal measures following implantation in intrabony defects [13]. The porosity and degree of sintering of synthetic HA ceramics primarily determine the rate of biodegradation.

The degradation rate of β-TCP, a porous form of CaP, can be controlled by changing the Ca/P ratio. Because of its degradation characteristics and its similar proportions of calcium and phosphate to cancellous bone, β-TCP is regarded as an ideal material for bone substitutes for periodontal repair [108,156]. Owing to the rapid degradation of β-TCP and its associated poor mechanical properties, research has focused on mixed CaPs, such as mixtures of β-TCP and HA or β-TCP and polymers. These hybrid materials appear to be reliable vehicles for cell delivery, with studies showing good tissue formation associated with the implanted cells [6]. The treatment of periodontal intrabony defects with a combination of two ceramics (calcium sulfate in combination with β-TCP) led to a favorable clinical outcome after two years [157]. The alloplastic material Osteon (Genoss, Suwon, Korea) has an HA surface coated with β-TCP (70% HA and 30% β-TCP). The pore size of Osteon is 300−500 μm, and its volumetric porosity is approximately 77%. It has recently been reported that it is suitable for use in sinus graft applications [158]. For periodontal regeneration in periodontitis models, HA/TCP composite is demonstrated to be effective, both as vehicles for cell delivery [38] and as supporting biomaterials for cell sheet implantation [39].

Biomaterial redesign for periodontal application

The selection of the specific biomaterial is a key variable in the design and development of scaffolding systems. It has recently been proposed that a combination of several materials may offer the best opportunity for beneficial clinical outcomes [122]. For example, a porous chitosan/collagen scaffold [144] and a glycidyl methacrylate derivatized dextran (Dex-GMA)/gelatin scaffold prepared through a freeze-drying process were developed specifically for periodontal tissue engineering. Furthermore, natural polymers can be strengthened mechanically by combining them with ceramics, such as HA [131,143], β-TCP [145] or polylactide (blends, copolymers and interpenetrated networks) [159]. Thus, for periodontal regeneration, one promising direction is the formation of hybrid materials that take advantage of both natural and synthetic materials by combining excellent mechanical properties and an intrinsic structure highly compatible with tissue ingrowth. However, in nearly every case, these materials were adopted from other areas of science and technology without substantial redesign [11]. Although a number of biomaterials, such as ASC and β-TCP, have had a long history of clinical use in periodontal therapy, very few interact with their surrounding host environment or promote regeneration of functional periodontal tissue such that a seamless integration with host tissue is created in an intelligent and proactive fashion. Alongside the rapid advances in stem cell biology, progress in biomaterials design and engineering is now converging to enable a new generation of instructive materials to emerge as candidates for periodontal regenerative medicine.

In terms of shape and geometry, preformed scaffolds are suitable for defects requiring mechanical strength or a predefined shape, such as one-walled bone defects, whereas injectable scaffolds may facilitate clinical use in two- or three-walled intrabony defects. Injectable types of scaffolds are expected to form the shape of any cavity or repair site, but must solidify within a clinically acceptable timescale *in situ*. Of note, such devices will shorten surgical operation time, minimize damaging effects of large gingival retraction and lessen postoperative pain. One critical disadvantage of injectable biomaterials is their poor mechanical strength and porosity; however, pores can be generated in scaffolds with rapidly degrading particles or gaseous bubbles as porogens to promote cell attachment, migration, proliferation, mass transfer, vascularization and material resorption [160]. These modified hydrogels are promising delivery systems for therapeutic cells.

The functional significance of the ECM has generally been defined in terms of the provision of a structural support for cell adhesion and the establishment of tissue physical integrity. Recent evidence has, however, led to a paradigm shift where the ECM is increasingly recognized as exerting a profound influence on cell behavior. Therefore, biomaterials are required to act as

the temporary ECM in the regeneration of a new tissue. To this end, a scaffold is not simply a mechanical support and initial cell anchorage site, but it must be 'informative' to the cells. That is, the scaffold should be capable of stimulating specific cellular response at the molecular level [161]. It follows that the design of new cell seeding scaffolds must take into account microenvironment design features that induce the appropriate gene expression in cells forming new tissues [162]. The complexity of this design process is exemplified by the dynamic states of the cells and tissues, which are regulated by the spatial and temporal coordination of multiple cell processes, each of which is regulated by multiple reciprocal interactions between cells and their ECM [162]. Biomaterial modification can take on different levels of complexity to produce increasing levels of physiological 'mimicry' and functionality. Their interactions with cells and tissues may be improved or fine-tuned by adequate chemical modifications, such as grafting with cell adhesion peptide Arg-Gly-Asp (RGD) or by surface treatments (e.g., surface plasma modification or surface chemistry modification through grafting with other polymers) [136,163]. The incorporation of RGD peptides in hydrogel matrices has been found to significantly enhance the attachment, spreading, survival and mineralization of encapsulated dental follicle progenitors, suggesting that RGD additives may promote the use of hydrogels for periodontal mineralized tissue engineering. The incorporation of peptide motifs recognized by cell receptors and the use of recombinant DNA technology have enabled the creation of naturally derived tissue-engineering scaffolds with new levels of biofunctionality [15]. Typical surface modifications include, but are not limited to, changes in hydrophilicity, functionalization with charged groups, the incorporation of insoluble ligands and peptide cell recognition sequences (e.g., RGD), the attachment of larger proteins, supramolecular self-assembly and the development of materials that bind and release soluble factors [164]. The wettability of the material surface, particularly in synthetic polymers, can be effectively regulated by physical treatments, e.g., by irradiation with ions, plasma or ultraviolet (UV) light. The irradiation-activated material surface can be functionalized by various biomolecules and nanoparticles. This characteristic further enhances its attractiveness for cells and its effectiveness in regulating cell functions [165]. Strategies based on physical cues include the reproduction of nanoscale topology, superposition of mechanical cues and control of degradation. Designing biological recognition into a biomaterial may also obviate the need for therapies based on the delivery of cells or recombinant GFs, which are subject to regulatory constraints [162]. To this end, scientists are creating new materials, including those with improved biocompatibility, stealth properties, responsiveness (smart materials), specificity and other critical properties.

We must be aware that sites of injury or diseased organs are characterized by heightened immunological surveillance and a high concentration of inflammatory cytokines. As such, these sites often present environments hostile to healthy cells attempting to establish and repopulate. This is extremely important in the management of periodontal disease. Therefore, to eliminate the need to promote a harsh immunosuppressive regime, an additional role for scaffolds needs take into account the insulation of their cellular cargos from the host immune system to, in turn, promote the survival of transplants [3]. Finally, in order to engineer functional periodontal tissues, the correct mechanical stimuli will need to be conveyed to the developing tissues within the scaffold, although very few studies have addressed this issue [6]. To date, biomaterials are rapidly being developed to display and deliver stem cell regulatory signals in a precise and near-physiological fashion within the tissue defect, fostering the regeneration of the missing tissue [75]. The transplant must participate in the regenerative process, considering the specific properties of each tissue interface, which can only be achieved through the design of scaffold materials accommodating the specific characteristics of periodontal hard and soft tissues, and providing stem cells with the necessary cues to satisfy cellular needs of both tissue types. Further synergism of cell biological and biomaterials technologies promises to have a profound impact on stem cell biology and to provide insights that will advance stem cell-based clinical approaches to tissue regeneration [166].

PERIODONTAL BIOENGINEERING STRATEGIES

Periodontal tissue-engineering strategies typically involve harvesting of suitable autologous donor tissues (e.g., the PDL) from which cells are isolated and expanded in a good manufacturing practice (GMP) facility. Expanded cells are then seeded onto a matrix and surgically implanted into the host's periodontal defects. Alternatively, the cell/matrix may be further incubated in a bioreactor, or other such system prior to implantation, in which the cells may be exposed to biological, chemical or physical stimuli that promote the formation of the appropriate tissue [5]. Considerable preclinical data and some clinical successes support the applicability of these cell-based approaches [40,64,66]. However, the process is very complex and not cost-effective. Additionally, the scarcity of the cell source is an issue. Although allogenic cell transplantation has shown success [39], the potential risks associated with transplanting exogenous (foreign) cells remain to be addressed. Even without any therapeutic intervention, living periodontal tissues can have a staggering capacity for regeneration. As such, a biomaterials/protein-based strategy that avoids the use of *ex vivo* cultured stem cells to facilitate endogenous repair is a novel approach to periodontal tissue engineering. Based on current understanding of periodontal tissue engineering, both cell-free and cell-based approaches have their own advantages and disadvantages, and both warrant further investigation (Fig. 72.3).

Cell-free approaches

As noted in the Introduction section, recent advances in periodontal bioengineering have shifted the focus from the attempt to recreate tissue replacements *ex vivo* (*in vitro* paradigm) to the development of an *in vivo* paradigm to achieve *in situ* periodontal regeneration [5]. However, a daunting question for the *in vivo* paradigm involves the requirement for transplanted cells, as this has been viewed as a core component for *in vivo* tissue engineering. Interestingly, but not surprisingly, cell-free *in vivo* tissue engineering has shown that appropriate stimuli can activate local cellular populations and direct their trafficking, leading them to contribute to endogenous tissue formation [4] (Fig. 72.4). The design of cell-free approaches for periodontal regeneration is based on the concept that relies on harnessing the intrinsic regenerative potential of endogenous tissues using a conductive/inductive scaffold, either alone or in combination with such molecular stimuli as GF or genes, to initiate reparative processes *in situ* [167].

In carefully selected cases, upon implantation, a conductive scaffold may guide the regeneration of the periodontal tissue by passively allowing the attachment and growth of vascular elements and progenitor/stem cells that reside in the tissue defect. Nevertheless, its regenerative potential is limited by the lack of biologically active factors and sufficient progenitor cells within the defect site. On the other hand, an inductive scaffold may guide the regeneration of the periodontal tissue by carrying one or more biologically active factors, such as stromal cell-derived factor (SDF)-1, BMP-2, PDGF-BB and/or FGF-2, which recruit vascular events and progenitor/stem cells from the immediate vicinity to the tissue defect (reviewed in [11]). The regenerative potential of an inductive scaffold is higher than that of conductive biomaterial because more progenitor cells can repopulate the tissue defect [168]. Trafficking of stem/progenitor cells requires the complex processes of mobilization of MSCs from their niche, recruitment to the desired target tissue or site, and integration and maturation at the target site. For cell-free tissue engineering to be successful, identification of specific cues that steer stem cells to their niche and increase the efficiency of the homing process is of critical importance. For stem cells to be recruited by injured tissues, implanted biomaterials must initiate a sequence of coordinated interactions between the cells and their environment and provide the signals and signposts that guide the cells along their journey [167]. Therefore, artificial biomaterial niches would need to incorporate appropriate navigation cues that could attract endogenous stem cells and have ECM-mimicking components and structures that could localize and accommodate the cells within the destination [5].

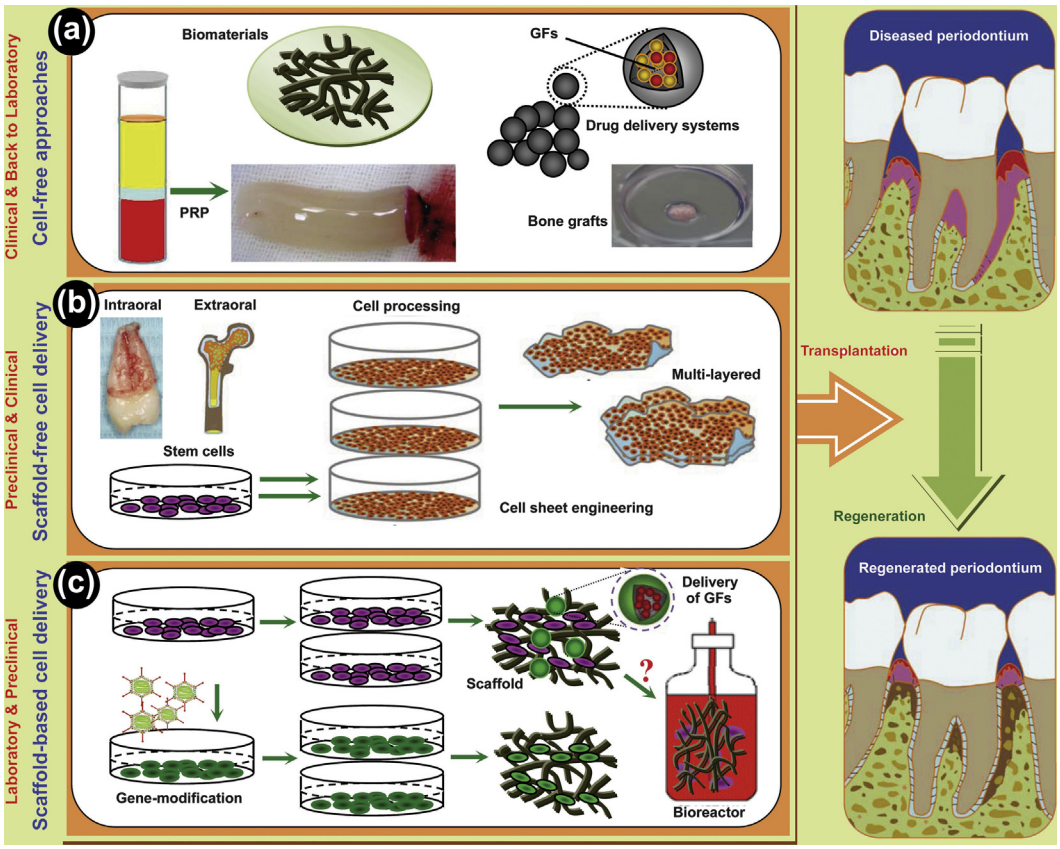

FIGURE 72.3

Cell-free (a) and cell-based (b & c) technologies for periodontal tissue engineering. (a) Cell-free approaches aim at achieving endogenous periodontal regeneration with the use of growth factors (GFs), well-designed biomaterials, platelet-rich plasma (PRP) and its associated formulations, and maybe other commercially available products, alone or in combination. In this regard, periodontal defects is likely to be restored by endogenously recruited cells and without the need of delivery of *ex vivo* manipulated cells. This concept has been employed in clinic for several decades and feedback based on clinical practice has led to new insights into and novel applications of such endogenous mechanisms for tissue regeneration [122,167]. Besides, both extraoral and intraoral stem cells represent a viable and accessible 'tool' for regeneration. Adequate cell density could be reached *in vitro* under a controlled environment and made readily available for reimplantation into a periodontal defect site via either scaffold-free (i.e., cell sheet engineering) (b) or scaffold-based delivery (c). Although the time is now ripe to move stem cell-therapy from preclinical studies to clinical trials, there are a number of biological, technical and translational hurdles that remain unresolved. For scaffold-based delivery, the expanded cells are seeded into a three-dimensional (3D) scaffold (normally containing GFs), and immediately implant into periodontal defects (*in vivo*); or to create tissue-like constructs in bioreactor before transplanting into patients (*in vitro*). In both cases, gene of interest may be incorporated into a therapeutic cell because a therapeutic gene has been shown to increase the regenerative potential and enhance the availability of therapeutic cells.

The use of PRP and EMD in periodontal reconstructive surgery provides evidence that cell-free approaches are useful in various experimental and clinical models, yet the outcomes have frequently been inconsistent or conflicting between studies [5]. If we just consider the current endogenous technique for routine clinical periodontal regeneration, it clearly needs to be improved and refined [169]. One pivotal aspect in the development of PRP technology, for example, has been the deep platelet characterization that has been developed to determine the most important GFs and cytokines contained within these cells, and the protocols that facilitate their safe manipulation and concentration [170]. Cell-free tissue engineering is still in its infancy. New insights from developmental biology and other biological disciplines are actively guiding the development of biofunctonalized materials that work with nature's own mechanisms of repair, where the scaffold should be considered as both a biochemical and biophysical signaling device, modulating and orchestrating cellular activity in the defective microenvironment to harness and direct a patient's own regenerative potential. To date, the cell-free approach offers a preferential route for tissue-engineering therapies to be utilized within clinical settings.

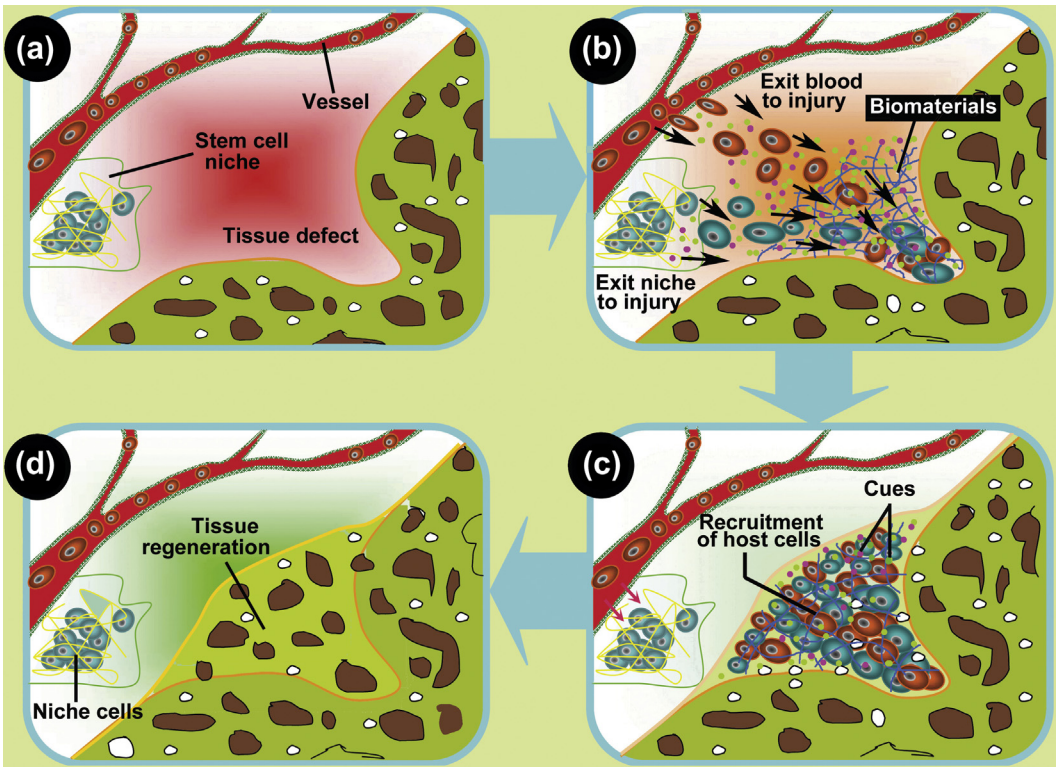

FIGURE 72.4
Schematic representation of the recruitment of host cells for endogenous tissue regeneration (a: tissue defect; (b: cell trafficking in response to implantated biomaterials and growth factors; (c: sufficient cell populations recruited to the defect; (d: defect restoration via endogenous tissue regeneration). The use of well-designed biomaterials in combination with molecular stimuli (growth factors or genes) may harness the body's innate ability to restore small or moderate tissue defects without the need of *ex vivo* cell delivery. This concept is generally termed cell-free tissue-engineering strategy (modified from Chen et al. [15].

Cell-based approaches

If an existing degree of periodontal dysfunction already exists and cannot be restored by endogenous mechanisms alone, stem cell implantation to enhance a regenerative outcome could be pursued as an alternative (Table 72.2). In addition to promoting tissue repair directly, MSCs have also been shown to modulate the immune system and attenuate tissue damage caused by excessive inflammation [5]. This immune modulation has been found to be an active process that renders stem cell therapy.

SCAFFOLD-FREE CELL DELIVERY

Without the use of scaffolds, cells can either be injected directly to the defect or form sheets/microtissues for surgical implantation (reviewed in Ref. [12]). The main drawback of single-cell injection is the high cell loss caused by washout and the low level of integration of the transplanted cells, in addition to the risk of unwanted neoplastic growth if the cells eventually settle in non-target sites. The development of advanced scaffold-free delivery systems is an emerging subdiscipline of regenerative medicine with broad applications and significant impact in orthopedics and reconstructive surgery. For example, cell sheets/microtissues allow for the generation of functional cell transplants in which cells can survive, grow and recapitulate native tissue characteristics. In this concept, fibroblasts, or other cell types that secrete significant amounts of ECM proteins, are cultured for a prolonged period to create sheet/tissue-like structures for therapeutic use [104]. Based on the presence of deposited ECM produced during *in vitro* incubation, the cell transplants can be easily transferred and attached to

TABLE 72.2 Delivery strategies in cell-based periodontal tissue engineering

Scaffold-free delivery

Design of Delivery	Therapeutic cells	Applications	References
Cell sheet engineering	Human PDL[a] cells	Dog — dehiscence defects on the buccal surface of the mesial roots of bilateral mandibular first molars	Akizuki et al. [35]
	Allogeneic PDLSCs[b]	Miniature pig — experimental periodontitis	Ding et al. [41]
	Autologous PDLPs[c]	Human — 16 teeth in three paitients with periodontitis	Feng et al. [37]
	Autologous PDL cells, iliac BMMSCs[d] or APCs[e]	Canine/one-wall intrabony defects	Tsumanuma et al. [41]
	Human PDLSCs	Rat/ectopic model	Iwata et al. [44] Washio et al. [171]
Cell pellets	Autologous PDLSCs, DPSCs[f], or PAFSCs[g]	Canine/Apical involvement defect	Park et al. [40]

Scaffold-based delivery

Design of delivery	Therapeutic cells	Biomaterials/applications	References
Injectable carrier or soft scaffold	Autologous PDL fibroblast-like cells	Autologous blood coagulum/Dog — artificial fenestration defects	Doğan et al. [34]
	Autologous cementoblasts	PLGA[h] polymer sponges/Rat — rodent periodontal fenestration model	Zhao et al. [50]
	Autologous BMMSCs	Atelocollagen (2% type I collagen)/ Dog — class III furcation defects	Kawaguchi et al. [64]
	Autologous cryopreserved BMMSCs	Collagen scaffold carrier/Dog — periodontal fenestration defects	Li et al. [72]
	Autologous ASCs[i]	PRP[j]/Rat — periodontal defects in the first upper molar palatal side	Tobita et al. [66]
	Autologous CDCs[k] and PDL-derived cells	Collagen sponges/Dog — periodontal intrabony defects	Nuñez et al. [175]
Hard scaffold	Autologous PDLSCs	HA-TCP[l]/Miniature pig — experimental periodontitis lesions	Liu et al. [38]
Cell encapsulation	Autologous BMMSCs	Microcarrier gelatin beads/Rat — surgically created periodontal defects	Yang et al. [177]

[a] *periodontal ligament;*
[b] *periodontal ligament stem cells;*
[c] *periodontal ligament progenitors;*
[d] *Bone marrow mesenchymal stem cells;*
[e] *alveolar periosteal cells;*
[f] *dental pulp stem cells;*
[g] *periapical follicular stem cells;*
[h] *poly-lactide-co-glycolide-acid;*
[i] *absorbable collagen sponge;*
[j] *platelet-rich plasma;*
[k] *cementum-derived cells;*
[l] *hydroxyapatite-tricalcium phosphate.*

other surfaces, such as tooth root and host bone tissues. The resulting cell sheets/pellets retain their original ECM and cell-cell contacts to facilitate cell integration and tissue formation.

Because cell sheet engineering can produce functional layers of cells, various types of cell sheet transplantations without scaffolds have been tested for periodontal regeneration, and these have shown significant potential to induce the reformation of the PDL and cementum

[35,39,41]. Thus, the application of cell sheets may be an effective clinical strategy, and the safety and efficacy were evaluated, both *in vitro* and *in vivo*, and presented no evidence of malignant transformation [44,171]. Very impressively, this concept has also been tested in a feasibility/pilot study in which autologous PDL cells were utilized in the treatment of human periodontitis [37].

Cell pellets or microtissues are groups of cells (cell re-aggregates) normally ranging in size between 100 and 500 μm in diameter. These cell aggregates gain structure and functionality resembling the native tissue when dispersed cells are grown under certain culture conditions [172]. Keeping this in mind, *ex vivo* expanded dental stem cells were grafted to the periodontal defect without the need of a scaffold, where the cellular pellets could cover the denuded root surface thoroughly and fill the defect [40]. Furthermore, bilayered cell pellet constructs comprising calcified bone-forming cell pellets (i.e., BMMSCs) and cementum/PDL-forming cell pellets (i.e., PDLSCs) can be fabricated *in vitro* via the intrinsic capacity of monodispersed cells to self-assemble into a microtissue (e.g., 3D spheroid). This strategy may provide an alternative to reconstruct extensive periodontal defects and achieve a predictable and complete regeneration of the periodontium. However, the possibility of microtissues turning into tumors based on their structural similarity is a great concern, especially when undifferentiated cell types are used [173].

SCAFFOLD-BASED CELL DELIVERY

From an engineering standpoint, current approaches to periodontal regeneration via scaffold-based cell transplants can be summarized into four possible strategies [12]:

- stem cells are amplified by *ex vivo* expansion and differentiated into the target cell type before being seeded into scaffolds to constitute the transplants;
- stem cells are amplified and differentiated directly in the scaffold before implantation, which is a strategy more suited to adult stem cells, but instructive signals need to be incorporated into the scaffolds to effect direct cell proliferation/differentiation *in situ* within the scaffolds;
- stem cells are partially differentiated into progenitor cells, either before or after seeding into scaffolds, to give rise to proto-tissues; following implantation, these constructs transiently release progenitors that migrate into surrounding regions where they undergo terminal differentiation, integrate and contribute to regeneration of the lesion areas (prolonged release of stem/progenitor cells may be achieved when a suitable scaffold is used to maintain them in a partially differentiated state;
- injectable transplants composed of pristine or stimulated stem cells encapsulated in biodegradable hydrogels; this strategy is attractive for soft tissue repair or treatment of solid tissues with critical size defects that are too fragile for surgical intervention.

All four strategies have been tested in periodontal tissue engineering. Injectable materials are considered to be the ideal delivery vehicles for cells and bioactive factors, because they can be delivered by a minimally invasive process and can fill irregular spaces of the defect. The injectable system can offer early mechanical stabilization by *in situ* polymerization, such as fibrin and alginate [174]. The rapid degradation of fibrin can be decelerated by modification with PEG, thus creating a hybrid material for robust cell delivery [149]. It is known that PRP may enhance new bone formation and accelerate existing wound healing process. Therefore, autologous injectable scaffold, was used to deliver BMMSCs to the periodontium [28]. Although cell transplantation via injectable matrices into sites of articular defects has been successful, it has not been tested in either preclinical or clinical studies to demonstrate whether injections of stem cells into periodontal defects can achieve significant improvement.

In contrast, hydrogel sponges as vehicles for cell and drug delivery for periodontal regeneration have been tested in various animal models and in clinical practice with success [136,175]. In terms of synthetic biomaterials, a pilot study has demonstrated that cementoblasts have

a marked ability to induce mineralization in periodontal wounds when delivered via PLGA polymer sponges [50]. Impressively, a collagen sponge scaffold has been shown to be an effective cell delivery vehicle for oro-maxillofacial bone tissue repair in patients [27].

Solid scaffold-cell transplants may stimulate the repair of damaged/diseased tissue while maintaining adequate integrity, where HA/TCP can be used directly as a cell delivery scaffold [38,39]. The requirements and design of scaffold systems for cell delivery have been discussed previously in this chapter. Many promising strategies for functional tissue engineering aim to replicate components of the natural cellular microenvironment by providing a synthetic ECM and by delivery of GFs [122]. A major clinical challenge in the reconstruction of large oral and craniofacial defects is the neogenesis of osseous and ligamentous interfacial structures where the natural 3D shape of the tissue needs to be recreated. In this regard, the multiscale computational design and fabrication of composite hybrid polymeric scaffolds (PCL-PGA) to carry genetically modified human cells to regenerate human tooth dentin-ligament-bone complexes have been tested *in vivo*. This approach offers potential for the clinical implementation of customized periodontal scaffolds that may enable regeneration of multi-tissue interfaces required for oral, dental and craniofacial engineering applications [9,10]. Recently, cell-based research has focused on the geometric design of scaffolds for tissue engineering. However, the orchestration of multiple tissue formation, spatial fibrous tissue organization, and endpoint functional restoration using a single *in vivo* scaffold system remains a significant challenge.

Stem cells can potentially be expanded on microcarrier beads in spin culture for direct transplantation into tissue defects. The process of encapsulation physically isolates cells from the outside environment and aims to maintain cellular physiology within a selectively permeable membrane [176,177]. By mechanically blocking the cells from immune attack, this technology significantly reduces post-transplantation cell apoptosis and offers a solution to the shortage of donors for functional cell transplantation, particularly since it allows xenobiotic or allogenic cells to be used. However, this therapy has not yet been introduced to periodontics, and it still faces many challenges, such as materials choice and design, before it can be moved into clinical reality [178].

In prospect, shape memory materials are potentially useful for periodontal regeneration, although they remain to be tested. These materials can deliver bulky scaffolds via minimally invasive surgery to the periodontal defects. Using these smart materials, future scaffolds may be fabricated in a condensed state before transplantation and then later acquire the desired shape *in vivo* [172]. Self-shaped hydrogels can be structurally collapsed into smaller, temporary shapes that permit their minimally invasive delivery *in vivo*. Scaffolds are rehydrated *in situ* with a suspension of cells or cell-free medium and delivered through the same catheter. The rapid recovery of the scaffold properties facilitates efficient cell seeding *in vivo* and permits neotissue formation in the desired geometries [178]. Improved delivery platforms coupled with enhanced understanding of the mechanistic/biological regulators of tissue formation should lead to better and more predictable therapeutics within the next decades [132].

CHALLENGES AND FUTURE DIRECTIONS

The currently available treatments, which are based on the 'damage to heal approaches', have had only limited success in periodontal medicine. With an increasing aging population, tissue-engineering strategies provide important cures and hope for the treatment of periodontal disease, and they have set the stage for successful regeneration of many other tissues. The development of biological transplants for reconstructive therapies has considerably improved the currently available treatment options for periodontal repair. Particularly, the accelerated pace of research in the stem cell field and the accumulated body of knowledge has spurred interest in the potential clinical use of stem cells [15]. This developing area is attracting

increasing attention from both the private and government sectors because of its considerable economic and therapeutic potential. However, there are critical steps in moving the field towards human clinical utility [179]. In particular, the events following cell transplantation are poorly understood, underscoring the considerable need for robust preclinical modeling for the evaluation of the safety and efficacy of stem cells. Although the clinical application of stem cells to the regeneration of periodontal tissue has begun, the risks of stem cell therapies should not be ignored or underestimated by clinicians and researchers [12].

There are two main criteria for successful tissue engineering [6]. First, there are the engineering principles, which relate to biomechanical properties of the scaffold, architectural geometry and space maintenance. In this regard, biomaterials are increasingly being developed as *in vitro* microenvironments mimicking *in vivo* stem cell niches. Future successful periodontal bioengineering may very well rest on the ability to effectively utilize these biomaterials as delivery platforms for appropriate cells and/or factors [132]. However, current macroscale methodologies to produce these niche models fail to capture the spatial and temporal characteristics of the complex native stem cell regulatory systems. The second criterion relates to the biological functions of the engineered construct, including cell recruitment, proliferation and survival in culture and at the site of implantation, neovascularization and delivery of GFs necessary for successful differentiation and tissue regeneration. However, control over stem cell fates within a complex *in vivo* milieu is extremely difficult and represents the most challenging issue faced by current periodontal bioengineering.

Tissue engineering is making an important impact on the concept of periodontal therapy, and several clinical trials involving transplantation of stem cells into human patients have already begun or are in preparation (reviewed in Ref. [12,179]). The use of protein- or cell-based therapy to enhance and direct periodontal wound healing into a more predictable regenerative path is being exploited in bioengineering efforts that aim at developing a new therapeutic paradigm for clinical application. As tissue engineering becomes more of a clinical reality through the ongoing bench-to-bedside transition, research in this field must focus on addressing relevant clinical situations [179]. Acknowledging that tissue regeneration alone is not the only answer to predictably securing a long-term stable treatment for the patient with a history of periodontal disease, host modulation therapies are therefore rising as an important aspect in the control of periodontal diseases and tissue reengineering [3]. Although most *in vivo* work in the area of periodontal tissue engineering focuses on tissue regeneration within sterile, surgically created defects, there is a growing need for the investigation of engineering approaches within contaminated or infectious wound beds, such as those that may be encountered following tissue damage by periodontitis. As discussed within the context of this chapter, there are a number of developing systems that have the potential to optimize tissue healing biology. A major obstacle that remains today is how to maximize the utility of therapeutics delivered to a passive or permissive environment where there is a context for the type of cell needed, but in which very few biological signals are given to encourage normal cell function [3]. In addition, the tissue-engineering field still needs to confront other hurdles, such as identifying the best cell sources, clinically relevant cell numbers, an effective mode of administration, the integration of new cells into existing tissue matrices, and the achievement of functional properties of tissue equivalents using an expanded repertoire of biomaterials. Finally, major constraints to the tissue-engineering fields remain in the context of practical, safety and regulatory concerns related to the application of these technologies in the clinical arena [3].

CLOSING REMARKS

Regenerative therapies for periodontal disease that use patients' cells to repair the periodontal defect have been proposed in a number of preclinical and clinical studies. Periodontal tissue-derived stem cells, such as PDLSCs, are committed toward all of the periodontal developmental lineages that contribute to cell turnover in the steady-state and would thus

be useful cell sources for treating periodontally destructive diseases, such as periodontitis. Treatments that partially regenerate damaged periodontal tissue through the localized administration of GFs have now been established. Although the clinical practice is not very successful, such regenerative therapies have provided very useful and feasible clinical study models for the future design of tissue engineering and stem cell therapies. Using currently available clinical strategies, partial regeneration of the periodontal tissue is becoming possible; however, methods to achieve the functional regeneration of large defects caused by severe periodontal disease are still lacking. To address this, it is essential to better understand the cellular and molecular mechanisms underlying periodontal development and, thereby, identify the appropriate functional molecules that induce the differentiation of stem cells into periodontal lineage cells for the successful reconstruction of periodontal tissue. The field of periodontal bioengineering has entered an exciting new developmental phase that will make increasingly important contributions to the patient. Particularly, a number of biological technologies are being aggressively explored for clinical translation, signifying a veritable "coming of age" of the field. However, such issues as appropriate delivery devices, immunogenicity, autologous cells *vs.* allogenic cells, identifying tissues that provide the most appropriate donor source, control of the whole process, and cost-effectiveness are all important considerations that should not be overlooked. The future of periodontal bioengineering is undoubtedly driven by technology. New applications and improvement upon current designs will depend heavily on innovations in biomaterials engineering. Progress in stem cell biology will be imperative in dictating advances in stem cell-based regeneration. A better understanding of the molecular mechanisms by which substrate interactions impact stem cell self-renewal and differentiation is of paramount importance for targeted design of biomaterials. Discoveries in the fields of developmental biology and functional genomics should also be exploited for broadening the repertoire of biological molecules that can be incorporated into biomaterials for fine-tuning stem cell activities. With the merger between the two powerful disciplines—biomaterials engineering and stem cell biology—a new drawing board now lies before us to develop therapies that promise to revolutionize periodontal tissue engineering.

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PART 18

Respiratory System

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CHAPTER 73

Tissue Engineering for the Respiratory Epithelium: Cell-Based Therapies for Treatment of Lung Disease

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INTRODUCTION: THE CHALLENGES FACING CELL-BASED THERAPY FOR TREATMENT OF LUNG DISEASE

The lung is a remarkably complex organ that evolved for the adaptation of vertebrates to terrestrial life. While embryonic development proceeds normally in the absence of lung tissue, life after birth is entirely dependent upon respiration, a process that, in turn, is dependent upon the structure and function of the lung. The respiratory tract consists of distinct anatomic regions from the nasal passages, pharynx, larynx, trachea, bronchi, lobar bronchi, bronchioles and peripheral airways that direct inhaled gases to the alveoli. Gas exchange occurs across alveolar epithelial and capillary endothelial cells. Ventilation is driven by mechanical forces dependent upon neuromuscular activity that is precisely controlled by neurosensory inputs to maintain normal pCO_2, pO_2, and pH. There is little evidence that the lung serves critical physiological roles other than gas exchange. Lung function is entirely dependent on its remarkable structure that exchanges millions of liters of environmental gases throughout our lifetime. Unlike other organs, for example endocrine organs that synthesize and secrete hormones critical for growth and metabolism of many target organs, respiration is an intrinsic property defined by lung structure and mechanics. Regeneration or replacement of functional respiratory tissues will depend upon creating and protecting the structures of the lung that bring environmental gases to an extensive alveolar surface across which oxygen and carbon dioxide are exchanged with the pulmonary vascular bed. Achieving these goals presents formidable technical challenges for regenerative medicine (Fig. 73.1). Repair/regeneration of tissues in conducting airways (for example, the repair or replacement of the larynx, trachea or bronchial cartilage), or the delivery of therapeutic cells to the lung parenchyma may represent a more achievable target for the application of regenerative medicine for the respiratory tract. Significant progress has been achieved recently in developing re-cellularized matrices for tracheal-bronchial engraftment. This chapter will consider issues regarding the application of cell-based therapy for prevention and/or treatment of lung diseases, as well as the role of

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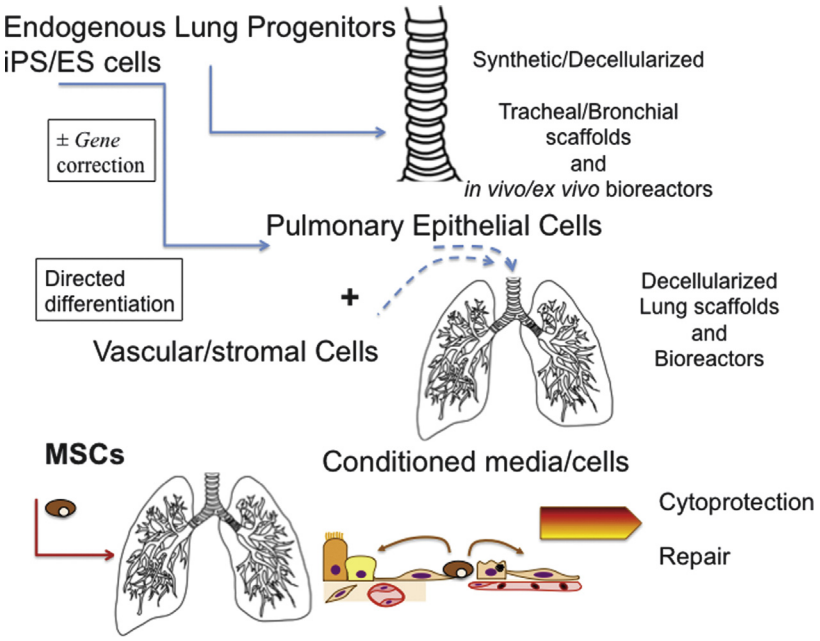

FIGURE 73.1

Strategies for cell-based therapies for the lung. Cell-based therapies for treatment of lung disease using distinct strategies are in active experimental stages. Embryonic stem cells (ES) and induced pluripotent cells (iPS cells) can be genetically modified and differentiated to specific lung cell types *in vitro*, producing respiratory epithelial or pulmonary vascular cells. Resident pulmonary stem/progenitor, ES or iPS cells have been identified, isolated, cultured on acellular or decellularized matrices and engrafted to create airway and alveolar-like tissue. Bone marrow tissue derived stem cells or cell conditioned media from progenitor cells are administered systematically or intratracheally to participate in cytoprotection after acute injury or for correction of pulmonary disease.

mesenchymal stem cells for treatment of lung disorders. The development of new therapeutic strategies, for example the use of endogenous lung stem cells, mesenchymal stem cells (MSCs), embryonic stem (ESs) or iPS cell-based treatments using tissue engineering will depend upon knowledge of pulmonary morphogenesis and repair. Understanding these processes will be useful in guiding studies in which the principles of lung cell biology can be applied to enhance the treatment of life-threatening pulmonary disorders.

LUNG MORPHOGENESIS

The lung is a highly complex organ that contains multiple cell types which are derived from the three layers of the early embryo, including the ectoderm, mesoderm, and endoderm. All germ layers contribute to the diverse cell types that form the respiratory tract. Lung morphogenesis begins as evagination of foregut endodermally derived cells along the anterior foregut. Epithelial cells of the lung primordia proliferate and migrate into the splanchnic mesenchyme influenced by paracrine and cell-cell signaling between the epithelium and mesenchyme. These interactions are regulated by numerous signaling and transcriptional pathways that instruct cell proliferation, migration, and differentiation (see reference [1] for review). Rapid progress is being made regarding the genes and processes by which the trachea, bronchi and peripheral airways are formed during lung morphogenesis, knowledge that has been useful in programming of ES cells or induced pluripotent stem (iPS) cells into lung epithelial cell lineages *in vitro*.

Understanding the early events directing the normal embryonic development are highly relevant to methods to program ES or iPS cells to produce progenitor cells that can be expanded for cell-based therapies. During early embryonic development, pluripotent cells are

sequentially programmed to produce endoderm, anterior foregut endoderm, further restricted to respiratory epithelial progenitor cells that produce a diversity of respiratory epithelial cells that line the conducting airways and the alveoli, (Fig. 73.2). High levels of Fibroblast Growth Factor (FGF) signaling from the cardiac mesoderm induce the commitment of foregut endodermal cells to produce lung epithelial cell progenitors. Suppression of Bone Morphogenetic Protein (BMP) signaling, receipt of high levels of FGF, and expression of specific Wnt ligands (Wnt2A/B) serve to specify lung epithelial cell progenitors from those destined to form other organs along the gut tube, e.g., thymus, thyroid, the gastrointestinal tract, liver, and pancreas [1–3]. The commitment, restriction, and differentiation of endodermal cells to form the lung buds, are first marked by the expression of the thyroid transcription factor-1 (also termed NKX2.1) [4]. While the trachea and main bronchi are found in TTF-1 gene deleted mice, branching morphogenesis and differentiation of pulmonary epithelial cell types fails to occur. Complete separation of the trachea and esophagus requires TTF-1, as dose formation of

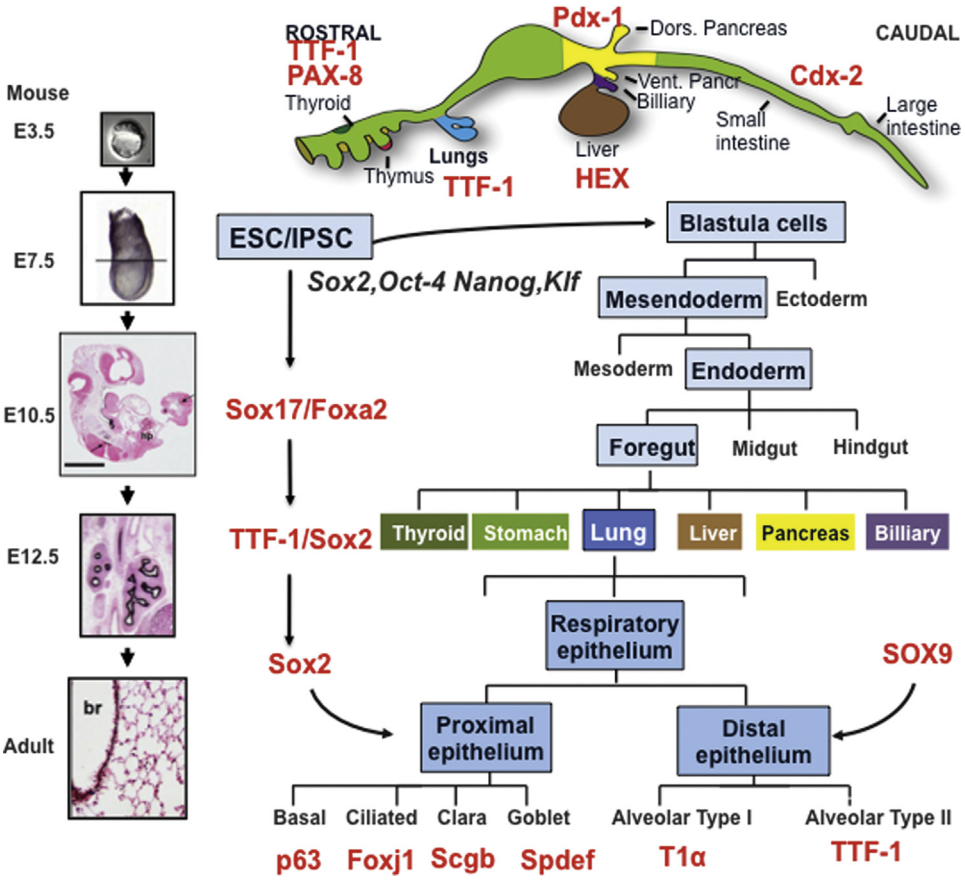

FIGURE 73.2

Stage-specific specification and differentiation of the foregut endoderm during lung formation. The tracheal-bronchial tree and alveolar regions of the lung are derived from progenitor cells located in the anterior (rostral) foregut endoderm. Shown (left) is staining of FOXA2 in respiratory epithelial cells during mouse embryonic lung development from E10.5-adulthood. Cells along the embryonic gut tube are sequentially programmed by inductive signaling between mesenchyme and endoderm to initiate organ formation at specific sites along its rostral-caudal axis. Endodermal cell commitment is marked by expression of SOX17 and FOXA2, as the A-P axis of the embryo is initially formed near the time of implantation. Lung cell specification (blue) from the esophagus (green) is marked by differential expression of TTF-1 (Nkx2.1) and SOX2. Conducting airways express SOX2 and are distinguished from progenitor cells that form the distal epithelium, the latter marked by expression of SOX9. Later in fetal lung morphogenesis, basal (Trp63), ciliated (Foxj1), serous/Clara cells (Scgb) and goblet cells (Spdef) differentiate from airway progenitors. Cells in the distal epithelium differentiate into type I and type II epithelial cells that form the alveolar, gas exchange region critical for respiration after birth. Sequential specification and differentiation of lung progenitor cells during normal lung morphogenesis provides the molecular pathways by which ES or iPS cells are being reprogrammed to differentiate into pulmonary cells *in vitro*. Lung epithelial selective markers are shown in red. Neuroepithelial cells are not shown. (E) represents embryonic day of mouse development.

conducting airways and the lung periphery. In the mouse, lung tubules, stromal, and vascular components typical of the normal lung are lacking in the absence of TTF-1 [5]. The activity of TTF-1 is influenced by its interactions with other transcription factors and co-factors that regulate gene expression and differentiation in the various cell types that line the respiratory tract. TTF-1 is co-expressed with a number of transcription factors, including FOXA2, FOXA1, GATA-6, NFATc3, NF-1, SOX and ETS family members (to name only a few) to regulate gene expression and cell differentiation during formation of the respiratory epithelium [6]. The proximal-peripheral patterning of the embryonic lung is established early in lung morphogenesis [7], SOX2 directing differentiation of conducting airway epithelial cells while SOX9 identifies cells that form the alveolar regions of the lung [1]. Signaling between the lung mesenchyme and respiratory epithelial cells, and between subsets of cells establishes the number and sites of distinct cell types that line conducting and alveolar regions along the cephalo-caudal and ventral-dorsal axes of the lung.

Integration of signaling and transcriptional pathways during lung formation

A number of signaling molecules mediate the autocrine and paracrine signals that are precisely regulated during lung formation. Wnt-β-catenin [8,9], FGF [10–12], SHH [13,14], BMP4 [15], Notch [16] and retinoic acid [17] pathways play critical roles during formation of the lung. The sites and functions of ligands and receptors that determine the multiple signaling centers required for precise temporal-spatial control of cellular behaviors during lung morphogenesis are complex and are in active study at present. Elucidation of these pathways will be relevant to understanding the control of proliferation and differentiation of embryonic stem cells, iPS, and ES and endogenous lung 'stem' or 'progenitor' cells that can be programmed to generate the pulmonary cells and tissue used for development of cell-based therapies for repair or regeneration of the lung.

The mature lung consists of diverse cell types

The respiratory epithelium itself consists of many distinct cell types that are characteristic of the proximal (conducting or cartilaginous) airways, as compared to peripheral airways and alveolar (gas exchange) regions of the lung, (Fig. 73.3) ([1,18] for review). The extent of the respiratory tract covered by distinct cell types varies greatly among species and during development. There are considerable species differences in the number of branches, extent of cartilaginous airways and submucosal glands, and the numbers and types of epithelial cells lining the airways. In general, similar types of respiratory epithelial cells are found in all vertebrate lungs. In human and mouse lung, cartilaginous, conducting airways are lined predominantly by a pseudo-stratified epithelium primarily consisting of ciliated, basal, goblet and other secretory cells, including Clara cells. Cartilaginous airways contain an abundance of submucosal glands consisting of multiple epithelial cell types, including myoepithelial cells and those secreting fluid, electrolytes, mucins, and host defense proteins. Conducting airways and the ducts of submucosal glands contain progenitor cells capable of rapid migration, proliferation, and differentiation to repair the airway epithelium after injury. Neuroendocrine cells are a relatively less abundant cell type and are found either as isolated cells, or as organized clusters termed neuroepithelial bodies (NEBs) found in precise anatomic regions of the conducting airways. NEBs are found in close apposition to a subset of non-ciliated airway epithelial cells that together may provide a cellular niche with unique repair capacity. Smaller, non-cartilaginous airways are lined primarily by a more simple columnar epithelium consisting of ciliated, serous and 'Clara cells,' and less abundant neuroepithelial cells. Squamous type I and cuboidal type II cells line alveolar surfaces in the peripheral lung. Type II cells synthesize and secrete pulmonary surfactant necessary to maintain lung inflation during the respiratory cycle. Squamous type I cells derived primarily from type II epithelial cells or other progenitors, are found in close apposition to alveolar capillaries to facilitate gas exchange.

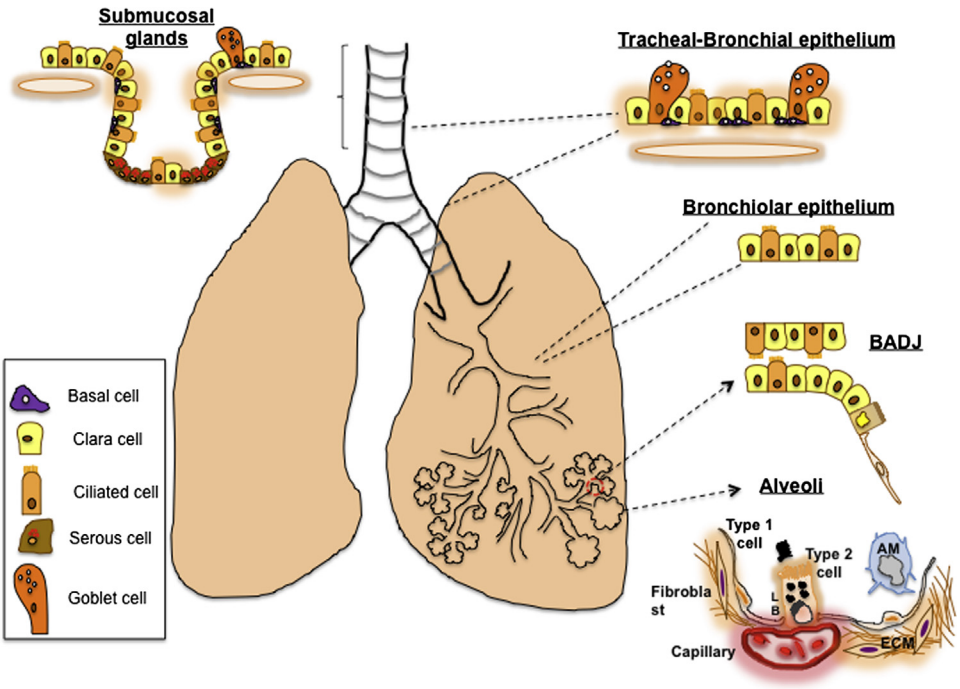

FIGURE 73.3

The complexity of the respiratory epithelium. Epithelial surfaces of the larger cartilaginous airways are generally lined by a pseudo-stratified columnar epithelium consisting of basal, ciliated, goblet, Clara (non-ciliated secretory cells) and less abundant neuroepithelial cells (not shown). Submucosal glands are present along the tracheal-bronchial tree and consist of numerous cell types including basal, ciliated, and various secretory cells. Smaller airways, including bronchioles and respiratory acinar ducts, are lined by a single columnar or cuboidal epithelium that consists primarily of ciliated and non-ciliated secretory cells. In the alveoli, peripheral lung epithelial cells become cuboidal (type II) or squamous (type I) as the peripheral saccules form.

Respiratory epithelial cells create a tight alveolar-capillary barrier that restricts fluid from the alveolar surface, a barrier critical for lung formation that is lost following severe lung injury. After birth, the diversity of cells lining the respiratory tract is strongly influenced by acute and chronic injury. For example, goblet cell and squamous metaplasia are associated with asthma, chronic obstructive pulmonary disease (COPD) and other pulmonary disorders. The pattern of stromal tissue and the pulmonary vasculature also varies greatly along the proximal-distal axis of the lung. Proximal regions of the lung are supported by cartilage, nerves, larger pulmonary arteries, veins, and lymphatics, as well as tracheal-bronchial glands. Pulmonary vessels (arteries, veins, capillaries and lymphatics) are precisely aligned with the lung tubules and are supported by smooth muscle and a diversity of lung fibroblasts or 'stromal' cells. In alveolar regions, mesenchymal cells are less abundant and endothelial cells within the microvascular network come in close contact with type I epithelial cells. The alveoli are supported by a network of elastin. Autocrine-paracrine and direct cell-cell interactions among the various cell types control proliferation and differentiation during lung formation. Mitotic activity of the lung parenchyma is generally high during embryonic and postnatal development of the lung. In contrast, proliferative rates in the mature lung are remarkably low, cell turnover of lung parenchymal cells occurring over many months in the absence of lung injury.

Evidence supporting lung regeneration

In general, cell proliferation in the mature lung is remarkably quiescent. Proliferative rates of all types of pulmonary cells are remarkably slow in stark contrast to the rapid and ongoing turnover of cells lining the gastrointestinal tract or skin, wherein stem cells proliferate, differentiate, migrate and senesce within days. Rates of cell proliferation in the lung are low unless subjected to injury, chronic disease or resection, after which the lung is capable of remarkable proliferative

responses. Perhaps the most dramatic of these is the post-pneumonectomy response after ipsilateral lung resection that is followed by rapid proliferation of many cell types in the remaining lung tissue, serving to regenerate lung volume and function within several weeks after resection (see [19] for review). Regeneration of well-perfused, functional respiratory units occurs in rodent models and is most efficient in younger animals. Lung regeneration depends on available space (chest volume), likely indicating that tissue stretch is an important component of the growth response. Although evidence of lung regeneration in human is less well documented, there is some evidence that the mature human lung is capable of regeneration [20]. Animal studies support the concept that retinoids and growth factor signaling can enhance or are required for efficient lung regeneration, respectively, providing the scientific framework for development of new therapies for lung disease [19].

The diversity of lung epithelial progenitor/stem cells

Reagents useful for immunohistochemistry, cell sorting, and *in situ* hybridization are being utilized to identify specific lung epithelial cell types and to identify progenitor cells useful for tissue-based therapy. Cell survival, proliferation, and capacity for self-renewal and differentiation differ amongst various cell types and regions within the lung [18,21,22]. In conducting airways, the epithelium is repaired primarily by proliferation of basal cells and other non-ciliated epithelial cells. Basal cells express p63 (Trp63); variably express cytokeratins (e.g., CK5, CK14). Basal cells are capable of self-renewal and differentiating into ciliated, goblet, and other secretory cells in the airways *in vivo* and *in vitro* [18,23,24]. After severe lung injury, basal cells lining the airways and within the ducts of tracheal-bronchial glands migrate along the airway surfaces, proliferate and differentiate to contribute to repair the respiratory epithelium [18,25,26]. 'Label-retaining cells' or LRCs are present in distinct anatomic regions where they are relatively protected, perhaps providing slow cycling progenitor cells for repair of the respiratory epithelium. Basal cells and various subsets of non-ciliated epithelial cells are capable of rapid migration, proliferation and differentiation during repair of the conducting airways and are likely to form a hierarchy of cells with varying stem/progenitor cell capabilities. In conducting airways, regional differences in proliferation and progenitor cell behavior occur in NEBs near bifurcations of airways, in bronchoalveolar ducts, and along vessels and stroma. Mitotic activity is induced throughout the airways following injury, although selective anatomic regions of cytoprotection or enhanced proliferative capacity may serve unique functions during lung repair and depend upon the nature and severity of injury [27]. Evidence indicating that many endogenous cells play a role in lung repair is derived from experiments in animals models exposed to toxicants (naphthalene, SO_2) [18]. Purified basal cells and/or Clara cells re-establish a complex respiratory epithelium consisting of numerous cell types *in vitro* [28] including ciliated, non-ciliated epithelial cells, and goblet cells. In the peripheral lung, type II alveolar cells express surfactant proteins, e.g., proSP-C, proSP-B, and other surfactant proteins, and are capable of rapid proliferation and differentiation into type I cells after alveolar injury. A distinct subset of $\alpha 6\beta 4$ integrin expressing airway and alveolar epithelial progenitor cells was identified recently in the mouse lung [29]. In the alveoli, there is evidence that both type II epithelial cells and $\alpha 6\beta 4$ integrin positive cells are recruited into the cell cycle following injury. Self-renewal and differentiation of airway basal cells is influenced by Notch signaling [18,30]. High levels of Notch activity induce secretory cell differentiation producing goblet and serous cells, while ciliated cells are produced in the absence or paucity of Notch activity.

In conducting airways, other subsets of 'stem cells' have been described in the lung including side population cells, LRCs and bronchoalveolar stem cells. 'Side population' (SP) cells are defined by their ability to actively efflux DNA-binding dye, Hoechst 33342. SP cells were identified in the embryonic and adult lung in distinct anatomic regions including the trachea and the alveolar epithelium [31–34]. SP cells are CD45 negative (non-hematopoietic), have

Hoechst efflux capacity, verapamil-sensitivity, low auto-fluorescence and are enriched in vimentin mRNA [35]. Analysis of the stem cell-like subpopulations demonstrated that SP cells present in the tracheal compartment represent a heterogeneous cell population that varies in clonogenicity, whereas alveolar SP cells are more homogenous. Some subsets of 'stem cells' in various organs have been defined by their low rate of proliferation and termed label-retaining cells (LRCs) because of their prolonged retention of BrdU or 3H thymidine after labeling. LRCs turn over slowly, are capable of self-renewal and may be an important source of progenitor cells that can further proliferate and differentiate during repair of the lung. LRCs populations are enriched in the necks of submucosal glands and in tracheal/bronchial folds in non-cartilaginous regions of the conducting airways [26,36–38]. Other potential populations of stem cells with label-retaining capacity are located near NEBs at branch points of conducting airways [39]. Stem cells within terminal bronchioles adjacent to the bronchoalveolar duct junction, termed bronchoalveolar stem cells (BASCs) may represent another subset of cells with unique proliferative and differentiation capacities [40,41]. BASCs were defined as CCSP- and pro-SPC-co-expressing cells that resist naphthalene injury and repopulate terminal bronchioles. These cells are not abundant and it is not clear at present whether the properties of self-renewal and production of daughter cells of BASCs are distinct from more abundant cells (e.g., Clara cells, alveolar type II cells) that are known to contribute substantially to repair of terminal airways and the alveoli. Lineage tracing experiments will be useful in defining the ability of these 'stem cells' to renew and differentiate into various cell populations.

In the peripheral lung, type I cells are sensitive to injury and do not proliferate. Repair of the peripheral lung depends upon alveolar epithelial type II cells. Proliferation of alveolar type II epithelial cells occurs two to three days following hyperoxia-induced injury. Subsets of these cells differentiate into type I cells to complete repair [42,43]. Recent studies support the presence of a distinct subset of alveolar cells, $\alpha 6\beta 4$ integrin positive cells, which do not express surfactant markers typical of type II alveolar cells and are capable of proliferation, self-renewal and differentiation into type II alveolar cells [29].

Thus, under physiologic conditions, repair is accomplished primarily by the proliferation of endogenous progenitor cells present in the various compartments/niches of the respiratory epithelium. There is increasing evidence that there exists a hierarchy of lung epithelial stem/progenitor cells. Identification of endogenous lung 'stem' or 'progenitor' cells, characterization of their proliferation and differentiation potentials and the development of processes to genetically repair progenitor cells capable of permanent engraftment and proliferation represent important scientific goals that will enable stem cell therapies for pulmonary diseases.

Since the respiratory tract is continuously exposed to pathogens, including viruses, bacteria, fungus, toxic particles, and toxicants, a remarkable system of innate defense has evolved to maintain pulmonary homeostasis throughout life. This is accomplished by the intrinsic integrity of the epithelial lining, mucocillary clearance, production of innate host defense molecules, and the instruction of acquired immune defenses that serve to maintain lung sterility after birth. Since many viruses and pathogens have developed strategies for targeting specific cells, vertebrates have also developed strategies to maintain cells capable of regeneration. Variation of surface molecules and distinct capacities for proliferation and differentiation of cells in protected anatomic regions provides multiple progenitor cells capable of repairing the lung. Under most circumstances, lung injury is followed by rapid repair with maintenance and restoration of normal lung structure and function. Repair of the respiratory epithelium occurs rapidly to maintain alveolar-capillary permeability and pulmonary homeostasis. Failure to repair is associated with loss of alveolar capillary integrity that can cause acute respiratory failure, as seen in adult respiratory distress (ARDS) or acute ling injury (ALI) syndromes. Repair of the respiratory epithelium primarily is dependent upon endogenous cells that line distinct regions of conducting and peripheral airways rather than bone marrow or EMT-derived progenitor cells. Epithelial cells that survive injury rapidly spread and migrate

to cover the epithelium surfaces. While the mature respiratory epithelium does not undergo rapid turnover, the respiratory epithelium responds rapidly to acute injury and many cell types are capable of re-entering the cell cycle during the repair process. While rare subsets of progenitor cells have been identified in the lung, rapid repair of the respiratory epithelium that occurs following the infection or other injury is likely mediated by the proliferation and re-differentiation of relatively abundant progenitor cells.

Selected anatomic regions of the lung likely serve as potential reservoirs of progenitor cells that are located along the airways and in the alveoli with important roles in regeneration of tissue following acute and chronic lung injury. In conducting airways, *in vitro* and *in vivo* experiments have demonstrated the ability of basal cells, Clara cells or other secretory cells to proliferate and differentiate into other respiratory epithelial cell types [18,24,37–40,44–47]. Ciliated cells contribute to repair by undergoing squamous metaplasia. Lineage tracing experiment indicate that ciliated cells are not proliferative and do not serve a role as progenitor cells after injury to airway epithelium [48]. There is evidence that both type II epithelial cells and α6β4 integrin positive cells are recruited into the cell cycle following injury [29,42]. While Trp63 positive basal cells were considered to be confined to conducting airways and ducts of submucosal glands, recent studies demonstrated expression of Trp63 in regenerating pods of epithelial cells that contributed to repair of alveoli following influenza pneumonia in the mouse [49]. Thus, there is increasing evidence of a diversity of lung progenitor/stem cells that variably contribute to repair at distinct anatomic sites following injuries of varying types and extent. Whether distinct activities of subsets of respiratory epithelial cells contribute uniquely to normal or pathological repair processes to cause chronic lung disease remain to be clarified. Understanding these normal repair processes will serve to inform the scarring and hyperproliferative responses seen following catastrophic or chronic injury that lead to airway epithelial metaplasia, dysplasia, and hyperplasia that are associated with lung fibrosis, remodeling, and cancer.

Cell plasticity and reprogramming

Studies in mouse models and recent major advances in ES and iPS cell technology have revolutionized our understanding of cell plasticity and reprogramming, concepts that are creating the foundations for regenerative medicine applicable to many organs including the lung [50]. Early in foregut endoderm differentiation, there is considerable cell plasticity among foregut epithelial cells. The anterior-posterior axis of the early endoderm is strongly influenced by BMP/Activin and Wnt signaling. Expression of β-catenin-LEF caused differentiation of embryonic pulmonary epithelial cells into cells with gastrointestinal tract selective markers [51]. Later in lung development, mesenchyme obtained from the peripheral lung can reprogram epithelial cells from the trachea to differentiate with peripheral alveolar cell types [52]. Likewise, expression of SOX2 in alveolar cells of the adult mouse reprograms cells in the peripheral lung to proximal epithelial subtypes, demonstrating remarkable cellular plasticity even in the mature lung [53]. While normal differentiation processes occur during defined developmental stages, foregut endodermal cells display remarkable plasticity during the embryonic period and *in vitro*. Recent findings, that embryonic stem cells (ESCs) and iPS cells can be directed to differentiate into respiratory, epithelial cell-like cells demonstrate the potential to produce lung progenitor cells by recapitulating some of the processes that direct normal differentiation of respiratory epithelial cells during lung formation [50]. Culture conditions including nutrients, hormones, matrices, transplantation, or co-culture with mesenchymal cells provide instructive signals that recapitulate aspects of normal lung development. Understanding these developmental programs has been useful in engineering iPS and ES cells capable of differentiation into lung epithelial cell lineages, see Fig. 73.3.

EXOGENOUS SOURCES OF STEM CELLS: ES, iPS, HSC, and Mscs

'Stem cell' is a term used to define cells that are capable of both self-renewal and production of daughter cells with capacity to differentiate and contribute to tissue formation and repair. ESCs retain the potential to differentiate into cells forming all germ layers, e.g., ectoderm, mesoderm and endoderm. Pluripotent stem cells, exemplified by ESCs, are derived from the inner cell mass of the embryo, and are capable of proliferation and contribution to the formation of multiple tissue types. The term 'stem cell' is used with varying precision amongst fields of study, for example, being more precisely defined for embryonic, bone marrow stem cells but perhaps more loosely in tissues in which precise cell types and lineage relationships are not known. Hematopoietic stem cells (HSCs) in the bone marrow are considered stem cells, when a single cell regenerates the entire hematopoietic system after transplantation. Bone marrow-derived MSCs are considered 'multipotent' when exhibiting capacity for differentiation into two of the germ layers. Distinct stem cell properties have been described for HSCs, MSCs, tissue-derived stem cells, circulating endothelial progenitor cells (EPCs) that are capable of differentiating into endothelial-like cells *in vitro* [54], SP cells, and multipotent adult progenitor cells (MAPCs) (see [55] for review, and various contributors in this book). Capacities of various stem/progenitor cells to contribute to the diverse cell types present in the lung are not well defined at present but are under intense investigation.

Embryonic and induced pluripotent (iPS) stem cells

Embryonic stem cells (ES cells) provide a remarkable source of cells that can be programmed by various *in vitro* cell culture conditions to differentiate to organ-specific cell types, including those with respiratory epithelial cell characteristics [50,56–58]. While ES cells are subject to clinical and ethical constraints and are extremely limited for investigative and clinical applications, recent advances in cellular reprogramming have revolutionized our concepts regarding cell plasticity, enabling generation of patient-specific, non-immunogenic cells for the study and treatment of human disease, Figs. 73.1 and 73.4. Differentiation of respiratory epithelial cells requires that iPS/ES cells acquire endodermal, foregut, then pulmonary specific characteristics following the normal developmental patterning of the foregut endoderm. For example, TTF-1 is useful cell markers for early differentiation of lung epithelial cells. ProSP-C selectively stains type II alveolar cells in the lung. Foxj1 and β-tubulin are useful in identifying ciliated cells. Serotonin, CGRP (Calcitonin gene related peptide), and bombesin are used as markers of neuroepithelial cells. Non-ciliated secretory cells express Secretoglobin1a1 and Secretaglobin3ac. Increasing numbers of cell markers are used to identify and sort lung tissue-specific progenitor cells and for study of differentiation of iPS/ES cells into pulmonary cell types. iPS/ES cells are cultured under various conditions to enhance respiratory epithelial cell differentiation. Cultures of iPS/ES cells in defined media, at air/liquid interfaces or in organoids are being used to produce a complex, highly differentiated epithelium with properties of conducting and peripheral airways that contain ciliated, basal, and secretory and alveolar-like cells [58]. Co-culture of ES cells with embryonic lung mesenchyme enhanced the differentiation into cells with respiratory epithelial cell characteristics [59]. Nuclear extracts obtained from A549 adenocarcinoma cells, and mouse lung epithelial (MLE)-12 cells (an SV40 large T-antigen immortalized cell line) increased pulmonary differentiation of ESCs *in vitro* [60]. Differentiated alveolar type II-like cells were produced after genetically modified ES cells were selected on the basis of expression of TTF-1 or grown under conditions that selected for the survival of respiratory epithelial cell phenotype [61]. Differentiation of ES cells into respiratory epithelial-like cells has been associated with the expression of pro-SP-C, CFTR, α1AT, cytokeratin, ZO-1 and others [59,61–63]. It remains unknown whether stem cells or 'differentiated' respiratory epithelial cells can be re-introduced into the lung to contribute to normal lung function or repair. Initial experiments in which ES/iPS derived cells are administered to mice following severe lung injury caused by bleomycin, supports their ability to engraft and ameliorate lung injury and fibrosis. While ES/iPS cells expressing markers

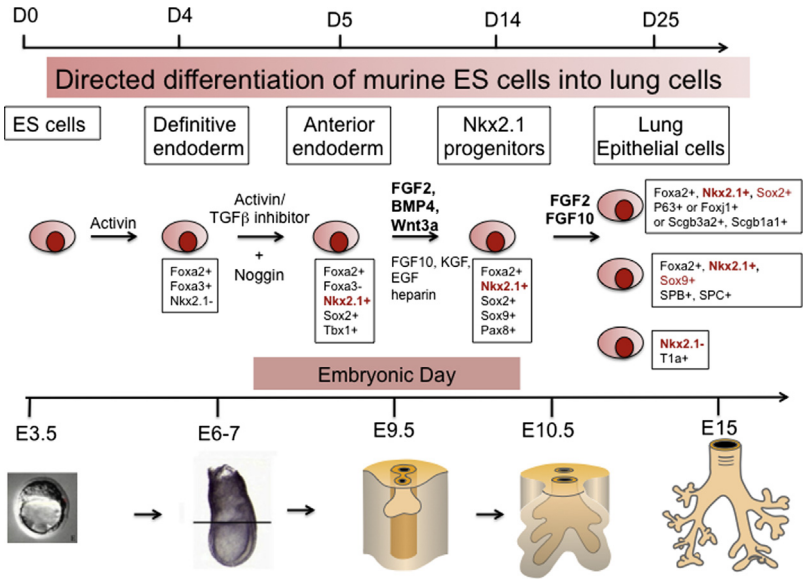

FIGURE 73.4

iPS/ES cells are reprogrammed into respiratory epithelial-like progenitors. Schematic representation of murine ES cells reprogramming adapted from the study by Mou et al. [57]. Directed differentiation of murine ES cells into lung epithelial cells was achieved by activation/inhibition of signaling and transcriptional pathways occurring during normal lung development. In general, the normal pattern of endoderm, foregut endoderm to lung formation is recapitulated during the *in vitro* differentiation of iPS/ES cells. Sequential expression of transcription factors marking and regulating specific stages of differentiation are indicated in association with the signaling molecules used in culture. Development stages of mouse embryogenesis are shown below each phase of differentiation. (E) is embryonic day. (D) represents day in culture.

specific for respiratory cell types have been produced, it is unclear whether these cells recapitulate gene expression or other biological functions comparable to endogenous lung epithelial cells. Engineering ES/iPS cells capable of engrafting, proliferating, differentiating and repopulating the respiratory epithelium represents a considerable technical challenge.

Advances in reprogramming of somatic cells into induced pluripotent stem (iPS) cells are transforming biology and medicine [64–67], enabling production of patient-specific iPS cells useful for study of the pathogenesis of disease and for cell-based therapies for many disorders. The success of exogenous stem cell-based therapies for the lung and other organs will depend on the ability to isolate, culture, and genetically modify cells capable of influencing tissue repair or directly engrafting, proliferating and differentiating into cells that can contribute to organ function. Because of their capacity for self-renewal and pluripotency, it may be possible to generate large numbers of iPS cells for cell-based therapy. Patient-specific stem cells will be non-immunogenic and therefore uniquely applicable for cell-based therapies. Reprogramming somatic cells into pluripotent stem cells overcomes social and religious barriers to the use of ES cells. The ability to reprogram iPS cells capable of safely contributing to organ function *in vivo* remains a considerable scientific challenge. Nevertheless, patient-specific stem cells can be genetically modified for cell-based therapy that will substantially bypass the immunologic barriers intrinsic to organ transplantation. iPS cells are now readily generated from patients' skin, blood or other cells, and can be differentiated into multiple cell types *in vitro*. Gene transfer or genomic modification, e.g., utilizing non-viral and viral vectors, TALENS or Zn-finger nucleases, provides the technology to repair genetic disorders in the future.

Application of mesenchymal stem cells (MSCs) for treatment of lung disease

MSCs have been derived from bone marrow stroma, umbilical cord blood (UCB) [68], whole blood and other organs (see also other chapters in this book) [68–74]. Adult bone

marrow-derived MSCs have limited capacity to proliferate *in vitro* but are able to grow and differentiate into bone, cartilage, fibrocytes and other cell types [68]. MSCs and HSCs isolated from umbilical cord blood (UCB) contain pluripotent hematopoietic stem cells that are readily isolated at birth. UCB stem cells have clinical and logistical advantages for cell-based therapy and are routinely used for bone marrow transplantation [71]. UCB cells are collected without risk to the donor and can be readily stored. UCB cells are amenable to Human Leukocyte Antigen (HLA) matching for allogeneic transplantation. MSCs are present in UCB and are relatively abundant (0.1%) compared to adult bone marrow, wherein the frequency of the MSCs ranges from 0.01% to 0.001% of total nucleated cells [70]. UCB-derived MSCs produce multipotent colony, and have longer telomeres than adult BM-derived MSCs perhaps providing an advantage for longevity as progenitor cells [71,72]. MSC derived from UCB are expanded *in vitro* and differentiate into multiple cell types (e.g., chondrocyte, osteocyte, myocyte and adipocyte lineages) [69,73,74]. 'Fibrocytes' are bone marrow-derived mesenchymal progenitors that express cell surface markers indicating hematopoietic origin but express collagen and other matrix proteins characteristics of fibroblasts [75,76]. Circulating fibrocytes migrate to wounds where they contribute to populations of fibroblasts, lipocytes and myofibroblasts [77]. Circulating epithelial progenitor cells (CEPCs) have been identified in bone marrow and blood by expression of CD45, cytokeratin-5 and CXCR4 [78]. CEPCs are recruited to the airways in response to a gradient of CXCL12 following lung injury. CEPCs enhanced re-epithelialization of tracheal transplants *in vitro*, inhibiting squamous metaplasia resulting from the excessive proliferation of the resident epithelial progenitor cells during repair of the airway epithelium [78]. FGF-7 enhanced the migration of CEPCs to proximal airways following injury [79].

DO BONE MARROW-DERIVED CELLS DIRECTLY CONTRIBUTE TO REPAIR AND PROVIDE PROTECTIVE FACTORS?

Stem cell-based therapies may be designed to provide cytoprotective factors to enhance repair by endogenous cells or alternatively to provide progenitor cells (or genetically altered progenitor cells) capable of engraftment and permanently contributing to the lung ([80] for review). Long-term engraftment and proliferation of stem cells for repair following injury has not been accomplished in the laboratory. Evaluation for Y-chromosome-containing type II pneumocytes using Fluorescence *In Situ* Hybridization (FISH) technology indicated that repopulation by bone marrow-derived stem cells or their progeny may occur at a low frequency in the lungs [81,82]. Early studies of potential bone marrow contribution to lung repair reported that bone marrow-derived cells from male donors readily engrafted, forming alveolar and bronchiolar epithelial cells into female lung [83]. In a model of bleomycin-induced lung injury, MSCs were proposed to differentiate into alveolar type I cells. However, later studies have not supported these initial concepts. Engrafted MSCs fail to proliferate and differentiate into pulmonary epithelial cells. Cell fusion between the MSCs and endogenous epithelial cells or aberrant co-localization of cell markers perhaps related to insufficient technical resolution may have accounted for these earlier findings [84–86]. While it is well known that hematopoietic cells migrate to the lung and repopulate alveolar macrophages following bone marrow transplantation, definitive data supporting engraftment of bone marrow-derived cells capable of proliferation and differentiation in the lung has not been provided.

Systemic or tissue directed infusion of MSCs has been proposed for therapies that do not depend on long-term engraftment but influenced by provision of MSCs or their products. MSC conditioned media ameliorated cell injury from cigarette smoke [87]. In the kidney, in a rat model of ischemia/reperfusion, MSCs infusion enhances the restoration of renal function [88]. Recovery after MSCs infusion was mediated via paracrine actions with decreased expression of proinflammatory cytokines, and increased anti-inflammatory cytokines [89]. Pulmonary hypertension and endothelial function improved after engraftment of MSCs

into the injured lung in a process likely related to transient paracrine effects rather than replacement of endothelial cells [90,91]. Recent studies suggest that epithelial cell survival following severe lung injury may be initiated by mitochondrial transfer from MSCs to lung cells [92]. The utility of MSCs for therapy of pulmonary injury, bronchopulmonary dysplasia and pulmonary hypertension is being actively studied in animal models and in the clinic at present [93].

Delivery of stem cells into the lung

The efficient recruitment or delivery of stem cells into the lung represents another challenge for the application of stem cell therapy to the lung. There is at present little evidence that exogenous cells readily engraft and permanently contribute to normal lung function. While normal and tumor cells readily enter the pulmonary vasculature and can embolize or metastasize to the lung, engraftment appears to occur with very low frequencies. Previous studies with MSCs or marrow-derived cells demonstrated extremely low efficiency of integration into the normal lung epithelium or vasculature after intravenous injection. In most studies, engraftment of bone marrow and MSCs into the lung does not occur unless the lung was severely injured. The mechanisms underlying this observation are unclear. Secretion of growth factors and cytokines may enhance recruitment of MSCs to sites of injury. *In vitro* experiments demonstrated that cell suspensions from the injured lung contain chemoattractants and growth-stimulating factors including hyaluronan (CD44), osteopontin and SDF-1α [94]. Repair of the lung after bleomycin injury was enhanced after intravenous administration of bone marrow-derived cells and was associated with an increased expression of anti-inflammatory cytokines (IL-10, IL-1ra, IL-13), G-CSF and GM-CSF [95].

Intratracheal delivery of stem cells

Since the surface of the lung is directly accessible via the trachea, stem cells may be directly injected intratracheally. Intratracheal injection of bone marrow-derived mesenchymal cells decreased bacterial and bleomycin-induced lung injury [96,97]. The beneficial effects of MSC were independent of the ability of the cells to engraft in the lung and were proposed to be mediated by paracrine effects modulating inflammation. Intratracheal injection of alveolar type II cells decreased fibrosis after induction by bleomycin [98]. Engrafted alveolar type II cells did not contribute to alveolar epithelium repopulation but reduced collagen deposition and fibrosis development. The remarkable structural integrity of the respiratory epithelium is likely to limit the ability to introduce exogenous cells and may require depletion of endogenous cells to provide 'space' for engraftment. The success of bone marrow transplant depends on marrow ablation creating space for engraftment. Inhibition of c-kit, the receptor for the stem cell factor, by ACK2 antibody treatment depleted 99% of the host HSCs enhancing the engraftment of donor HSCs in the host stem cell niches [99]. Development of safe methodologies for providing the cellular space required for the engraftment of stem cells into the lung will likely be required for success of cell-based therapies dependent upon engraftment of cells into the respiratory epithelium.

Recruitment of MSCs to tumors

The use of genetically modified mesenchymal cells has been proposed for treatment of pulmonary tumors [100–102]. 'Umbilical cord matrix stem cells' (UCMS) transplanted into Severe Combined Immunodeficiency (SCID) mice bearing lung tumors, migrated to pulmonary carcinomas [102], indicating that mesenchymal cells may represent a vehicle for delivery of therapeutic molecules to the lung. Mesenchymal cells expressing therapeutic proteins (IFN-γ, NK4, CX3CL1) decreased the growth of pulmonary tumors [103–107]. The enhancement of recruitment of MSCs to pulmonary tumors represents a promising application of cell-based therapies for the lung.

BIOENGINEERING OF LUNG TISSUES

Acellular Matrix, Bioreactors and Synthetic Biomatrices for Study of Lung Biology

Advances in the identification and purification of endogenous lung progenitor cells and the ability to reprogram iPS or ES cells into respiratory epithelial-like progenitors enable experiments designed to seed these cells into organ-like scaffolds prepared from decellularized lung tissue or on synthetic matrices. The relative simplicity of the conducting airways has enabled the clinical use of both decellularized and synthetic tracheal-bronchial scaffolds capable of supporting the airways. Macchiarini et al. seeded the grafts with MSCs and applied growth factors and chemokines to enhance cell recruitment and proliferation to both bioengineered or decellularized tissue grafts that have been used to replace diseased airways in selected patients [108]. These airway grafts are rapidly vascularized and less rapidly epithelialized. Though considerably more complex in cellularity and architecture, similar procedures are being utilized to create acellular lung scaffolds that are seeded with lung cells, reperfused and ventilated in bioreactors or transplanted to sites compatible with continued growth and differentiation [29,109–113]. Lung epithelial progenitor cells bind to the de-cellularized lung scaffolds produced from cadaveric human or animal lung tissues. Endothelial cells are instilled into the vascular compartment and perfused to generate a capillary bed. Diverse epithelial cell types bind to and proliferate on the scaffolds. Conducting airways and alveolar surfaces become populated with the appropriate cell types characteristic of each region of the lung. Short-term physiological activity was demonstrated after orthotopic transplantation of lung tissue prepared on decellularized matrices, providing a model system with which to study lung cell biology and to test the feasibility of using various lung cell preparations ES/iPS cells as precursors [112]. Such experimental approaches seek to generate functional lung tissue on the decellularized lung matrices. Completely synthetic scaffolds and microfluidic chambers in which pulmonary cells are subjected to shear forces consistent with both ventilation and perfusion also hold promise for study of lung regeneration and repair.

Differentiation of iPS and ES cells to pulmonary epithelial cell lineages

Successful cell-based therapy for many genetic diseases affecting epithelial cell function (e.g., cystic fibrosis, genetic defects in genes controlling surfactant homeostasis and others) may require the permanent introduction of cells capable of maintaining stem cell activity as well as the ability to contribute to highly differentiated lung cell function. Differentiated respiratory epithelial cells have been produced from iPS, ES, and endogenous lung cells on various biomatrices, decellularized lung and tracheal-bronchial grafts, in air-liquid interface cultures and after transplantation under the renal or testicular capsules of SCID mice [57,110–113] supporting the feasibility of directing both lung epithelial cell differentiation and organ-like structure. Cell sorting or selectable markers may be used to enrich differentiating iPS cells with lung epithelial cell characteristics during preparation. For example, ES cells transfected with a Surfactant Protein C (SFTPC) promoter/NEOr fusion gene were selected with gancyclovir, enhancing the numbers of cells with respiratory epithelial cell characteristics [61]. Insertion of selectable markers directed by lung epithelial specific gene promoters, from the *TTF-1* or *SFTPC* gene loci have been used to search for lung cell differentiation [56,114]. Use of cell selection strategies and the expression of genes known to regulate differentiation of stem/progenitor cells into specific lung epithelial cell types may be used to search for specific cell types needed for treatment of pulmonary disorders. Thus, the elucidation of the signaling and transcriptional networks controlling lung epithelial cell differentiation will be useful in guiding the production of stem cells for therapy of lung diseases in the future.

CONCLUSION

Knowledge regarding the depth of physiology, genetics and cell biology of life-threatening diseases of the lung is expanding rapidly. Likewise, our understanding of development, lung morphogenesis and stem cell biology has accelerated. Together, these technical and scientific advances raise the hopes that novel therapies may be developed for life-threatening disorders in many organs including the lung. Major barriers to successful application of cell-based therapies for the lung remain. The ability to engineer lung tissue capable of enhancing respiratory function remains conceptual rather than actual, see Fig. 73.1. Nevertheless, isolation, culture and genetic manipulation of cells able to differentiate into pulmonary cell types have been achieved *in vitro*. A multiplicity of lung stem and progenitor cells has been identified and introduced into the various tissue compartments and bioreactors, providing the scientific basis for cell-based therapies for pulmonary diseases in the future.

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CHAPTER 74

Lungs

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INTRODUCTION

Pediatric and adult lung diseases are on the increase globally and represent one of the major challenges facing healthcare systems in the 21st century. In the pediatric realm, significant advances in preterm clinical care have increased the population of premature infants at risk of defective postnatal lung development due to 'chronic lung diseases of prematurity' [1], such as bronchopulmonary and alveolar capillary dysplasia [2]. In the adult realm, chronic obstructive pulmonary disease (COPD), which includes emphysema and chronic obstructive bronchitis, was responsible for more than 120,000 deaths in the US alone in 2008 [3].

Pharmacological intervention treats symptoms at the various disease stages; however, the only current viable option for patients with end stage diseases is lung transplantation. Unfortunately many patients on the transplant list die while waiting for a suitable donor organ, and those that are fortunate enough to receive a transplant have a life-long need for immunosuppression. Providing life-saving solutions for these daunting clinical challenges with cell therapies and implantable tissue constructs is the long-term goal of pulmonary regenerative medicine and tissue engineering. For the sake of simplicity, a regenerative medicine or tissue-engineering strategy can be thought of as any combination of four core elements: cells, matrices and scaffolds, growth factors or other signaling molecules, and 3D cell culture systems, aka bioreactors. In this chapter we will briefly introduce key elements of lung biology, and then address all four of these core elements, with particular emphasis on integrated strategies toward the generation of three-dimensional pulmonary tissue constructs.

LUNG FUNCTION AND ANATOMY

The lungs facilitate oxygenation of the blood by respiration, and therefore sustain life. The focus of this chapter is on the distal gas exchange units; however the lung is also an immunological interface under constant assault by inhaled particulates and pathogens [4]. The airways are the central component of the lung, organized in a branched network of conduits which decreases in diameter with each generation until reaching the terminal alveoli in the distal lung (Fig. 74.1), which have an average diameter of 200 micrometers in humans, while mice alveoli are ~50–70 micrometers in diameter [5]. Region-specific epithelial

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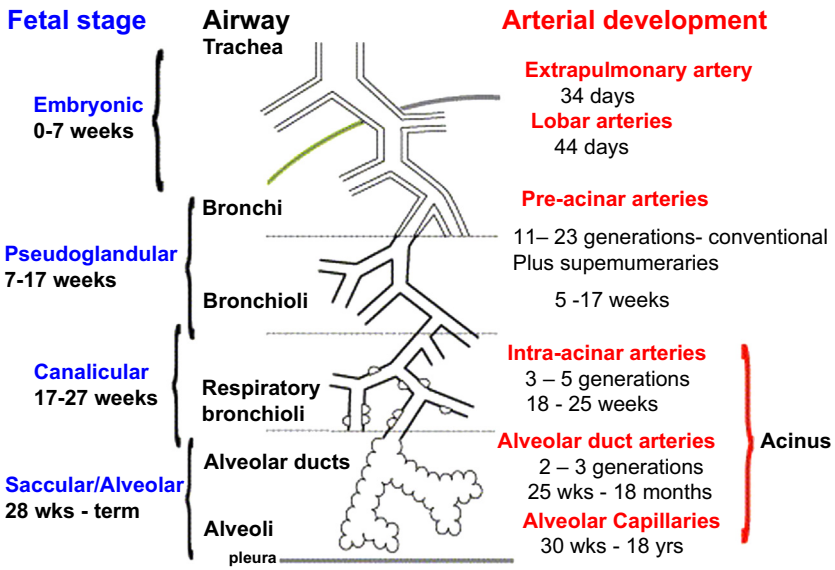


FIGURE 74.1
Temporal progression of lung epithelial branching in humans indicating the timing in weeks and transition from fractal branching of tubules into sacculation of terminal buds in the alveolar stage. *(Reproduced with permission from http://www.embryology.ch.)*

differentiation occurs along the proximal-distal axis, resulting in distinct local phenotypes that facilitate required physiological functions (Fig. 74.2a). Tracheal and upper airway epithelium is composed of columnar ciliated and mucous secretory cells, which function in concert to entrap inhaled particles in mucus that is then removed by upward beating of cilia. Pulmonary neuroendocrine cells (PNECs), comprising neuroepithelial bodies, are found throughout the upper airways, sense gas content of the air and are believed to play a potential role in mediating developmental and regenerative processes through paracrine activity of molecules such as calcitonin gene related peptide (CGRP) and gastric releasing peptide (GRP), both of which are classic markers of PNECs [6]. Clara cells are cuboidal secretory cells that reside in the bronchioles, functioning to protect the bronchiolar epithelium via secreted substances including Clara cell secretory protein (CCSP). Terminal bronchioles transition into the alveoli, marking the beginning of the distal respiratory zone (Fig. 74.2a).

In the alveoli, there are type I (AE1) and type II (AE2) alveolar epithelial cells. AE1 cells constitute the squamous, attenuated epithelium that forms the blood-air barrier interfacing

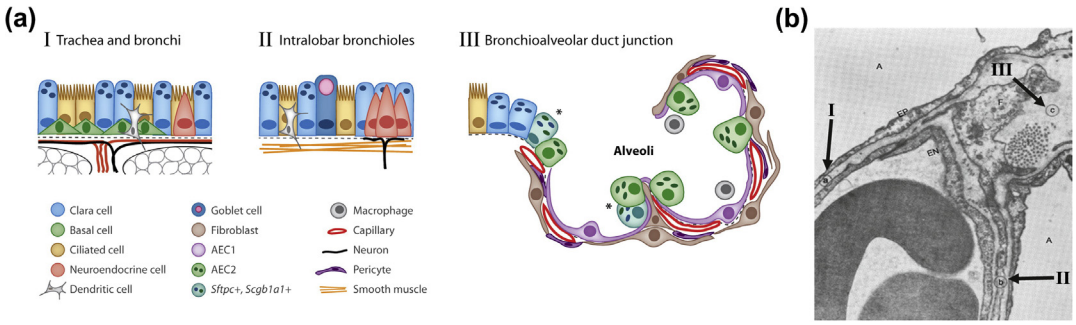


FIGURE 74.2
Cellular composition of the lung epithelium and surrounding mesenchyme at various depths in the respiratory tree. (a) Illustration of the trachea/bronchi, intralobular bronchioles, and the bronchioalveolar duct junction transition into the alveoli with all epithelial and mesenchymal phenotypes indicated. Note the transition in epithelial phenotypes as well as the tight association of capillaries with alveolar type I (AEC1) cells. *(Reproduced with permission from Rock and Hogan [9].)* (b) Transmission electron micrograph of the alveolar-capillary barrier illustrating ultrastructure that facilitates efficient gas exchange, i.e., O2 and CO_2 transport. Type I epithelial cells (EP) are seen in direct contact with a red blood cell-containing capillary lined by an endothelial cell (EN). Note the intervening basement membrane at 'i', the presence of a fibroblast 'F' with extension at 'ii', and collagen fibers in the alveolar septum at 'iii'. *(Reproduced with permission from Weibel, E.R. 1969 [10].)*

with the alveolar capillary bed. Aquaporin-5 and T1-alpha are commonly used markers of AE1 cells. AE2 cells, cuboidal and far fewer in number, produce and secrete lung surfactants, a lubricant mixture of phospholipids and proteins that regulates alveolar surface tension during inflation and deflation. Anatomically, the key functional feature of the pulmonary vasculature is its close association with the airways throughout the proximal-distal axis [7]. The vasculature accompanying the upper airways is smooth muscle-enrobed, while the alveolar capillary bed, the densest in the entire body, is comprised of attenuated capillary endothelial cells with occluding junctions and no fenestrations — conferring tissue-specific regulation of fluid transport [8]. The specialized gas exchange function of the distal lung is facilitated by seamless interfacing of the alveolar epithelium and capillaries (Fig. 74.2b).

During the process of epithelial branching morphogenesis and cytodifferentiation, the surrounding mesenchyme also differentiates in a region-specific manner. Sub-tracheal mesenchyme gives rise to cartilage, while the mesenchyme beneath the bronchi gives rise to smooth muscle and hyaline cartilage [8]. Mesenchymal cells surrounding the bronchioles are comprised of subsets of myofibroblasts and connective tissue fibroblasts that synthesize the collagenous and elastic interstitial matrix [11].

Lung mechanical properties

The lung possesses unique mechanical properties associated with respiration. Lung compliance and elastance[1] are conferred by collagenous and elastic fibers of the interstitial extracellular matrix (ECM) and are altered in various pulmonary diseases [12]. Fibrotic diseases are characterized by increased deposition of collagenous ECM, resulting in decreased compliance, i.e., a stiffer lung; thus increasing the amount of work required inflating the alveoli [11]. In emphysema there is a loss of elastin protein in the alveolar walls, decreasing lung elastance [13]. This results in a higher compliance and therefore increased difficulty exhaling. The elastic modulus of the tissue, i.e., the stiffness sensed by parenchymal lung cells, is of interest for the selection of suitable materials for three-dimensional culture of lung cells *in vitro*. The generally accepted range for elastic moduli of healthy mammalian lung tissue at rest is 5—30 kPa [14,15], a range of elastic moduli that can easily be achieved using 'soft' hydrogels. Elastic moduli for acellular collagen gels across a broad concentration range is 0.5—12 kPa [16], while that of Matrigel™ is approximately 450 Pa [17]. Other natural polymers such as alginate have been used to create hydrogels with Young's moduli on the order of 20 kPa [18]. For comparison the elastic moduli of synthetic polymer matrices, such as polylactide-co-glycolides, are about three orders of magnitude higher (in the MPa range) [19], which is less suitable for lung tissue-engineering applications. To date, no focused studies have been reported that test the effect of matrix stiffness on lung tissue formation *in vitro*.

LUNG DEVELOPMENTAL BIOLOGY 101: PRIMER FOR TISSUE ENGINEERS

Our increasing knowledge of the intricacies of embryonic development provides a sound basis for tissue-engineering strategies. Lung development begins with specification of a field within the ventral foregut endoderm. Among other early signals, cardiac mesoderm-derived FGF-2 is a key factor driving the commitment of the anterior foregut endoderm to the lung epithelial lineage, which will eventually give rise to all the diverse cell types comprising the pulmonary epithelium (Fig. 74.2a; Fig. 74.3) [20]. Thyroid transcription factor-1 (TTF-1), also known as Nkx2.1, is a definitive marker of commitment to the lung epithelial lineage. Following specification, the epithelium buds into the surrounding splanchnic mesenchyme,

[1] Lung compliance impacts how much work is required to inhale air. Lung elastance is a measure of how easily the lung recoils to its resting position after inflation, and is conferred primarily through elastin present in the alveolar walls.

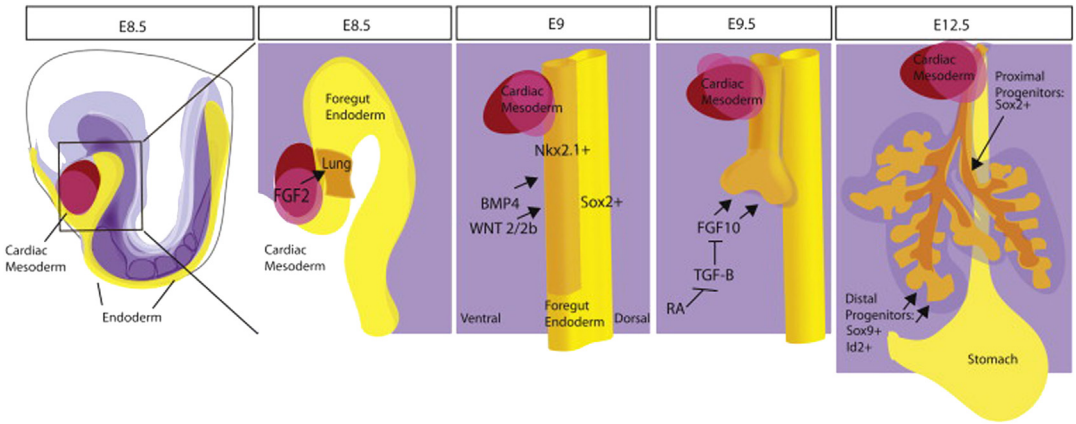
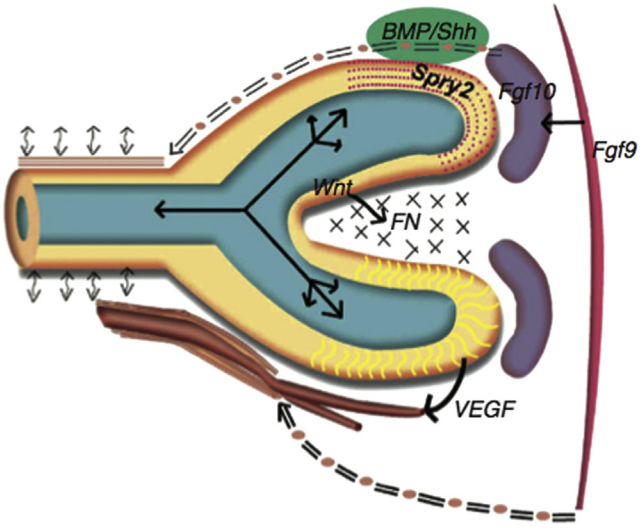


FIGURE 74.3
Specification of the foregut endoderm and important signals in the early stages of murine lung development. *(Reproduced with permission from Kadzik and Morrisey [22].)*

FIGURE 74.4
Molecular regulation of epithelial branching morphogenesis and capillary network interfacing. Fibronectin (FN) deposition occurs in epithelial clefts, the regulation of which is known to involve Wnt signaling. FGF10 expression in the distal mesenchyme guides the pattern of lung budding and is known to be regulated by mesothelial-derived FGF9. Bone morphogenetic protein (BMP) and Sonic hedgehog (Shh) signaling, as well as transcriptional activity of Sprouty-2 (Spry2) in the distal epithelium are all known to contribute to refinement of epithelial-mesenchymal interactions. VEGF released from the distal epithelium patterns interfacing with capillaries. *(Reproduced with permission from Warburton 2010 [45].)*



giving rise to the airway tree through the highly choreographed process of epithelial branching morphogenesis [7], the pattern of which is known to be regulated by mesenchymal FGF10 expression [21][2]. The upper airways undergo a series of fractal bifurcations that occurs in parallel with branching of the proximal vasculature; decreasing in diameter with each generation (altogether there are some 23–25 generations of branching) through the pseudoglandular and canilicular stages until the saccular stage after which sacculation of the terminal epithelial buds gives rise to the alveoli. The alveoli are refined and further increased in surface area through the process of secondary septation during the alveolar stage (Fig. 74.1).

[2] The lung developmental biology literature is almost entirely based on animal models, primarily mice. The precise timing and spatial patterning of the signals involved in specification and subsequent lineage commitments in humans is not known due to the inability to perform exhaustive time course embryological studies. It isn't clear whether all of the signals discovered in mice will have direct functional translation in human lung development, but the importance of some of the major players discussed here has been confirmed.

In addition to secreting morphogens that pattern epithelial branching morphogenesis, the mesenchymal compartment gives rise to fibroblasts which synthesize and deposit the fibrous interstitial matrix, smooth muscle cells of the upper airways and proximal vasculature, pericytes, and endothelial cells. The development of the proximal vasculature occurs by sprouting angiogenesis and coordinated fractal branching in parallel with the airway epithelium [23,24]. By contrast, the distal vascular plexus forms by vasculogenesis from differentiating resident endothelial progenitor cells and subsequently anastomoses with the expanding pulmonary circulation [25–27]. Also found within the interstitium are the pulmonary veins, nerves, and lymphatics. The lungs are innervated by extrinsic parasympathetic (Vagus nerve) and sympathetic (pulmonary nerve root) sources, as well as intrinsic neural crest cells during development [28,29]. The pulmonary lymphatics are believed to emerge exclusively from sprouting of pre-existing extrapulmonary sources [30]. While nerves and lymph vessels are largely absent in the distal airways, pulmonary nerves are required for autonomic control of the upper airways and relay of sensory information to the central nervous system, while the lymphatics perform essential fluid clearance functions. Re-innervation and lymphangiogenesis in tissue-engineered lungs are uncharted areas which should be of keen interest going forward.

Epithelial-mesenchymal interactions are known to guide lung morphogenesis and cytodifferentiation [31]. Members of the FGF family of cytokines are amongst the key molecular regulators of these interactions [32–34], along with bone morphogenetic proteins [35], Wnts [36], Sonic hedgehog (Shh) [37,38], and ECM molecules such as fibronectin [39], tenascin-C (TN-C) [40], and chondroitin sulfate proteoglycans. Some key signals are summarized in Figures 74.3 and 74.4. SHH-FGF crosstalk regulates VEGF-A expression [41], a key factor in patterning vascular development. Whole organ explants studies using rat lungs demonstrated increased branching morphogenesis and TN-C deposition in hypoxia (3% O2) compared to normoxia (21% O2) [40]. In a similar experimental system Van Tuyl et al. [42] reported increased VEGF expression and vascular development in explants cultured in hypoxia. Alveolarization occurs postnatally, i.e., in normoxia, however hypoxia can be a powerful driving force for the differentiation and morphogenesis of fetal lung cells and embryonic stem cell-derived progenitors. An exhaustive discussion of these developmentally relevant factors is beyond the scope of this chapter, therefore the reader is referred to the numerous textbooks and excellent literature reviews on the subject [43–46].

CELL SOURCES FOR LUNG REGENERATIVE MEDICINE AND TISSUE ENGINEERING

The field of pulmonary regenerative medicine and tissue engineering has focused heavily on identification, characterization, and application of stem and progenitor cell populations for clinical application. Some candidate stem/progenitor cell types that have been used to generate pulmonary tissue constructs are: embryonic stem cells, tissue-specific lung stem cells (fetal and adult), circulating endothelial progenitor cells, and mesenchymal stem cells derived from sources such as bone marrow and adipose tissue.

The earliest reports of differentiating rodent embryonic stem (ES) cells into pulmonary epithelium date back to 2002 [47]. Several approaches have been successfully applied to generate pulmonary epithelial cells from ES cells, such as: co-culture with mesenchyme [48], ES permeabilization and exposure to mature type II cell extracts [49], and culture with medium conditioned by A549 cells, a human pulmonary adenocarcinoma cell line, or in defined medium in with high dosages of FGF-2 [50]. More recently, researchers focused on defining culture conditions for optimal generation of definitive endoderm [51], in order to enrich the amount of cells competent to become lung. For example, in extending previous studies that emulate paracrine signaling during embryonic development, Longmire et al. [52] and Mou et al. [53] developed stepwise differentiation protocols that mimic the temporal progression of key signaling factors during lung development *in vivo*. With all of the incremental progress

many challenges still remain: The efficiency of differentiation protocols needs to be improved, functional efficacy and, more importantly, safety of derived populations needs to be assessed. The vast majority of work has focused on lung epithelium; however, directed, efficient differentiation of the pulmonary vasculature and connective tissue cells will also be required [54]. Furthermore, the entire repertoire of proximal-distal cytodifferentiation in the various tissue compartments and up to 40 different cell types of the lung may ultimately have to be accounted for.

The existence of peripheral airway stem cells, both bronchioalveolar stem cells (BASC) and AE2 progenitor cells, in rodents and humans has been extensively documented and reviewed [55–58]. A recent report of the existence of a subset of multipotent human lung stem cells, described as $CD117^{+}/CD90^{-}$ interstitial cells, that are putatively capable of generating all cell types and tissues in the lung remains highly controversial [59,60]. When injected into the cryo-injured lungs of recipient mice, these cells reportedly give rise to human airway and vascular structures integrated within the host tissue. If confirmed this finding might have tremendous therapeutic implications, representing an autologous source of cells, which could potentially be harvested from small biopsies, expanded, and used to reconstitute diseased or damaged tissue. In an exciting recent report, human iPS cells differentiated into proSpC+ alveolar type II lineage cells were shown to engraft in a murine model of acute lung injury [61].

Bone marrow-derived mesenchymal stem cells (MSCs) are the only class of stem cells, to date, that have been investigated as candidates for pulmonary regenerative medicine in humans. Osiris Therapeutics recently completed a phase II clinical trial testing the safety and efficacy of intravenous infusion of *ex vivo* cultured adult human MSCs (Prochymal™) for the treatment of moderate to severe chronic obstructive pulmonary disease (COPD) (http://clinicaltrials.gov/ct2/show/NCT00683722). MSCs are believed to function primarily as support cells, secreting paracrine factors that may stimulate both epithelial regeneration [62] and angiogenesis [63]; as well as playing an immunomodulatory role, acting to reduce inflammation and promote tissue healing [64,65]. Major advantages of MSCs include: relative ease of autologous sourcing [66], documented immune tolerance of allogeneic sources, and lack of tumorigenecity. An additional class of potentially relevant marrow-derived progenitor cells is the so-called 'circulating endothelial progenitor cells' [67], though contributions of these cells to lung vascular development and regeneration are not well-defined.

SCAFFOLDS AND THREE-DIMENSIONAL PULMONARY TISSUE CONSTRUCTS

In addition to development of cell therapies based on infusion of stem and progenitor cells, there has been significant progress toward the generation of three-dimensional pulmonary tissue constructs. Tracheal and upper airway tissue engineering have enjoyed major translational successes. For example, there have been several reports of creating bronchial tissue equivalents *in vitro* as models for studying topics such as stem cell differentiation and asthma [68,69]. The first tissue-engineered tracheal replacement in human was performed in 2008 utilizing allogeneic decellularized trachea seeded with the patient's own epithelial cells and mesenchymal stem cell-derived chondrocytes [70]. As the next consequential step, Macchiarini et al. have also performed a successful transplant of tissue-engineered trachea generated using an elastic nanocomposite scaffold [71]. This year (2012), Elliot et al. reported a two year follow-up study on a 12 year old boy that received a tissue-engineered trachea comprised of decellularized donor trachea seeded with autologous MSCs and patches of bronchial epithelium [72]. Trachea and the primary bronchus are among the few examples of engineered tissues successfully implanted into humans; however, the remainder of this chapter will focus on the ongoing efforts to engineer the lung as a whole, with particular emphasis on the alveolar compartment.

Naturally-derived scaffolds

The earliest reports of three-dimensional culture of lung cells were published by Douglas et al. in the 1970s [73]. These seminal experiments demonstrated that quasi 3-D culture of rat fetal lung cells on a collagen matrix with appropriate growth factor supplementation drove formation of alveolar-like cystic structures with extended maintenance of epithelial cell differentiation *in vitro*. The goal of the work was to develop models for studying pulmonary epithelial cell biology; however, these systems were largely overlooked in favor of considerably less complex 2D culture models.

Some 30 years later, Andrade et al. utilized Gelfoam™, a gelatin-based porous sponge as a model matrix for engraftment of rat fetal lung cells into the lung parenchyma of adult rats [74]. Survival and continued proliferation of pre-labeled fetal lung cells was maintained for up to 35 days *in vivo*, both within the Gelfoam™ sponge and at the interface with host lung parenchyma. The authors reported low levels of local inflammatory response and the presence of graft-derived epithelial and endothelial cells, as well as neovascularization within the Gelfoam™ construct. In a related set of experiments, Chen et al. showed that porous collagen type I-chondroitin-6-sulfate scaffolds supported the formation of alveolar-like structures by disaggregated rat fetal lung cells *in vitro* [75]. In line with the known role of chondroitin sulfate proteoglycans (CSPGs) in lung development [76], these *in vitro* findings suggest that incorporation of CSPGs into biomimetic matrices might be beneficial for driving lung epithelial morphogenesis.

Compliant natural ECM hydrogels such as Matrigel™ and collagen type I gels possess biochemical cues and mechanical properties that are permissive to three-dimensional organotypic morphogenesis of lung cells and maintenance of epithelial cytodifferentiation. Mondrinos et al. demonstrated that growth factor depleted Matrigel™ in combination with basal serum-free medium devoid of exogenous growth factors supported the formation of lumen-containing spherical cystic epithelial structures by mouse fetal lung cells, while addition of FGFs 10, 7, and 2 to the serum-free culture medium stimulated budding and sacculation of epithelial structures, a process akin to the early saccular stage of lung development [77]. Subsequently, Mondrinos et al. went on to show that simple collagen type I hydrogels in conjunction with the above mentioned combination of growth factors of the FGF family supported extensive epithelial budding and sacculation and robust endothelial network formation [78]. As an important step toward recreating the respiratory unit *in vitro*, this combination of matrix and growth factors also facilitated the interfacing between epithelial alveolar forming units and capillary-like tubes with continuous lumens (Fig. 74.5). Utilizing a subcutaneous implantation model, these authors further demonstrated that graft-derived endothelial cells contributed to the formation of perfused vessels that had anastamosed with the host vasculature *in vivo* [79].

Synthetic (polymer) scaffolds

Degradable polymeric biomaterials, such as poly(lactic-co-glycolic acid) (PLGA) can easily be engineered into scaffolds with fibrous or porous geometries. Lin et al. demonstrated that coating the surface of synthetic polymers with natural ECM proteins facilitates enhanced initial attachment [80] but might not suffice to overcome the inherent stiffness-driven dedifferentiation of the seeded (epithelial) cells towards the mesenchymal phenotype [77]. Utilizing surface modification strategies, such as integrating adhesive or instructive peptide domains, could enhance the differentiative properties of synthetic materials. In addition, elastomeric polymer chemistry could facilitate the design of materials that satisfy the mechanical requirements for organotypic cell growth and differentiation, as well as the need to support cyclic strain of respiration.

An additional limitation of synthetic materials is that they often induce foreign body reactions, limiting tissue development and integration *in vivo*. For example, Cortiella et al. tested the

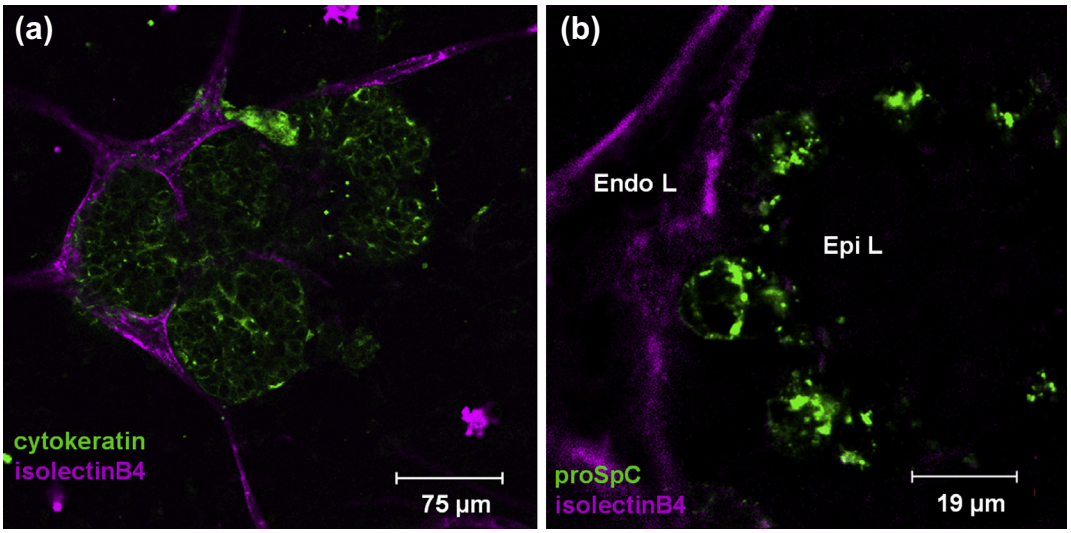


FIGURE 74.5
Interfacing of epithelial and endothelial tissue components in three-dimensional pulmonary tissue constructs. (a) A sacculated epithelial alveolar forming unit (AFU), shown with cytokeratin staining (green), enrobed by a developing capillary-like endothelial network, shown with isolectinB4 staining (magenta). (b) A single epithelial saccule of a developing alveolar forming unit (AFU) with a defined lumen (Epi L), comprised of proSpC-positive alveolar type 2 lineage cells (green) directly contacting an endothelial capillary-like tubule (isolectinB4 labeling, magenta) with a defined lumen (Endo L). Embryonic day 17 murine fetal pulmonary cells were admixed into collagen type I hydrogels and cultured for 1 week in a serum-free defined medium containing FGFs 10, 7, and 2. *(Reproduced with permission from Mondrinos et al. 2007 [78].)*

ability of polyglycolic acid (PGA) scaffolds and pluronic F-127 hydrogels to support *in vivo* lung tissue formation by ovine 'somatic lung progenitor cells' [81]. While these lung progenitor cells proliferated on PGA scaffolds *in vitro* and continued to express lung-specific markers, the scaffolds induced a foreign body response *in vivo*, limiting tissue development and integration. This limitation was partially overcome through the use of pluronic F-127. Shigamura et al. used a lung volume reduction surgery (LVRS) model in rats to test the efficacy of PGA felt sheets seeded with adipose-derived stem cells (ASCs) as a sealant graft construct [82]. The ASC-containing PGA grafts significantly improved compensatory growth following surgical resection, as evidenced by both alveolar and vascular regeneration. This improvement in tissue restoration resulted in enhanced as exchange and exercise tolerance when compared to control animals, which had undergone LVRS without any further treatment.

Decellularized lung tissue scaffolds

A potential paradigm for whole organ engineering has emerged recently in the form of decellularized cadaveric donor tissue. Paradigms of decellularized organs are heart, lung and liver [83]. Decellularized lungs are typically prepared using combinations of hypotonic lysis, detergent solutions – by diffusion or perfusion, and DNAse [84]. Petersen et al. were the first to demonstrate the ability of decellularized rat lungs subsequently repopulated with fetal lung cells to participate in gas exchange upon *in vivo* implantation for short periods (<120 minutes) [85]. The feasibility of this concept was confirmed by Ott et al., with slightly longer periods (up to six hours) of gas exchange *in vivo* [86], followed by a paper by Song et al., who described enhanced *in vivo* function of these decellularized/recellularized lungs [87]. In the latter study, decellularized rat lungs were recellularized with rat fetal lung cells and human endothelial cells, cultured for seven days in biomimetic bioreactor culture under dynamic conditions, then implanted following left pneumonectomy and maintained *in vivo* for up to 14 days. Gas exchange function was comparable to transplanted lung allograft controls up to day 7, however, the function of engrafted bioartificial lungs declined over the subsequent seven days due to graft immune rejection. The key component in the preparation of these

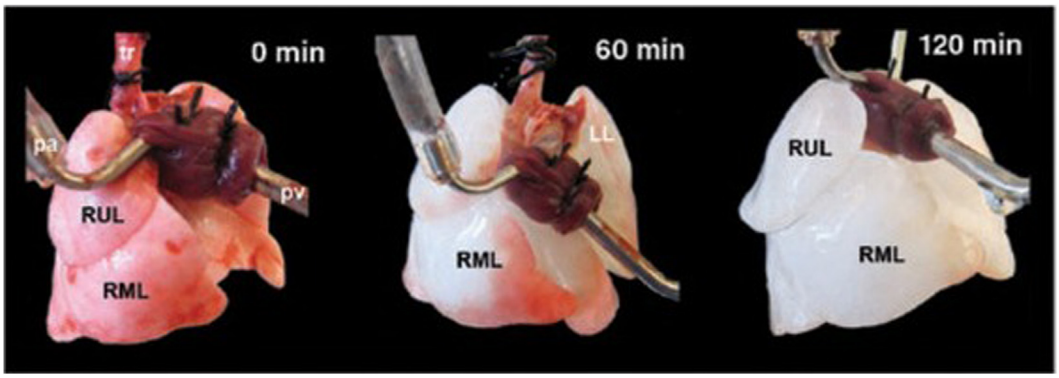


FIGURE 74.6

Rapid decellularization of whole rat lungs using a perfusion bioreactor and detergent solutions. Removal of the cellular contents is visible as the tissue loses its color and becomes more translucent. Decellularized lungs may then be re-seeded using the same perfusion approach, and subsequently mechanically conditioned with both ventilation of the tracheal conduit and perfusion of the reconstituted vascular tree at appropriate pressures. RUL = right upper lobe; RML = right middle lobe. *(Reproduced with permission from Ott et al. [86].)*

engineered whole organ systems is the independent perfusion of both the airways and vasculature, which allows for perfusion decellularization, seeding, and culture for mechanical conditioning of both these compartments *in vitro* (Fig. 74.6).

Some investigators have looked into re-seeding decellularized lungs with stem cells derived from various sources. Jensen et al. seeded mouse ES cells which were pre-differentiated into prosurfactant protein C-expressing alveolar type II-like cells into decellularized mouse lungs [88]. Inoculated cells maintain differentiation both *in vitro*, and *in vivo* – where vascularization of subcutaneous allografts was observed. Longmire et al., seeded murine ES cells that were pre-differentiated in a multi-step process first toward the definitive endodermal lineage and then into immature lung epithelial cells. Upon seeding into decellularized lung matrices, structures reconstituted by these cells demonstrate morphologies and expression of proteins indicative of both type I and type II alveolar epithelial cell differentiation [52]. This latter study suggests that a decellularized ECM may serve as a natural bioreactor that can enhance directed tissue-specific differentiation of competent progenitor cells.

Daly et al. reported preservation of ECM components following decellularization, along with the ability to support attachment and survival of MSCs for up to one month *in vitro* without, however, expressing any alveolar or vascular genes [89]. The re-seeding of decellularized mouse lungs with ES-derived lung cells [52], taken together with ability to generate patient-specific lung cells with iPS technology [53], provides the proof of concept for potentially utilizing autologous cells for the recellularization of whole decellularized lungs, although at this time this has only been accomplished in small animal models. As a next step forward towards clinical feasibility, Bonvillain et al. demonstrated effective decellularization of rhesus macaque monkey lungs using protocols developed with rodent lungs [90]. Importantly, this study demonstrated for the first time in a larger animal model, that ECM structure and composition were largely preserved, including the proteins collagens type I and IV, laminin, fibronectin, and sulfated glyosaminoglycans. As an additional proof of concept, decellularized primate lungs supported the adhesion, integration, and *in vitro* proliferation of allogeneic rhesus MSCs derived from adipose tissue and bone marrow.

The paradigm of combining decellularized lung scaffolds with stem cell-derived populations of lung cells represents the current 'state of the art' in the field. However, many practical challenges facing clinical translation remain. First, it is known that decellularized lung scaffolds retain cytoplasmic and nuclear contents, up to 8% of the original total DNA in the lung according to one report [90], which may potentially elicit immune rejection. Once

short-term functional integration of tissue-engineered lungs using autologous or otherwise known to be non-immunogenic cells is achieved (e.g., avoiding leakage of the vasculature), it will be possible to assess the long-term immune response specific to the allogeneic lung matrix and the seeded cells. In addition to determining the safety, immunogenicity, and long-term functionality of decellularized/recellularized matrices, the availability of healthy cadaveric human lungs will remain a problem.

Bioreactors and cell culture systems

Bioreactors are cell culture systems for the three-dimensional assembly, growth and differentiation of cells and tissues under tight control of key physicochemical factors such as pH, oxygen tension, nutrients and metabolite levels, as well as growth factors and hormones [91]. Importantly, these physicochemical parameters can be measured and controlled in real time [92], providing a level of complexity that could potentially facilitate more suitable bioprocesses to mimic the pulmonary microenvironment. Currently available real-time monitoring systems include: sensors for measuring pH, pO_2, and pCO_2 [93], protein quantification sensors [94], as well as *in situ* microscopy capable of measuring cell size, aggregation, and differentiation state [95].

The lung presents a uniquely challenging bioreactor design problem due to the need for independent perfusion of the airway and vascular trees, concomitant with the ability to ventilate tissue-engineered lungs for mechanical testing and conditioning. In their seminal work, Price et al. developed a simple bioreactor based on these principles that facilitated ventilation of decellularized mouse lungs suspended in culture medium [96]. The trachea was cannulated and tied off with sutures, serving as a path for delivery of fetal lung cells, and also allowing for mechanical ventilation. Subsequent designs include separate perfusion loops for the airways and vasculature [85–87] (Fig. 74.6). A similar but more sophisticated design, which is now being offered commercially (Harvard Instruments), satisfies the need to mechanically condition both compartments independently; the airways through ventilation, and the vasculature with constant perfusion recreating physiological flow rates, shear stresses and pressures. A major challenge will be to scale-up these bioreactors to the size of human lungs and make them a broadly applicable technology in a clinical setting.

In a different approach, perfused rotating wall vessel (RWV) bioreactors have been used as a venue for efficient expansion and differentiation of mouse ES cells into pulmonary alveolar cells [97] (Fig. 74.7a). When murine and human ES cells were encapsulated in alginate and cultured for 11 days in high aspect ratio vessel (HARV) bioreactors with A549 cell-conditioned medium, which has been previously shown to promote efficient pulmonary differentiation of mES cells [50]. Approximately 50% of the resulting cells were SPC-positive type II alveolar cells following removal from the RWV environment and de-capsulation. The system described by Siti et al. [97] is amenable to stringent process design/control, automation, scale-up, and adaptation to GMP manufacture, thus opening the prospect for generating larger reproducible batches of ES derivatives that might be used in future clinical applications. Recently, Botta et al. [98] developed an optical monitoring system capable of visualizing individual fluorescent-labeled cells and cell aggregates in real time in HARV bioreactors (Fig. 74.7b). Thus, using ES cell lines with GFP reporter genes [52] that indicate expression lineage-specific markers, this system could be applied to monitor stem cell differentiation in real time, all this within the context of a system that is scalable to larger batch processing.

Biohybrid mechanical devices

The highly complex alveolar architecture is a product of biological evolution; however a functional respiratory analog does not necessarily need to reproduce this complexity of structure. Extracorporeal membrane oxygenators are used for short-term support of patients with respiratory failure; however they do not offer a long-term solution. Paracorporeal artificial

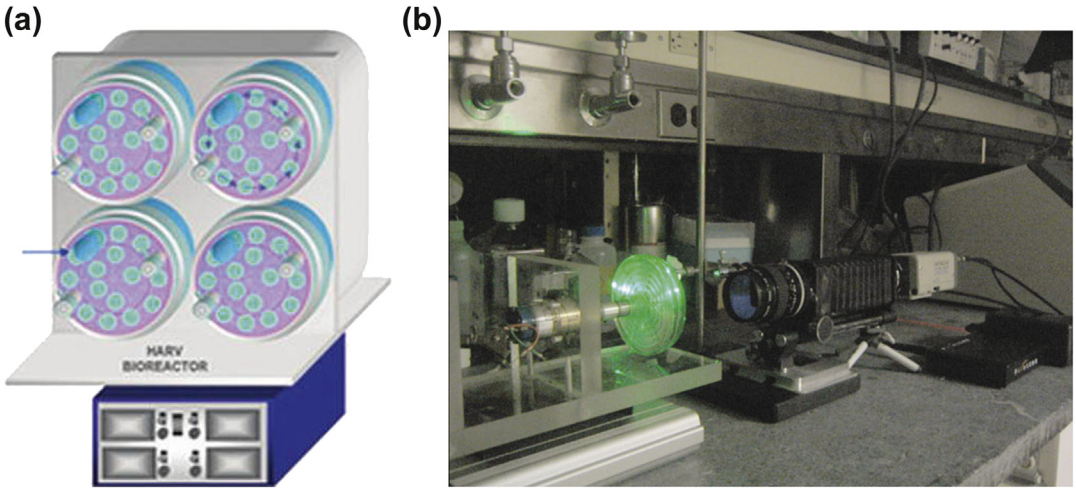


FIGURE 74.7

High aspect ratio vessel (HARV) cell culture systems. (a) HARV bioreactor schematic depicting alginate beads containing embryonic stem cells cultured with a medium that promotes lung epithelial differentiation. Taken from Siti et al. [97]. (b) Real-time optical system capable of monitoring the fluorescence of single cells or aggregates in HARV bioreactors. [97] *(Reproduced with permission from Botta and Lelkes [98].)*

lungs (PALs) have been in development for decades [99], with design features such as rotating microporous fibers that simultaneously drive blood blow and facilitate gas exchange. Polk et al. created a biohybrid version of traditional PALs which incorporated endothelialized microporous fibers, reporting improved blood biocompatibility and enhanced gas transfer [100]. Fritsche et al. employed microfabrication techniques using polydimethylsiloxane to create dual compartment scaffolds mimicking the interfaced airway and vascular trees [101]. Hoganson et al. developed a prototype implantable ambulatory lung assist device based on tissue-engineering scaffold platform technology [102], demonstrating physiological blood flow in terms of pressures, flow rates and shear stresses, with gas exchange rates comparable to the traditional hollow fiber devices. A more compact device (NOVALUNG iLA) is in clinical use and has been tested successfully [103].

Current bioengineering efforts are aimed at miniaturizing these devices and enhancing their functionality by incorporating tissue-engineering approaches that enhance the gas exchange across the epithelial/endothelial permeability barrier. Beyond the realm of clinical applications, similar technologies are being used on the microscale to develop miniaturized analogs of a respiratory unit for pharmacological and toxicological studies. Huh et al. created a prototype of a 'lung on a chip', demonstrating the utility of a microfluidic respiratory analog for modeling lung clearance of inhaled toxic particles [104] (Fig. 74.8). This system can, for example, be used to test the effects of cyclic strain on transport of silica nanoparticles *in vitro* from the 'alveolar' space of the device – across an epithelial layer, an interfacial membrane, and an endothelial layer – into the 'capillary' space, demonstrating similar biological responses to macro-scale experiments using whole mouse lungs. Currently (2012) a multi-institutional effort have been launched to expand the 'lung-on-a chip' into a more complex microfluidics-based 'human-on-a-chip' that is slated to model complex interactions between up to 10 vital organs, such as lung-heart-liver-kidney and the brain.

CONCLUSIONS AND CHALLENGES

Over the last decade the field of pulmonary regenerative medicine has advanced out of the realm of science fiction. In parallel with significant advances in human stem biology, the use decellularized cadaveric lung tissues as scaffolds in combination with bioreactor systems that facilitate ventilation of the airways and perfusion of vascular components in animal models

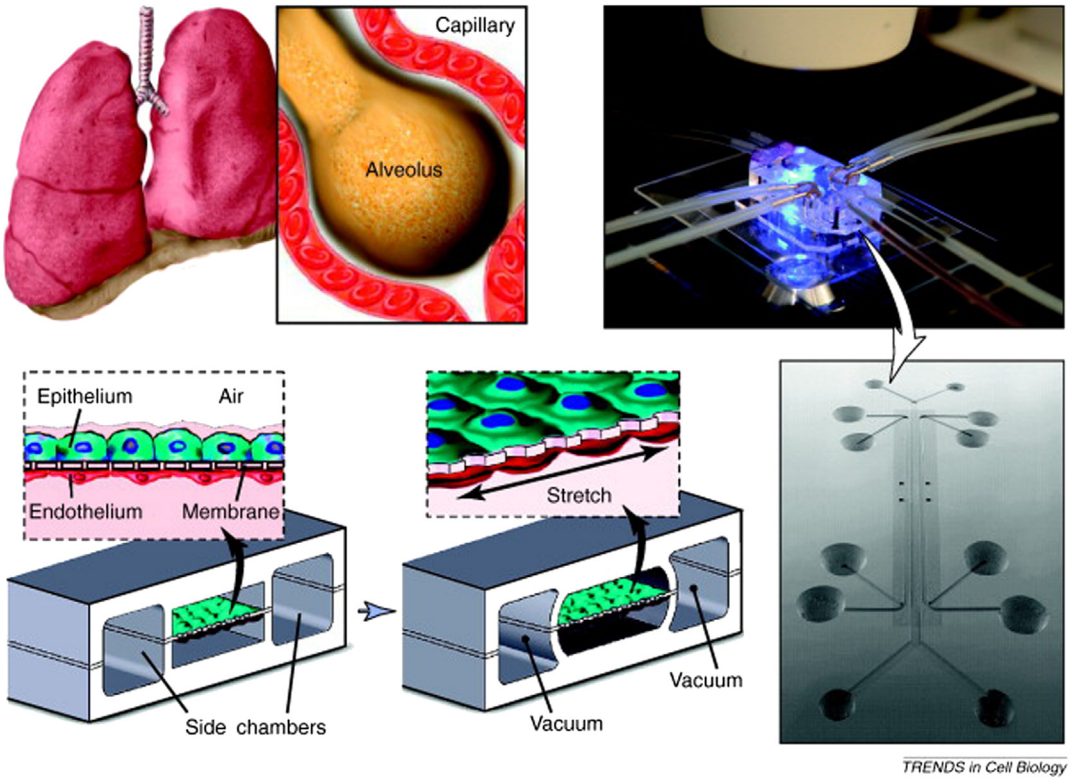


FIGURE 74.8

Microfluidic 'lung on a chip' device. Recreating the alveolar-capillary interface on a single sub-unit microscale allows for separately perfusable alveolar and capillary spaces on either side of the interface, as well as the ability to stretch the cell layers by applying vacuum to parallel side chambers. *(Reproduced with permission from Huh et al. [105].)*

has provided a functional paradigm for the field. Tissue-engineered lungs generated *in vitro* demonstrated short-term life-sustaining efficacy *in vivo* in small animal models. These landmark steps set the stage for a productive future in pulmonary regenerative medicine and tissue engineering, however there are several challenges remaining in each of these core areas.

In regards to stem cell differentiation, multiple protocols have been established for derivation of pulmonary epithelial cell lineages from human ES cells. The efficiency of these protocols can be improved, as well as expanded to more clearly define the conditions required to generate the milieu of epithelial cell types – as well as the as of yet largely ignored pulmonary-specific endothelial and mesenchymal lineages. Tissue-specific adult lung stem cells capable of forming cells from multiple germ layers have been described, but the *in vitro* differentiation repertoire and therapeutic efficacy of these cells *in vivo* needs to be more extensively validated. Once the optimal cell source has been established, the scalability of protocols for economically feasible generation of the numbers of cells required for therapies in humans (both infusion/injection and seeding of sufficiently large scaffolds), on clinically relevant time scales, is a challenge for the development of cell culture systems aimed at both expansion and multi-lineage differentiation. Along with progress in all of these areas, testing the efficacy of cell-based therapies for the treatment of lung disease in animal models and eventually clinical trials in humans will provide the ultimate measuring stick.

Decellularized lung tissue scaffolds are the current 'state of the art', providing nature's own matrix for rebuilding this highly complex organ. Once optimal preservation of ECM components and tissue-mechanical properties are attained, and methods for re-seeding of the epithelial, vascular, and interstitial compartments are established, the challenges of available

donor tissue and potential immunogenicity will need to be tackled. After resolving these core issues, further refinement of bioreactor systems for *in vitro* conditioning, and demonstration of the long-term efficacy of implanted tissue-engineered lungs in large animal models, will be required to move the field forward. Natural ECM hydrogels remain promising as *in vitro* models, and as a basis for the design of bioengineered matrices or injectable therapeutics – which may or may not contain cells – aimed at facilitating regeneration of pulmonary tissue *in situ*. Methods for engineering natural and synthetic materials into architectures that facilitate creation of the global interconnectivity of the airway and vascular systems, as well as the interstitial tissues, will be required to move beyond the use of decellularized lung tissue. The road to clinical translation will be long and arduous, however the numerous advances discussed herein lay the foundation for pulmonary regenerative medicine to grow and make an impact on the treatment of human lung disease in the coming decades.

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PART 19

Skin

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CHAPTER 75

Cutaneous Epithelial Stem Cells

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INTRODUCTION

The epidermis consists of multiple cell types and layers. The outermost layer, called the stratum corneum is composed of dead 'corneocytes' that adhere tightly to each other to form a hydrophobic barrier that protects us from the environment and prevents water loss. The innermost layer at the base of the epidermis generates new cells that migrate to the surface while terminally differentiating and eventually forming the stratum corneum. In addition to keratinocytes, which produce intermediate filament proteins called keratins and constitute the majority of the cells, the epidermis also houses melanocytes, fabricators of pigment, and Langerhans cells, sentinels against invaders, whose primary job is that to present foreign antigen to roaming T cells.

The epidermal surface is interrupted by orifices arising from adnexal structures, such as the hair follicles and sweat glands. The epidermal basal layer constitutes the outer cell layer of these structures and it has been shown that such basal cells from hair follicles and sweat ducts can move out and repopulate the epidermis after wounding. The adnexal structures possess a greater degree of tissue complexity compared to the epidermis. For example, in contrast to the stratified squamous epithelium of the epidermis, the hair follicle consists of at least eight different concentric layers of epithelia, which undergo degeneration and regeneration with each hair follicle cycle.

Because the epidermis and hair follicles continuously generate new cells to replenish dead corneocytes and hairs, which are sloughed into the environment, their homeostasis and repair were thought to depend on epithelial stem cells. Work by many investigators has proved the existence of such cells, and indeed provided a leading example for discovery of stem cells in other regenerative tissues. Epithelial stem cells, found in interfollicular epidermis and the hair follicle, fit a broader definition of adult stem cells, as they are quiescent in nature, with the unique capacity for self-renewal as well as differentiation. In recent years, the concept of interfollicular epithelial stem cells to replenish skin has come into conflict with a new theory. Hair follicle stem cells, once thought to be a single population arising from the bulge region, are now competing with several newly described follicular populations. In this chapter, we will review old and new evidence to integrate historical dogma with newly emerging concepts in the evolving tale of skin regeneration.

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INTERFOLLICULAR STEM CELLS

Physiological renewal of epidermis is supported by proliferation of cells in the basal layer. Since epidermal renewal continues throughout a person's lifetime, it has been postulated that at least a portion of epidermal basal cells behave like stem cells (Fig. 75.1a for location of putative stem cells in interfollicular and follicular epidermis).

Bickenbach and Mackenzie, in pioneering work, devised 'label-retaining cell' methods for detecting quiescent cells in the epidermis [1]. Further, Morris and Potten showed that these cells retained carcinogen and possessed the quiescent characteristic of stem cells (reviewed in [2]). To test the concept that these cells give rise to all skin layers, a replication-deficient retroviral vector carrying the beta galactosidase gene was transduced with low frequency into the skin basal layer *in vivo* [2a]. Over a period of one month, discreet blue columns of cells could be visualized arising from the basal layer and progressing to the skin surface, thus supporting the existence of clonal epidermal proliferative units (EPUs), at least in the mouse epidermis. To further support this hypothesis, bromodeoxyuridine (BrDU) pulse labeling of the basal layer revealed the presence of a small number of quiescent (label-retaining) cells. Taken together, these data suggested that quiescent stem cells in the basal layer serve to replenish the upper layers during homeostasis and following wounding.

Two models for skin renewal (EPU vs. Committed Progenitor (CP) model)

Historically, the favored model has been that basal layer stem cells provide 'transit amplifying' progeny which undergo a limited number of divisions to generate the upper strata of the

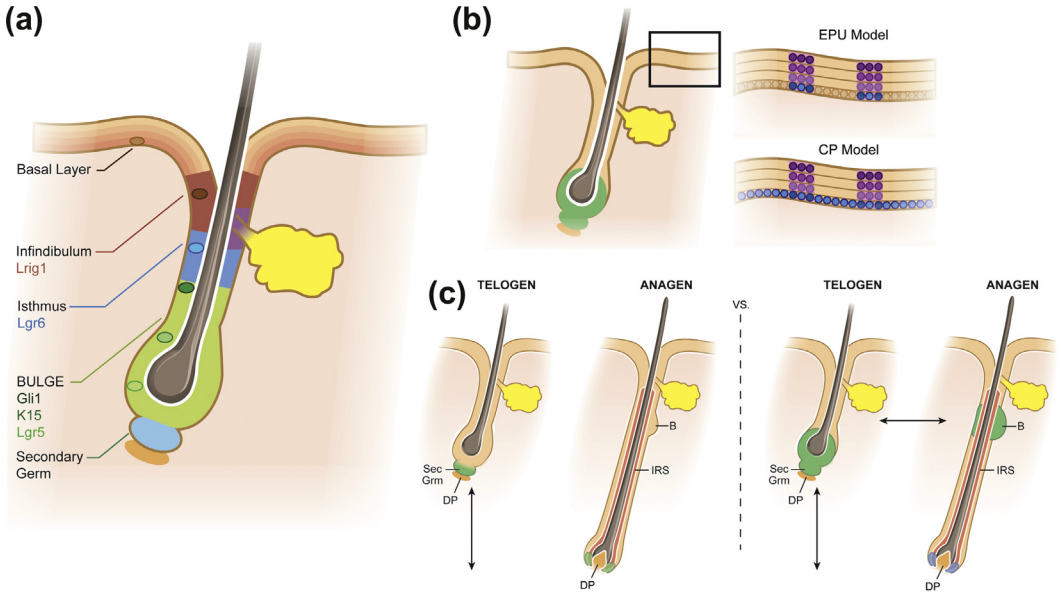

FIGURE 75.1

(a) Location of putative epidermal stem cells in the hair follicle and interfollicular epidermis. (b) Two models for interfollicular epidermal homeostasis. EPU model: In this model, rare stem cells (light blue) in the basal layer of the skin give rise to new progeny identical to itself (adjacent dark blue cells) or more differentiated progeny (pink and purple cells in upper layers). Each unit with a single central stem cell is termed an Epithelial Proliferative Unit (EPU). CP model: In this model, all cells in the basal layer have the same potential to make new identical progeny or more differentiated progeny. Therefore all cells in the basal layer are the same color. (c) Two models for hair follicle regeneration during cycling. Historic model (left panel): 'secondary germ cells,' (named for their similarity to primary germ cells present during development) were thought to contain the stem cells for the follicle. It was thought that these cells migrate from the base of the telogen follicle to the bulb during anagen onset, and then migrate back up during catagen. Current model (right panel): The secondary germ cells found at the base of the telogen follicle arise from the lowermost portion of the bulge at the end of catagen [15]. These cells migrate down into the secondary germ from their niche region where they alter their gene expression profile, proliferate and ultimately provide all the cells for the new lower half of the anagen follicle [49].

epidermis [2b,2c]. According to this model, a slower cycling central cell and more rapidly proliferating surrounding cells constitute approximately 10 basal cells and are roughly organized into a hexagonal unit, which lies beneath a single keratinocyte (reviewed in [3], Fig. 75.1b). Based on these proliferative and morphological characteristics, the term 'epidermal proliferative unit' (EPU) was coined to describe this architecture [2,3]. Without the benefit of direct lineage analysis, it was assumed that the central cell within the EPU generates the rapidly proliferating cells, termed transient or transit amplifying (TA) cells, which move laterally and then differentiate and move upward. Thus, within the epidermis, the main source of cells, i.e., the stem cells, responsible for continual epidermal renewal appear to reside in the center of the EPU.

The size of any EPU would be dependent on the total number of mitotic cycles that transiently amplifying progenitors derived from a single stem cell are able to undergo prior to terminal differentiation. The entire epidermal sheet would thus be maintained by a collection of co-existing stable EPUs with one stem cell at the center of each.

In recent years, the EPU-based model of epidermal regeneration has been challenged. Using a low frequency cre-inducible genetic model, individual proliferating basal cells were marked and followed for one year in a long-term fate mapping study [4,5]. In contradiction to the canonical EPU model, which predicts the size of each EPU to be finite, it was shown that some epidermal clones continuously expand in size over a period of one year, while others shrink and disappear, and yet others behave like typical EPUs. Mathematical modeling of these clone patterns suggested a stochastic model for epidermal renewal, in which each proliferating basal cell can give rise to two new proliferating basal cells, two differentiated progeny or both [4]. According to this CP model, epidermis is maintained by a uniform population of basal progenitors via stochastically distributed symmetric divisions to maintain the basal layer and asymmetric divisions to generate more differentiated progeny [6]. In support of this model, data showed that a single basal epidermal cell could indeed divide both symmetrically to produce two new basal cells and asymmetrically to generate more differentiated progeny [7].

New data challenge the CP model and reconfirm the existence of at least two populations within interfollicular epidermis; a slow cycling 'stem cell' and a rapid cycling 'progenitor' pool [8]. It has long been known that heterogeneity in marker expression defines multiple populations within epidermis. Indeed, this has provided a compelling argument against the possibility of a uniform basal population as proposed by the CP model. Mascre et al. [8] have capitalized upon this knowledge to follow the fate of two distinct basal populations, as defined by expression of involucrin. Their work conclusively demonstrates that involucrin-expressing cells divide rapidly (once per week) and quickly contribute to all layers of the epidermis. Thus, these cells are comparable to those described by Jones et al. as 'committed progenitors' [6]. However, some cells within the involucrin-negative population divide infrequently (4–6 times/year), have a distinct gene expression profile and can also contribute to skin homeostasis. In contrast to 'progenitors', these 'stem cells' are the primary contributors to re-epithelialization following wound repair (see below).

In reconciling the EPU and CP models, it thus appears that two populations maintain skin homeostasis, a rapid cycling progenitor and a slow cycling stem cell. Tissue damage invokes the aid of the stem cell population *only* for re-epithelialization. A similar phenomenon has been observed in hematopoiesis in which distinct rapid cycling and slow cycling cells contribute to homeostasis of immune cell lineages but only slow cycling cells are called upon for rapid repair as a stress response [9].

HAIR FOLLICLE STEM CELLS

Similar to the epidermis, the hair follicle generates a terminally differentiated keratinized end product, the hair shaft, which is eventually shed. Tracing back a hair shaft cell to its origin in

adult skin is not straightforward. In contrast to epidermis, the follicle undergoes cyclical regeneration, and has a more complicated proliferative profile and architecture with at least eight different epithelial lineages (Fig. 75.2). Hair is formed by rapidly proliferating matrix keratinocytes in the bulb located at the base of the growing (anagen) follicle. The duration of anagen varies drastically between hairs of differing lengths. For example, mouse hair and human eyebrow hair follicles stay in anagen for only 2–4 weeks while human scalp follicles can remain in anagen for many years. Nevertheless, matrix cells eventually stop proliferating, and hair growth ceases at catagen when the lower follicle regresses to reach a stage of rest (telogen). After telogen, the lower hair-producing portion of the follicle regenerates, marking the new anagen phase (Fig. 75.2).

Because the lower portion of the follicle cyclically regenerates, hair follicle stem cells were thought to govern this growth. Historically, hair follicle stem cells were assumed to reside exclusively in the 'secondary germ', which is located at the base of the telogen hair follicle (Fig. 75.1c). It was felt that the secondary germ moved downward to the hair bulb during

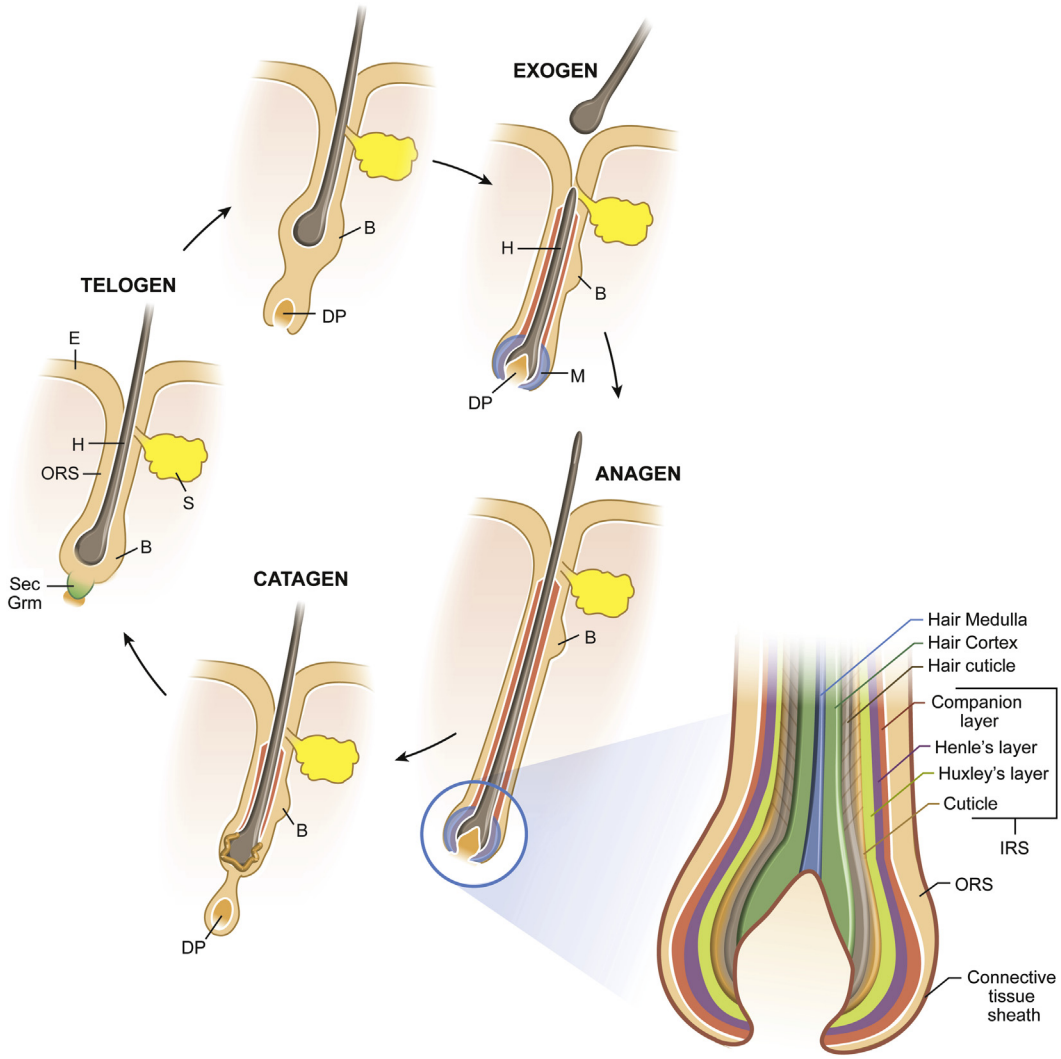

FIGURE 75.2

Hair cycle and anatomy. The hair follicle cycle consists of stages of rest (telogen), hair growth (anagen), follicle regression (catagen) and hair shedding (exogen). The entire lower epithelial structure is formed during anagen, and regresses during catagen. The transient portion of the follicle consists of matrix cells in the bulb that generate seven different cell lineages, three in the hair shaft and four in the inner root sheath (IRS).

anagen and provided new cells for production of the hair. At the end of anagen, the secondary germ was thought to move upward with the dermal papilla during catagen to come to rest at the base of the telogen follicle.

This scenario of stem cell movement during follicle cycling was brought into question when a population of long-lived presumptive stem cells was identified using label-retaining cell methods in an area of the follicle surrounding the telogen club hair, and not in the hair bulb [10]. That the presumptive stem cells localized to a previously defined area called the bulge was not appreciated until this description of the human embryonic follicle by Hermann Pinkus, [11] was rediscovered.

> 'This *bulge*, often the most conspicuous detail of the young germ, is as large as the bulb… The function of the bulge is obscure. While it serves as the point of insertion of the arrector muscle later in life, it develops much earlier than the muscle and the latter seems to originate quite independently in the skin near the sebaceous gland, and in many instances streaks by the bulge before approaching the lower follicle below it. Unna (1876) named the bulge area of the adult follicle the *hair bed* (Haarbett) believing that the club hair became implanted there and derived additional growth from it. Stöhr gave it the neutral name 'Wulst' (bulge or swelling). Some texts state that this is an area of marked proliferative activity, but no mitotic figures were observed in the bulge even if other parts of the follicle contained them. Whatever its function, the bulge marks the lower end of the 'permanent follicle' later in life. Everything below it is expendable during the hair change [cycle].'

This remarkable description, based purely on morphological observations, portended the characterization of the bulge cells in both human and mouse follicles as an area containing quiescent cells important for hair follicle cycling [10,12,13]. In the mouse pelage follicle, the area analogous to the human bulge becomes morphologically apparent in the postnatal period during the first telogen stage at the site of arrector pili muscle attachment. The shape of the bulge in the mouse follicle results from displacement of the outer root sheath (ORS) by the club hair. In the human follicle, the bulge appears as a true thickening of the ORS, but generally becomes much less apparent with age (Fig. 75.3).

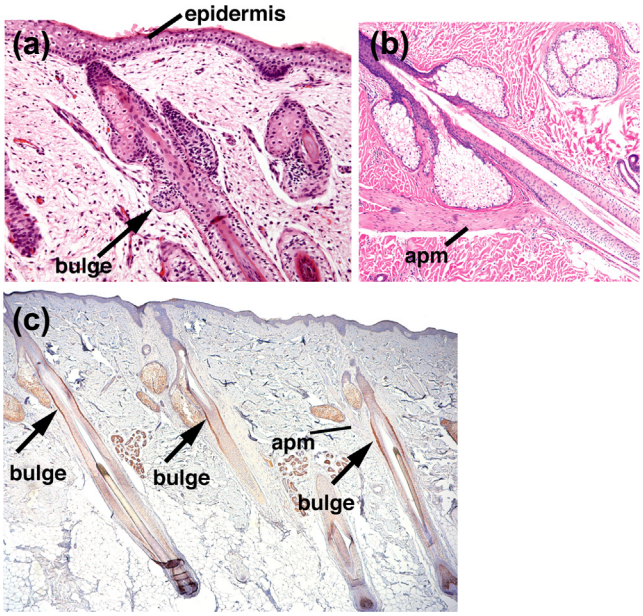

FIGURE 75.3
The bulge is a prominent structure in fetal skin (a), but generally is not morphologically distinct in the adult (b). Immunostaining for keratin 15 in scalp preferentially detects bulge cells (c). apm, arrector pili muscle.

THE BULGE AS STEM CELL SOURCE

Multiple criteria define the bulge as the true stem cell source for hair follicle regrowth. These include quiescence, gene signature and the ability of single cells to regenerate the entire hair follicle in skin reconstitution assays.

Quiescence: A salient feature of bulge cells in general is their quiescence. In both adult mouse and human skin grafted onto immunodeficient mice, the administration of nucleoside analogs, such as tritiated thymidine or BrdU, which are taken up by cells in S-phase, does not result in labeling of the bulge cells except at anagen onset [14,15]. Once labeled as either neonates or during anagen onset, when stem cells are proliferating, bulge cells can remain labeled for 14 months in the mouse and at least four months (the longest period examined) in the human [12]. This prolonged quiescence is remarkable given that the surrounding cells proliferate at a much higher rate. These results suggest that bulge cells persist for the lifetime of the organism.

Molecular signature: Understanding the genes that distinguish bulge cells from proliferating TA cells, as well as the genes that convert resting bulge cells to differentiating cells, provides insights into the precisely orchestrated events of hair follicle formation at anagen onset. With the advent of microarrays, large-scale comparisons of gene expression in bulge cells versus non-bulge basal keratinocytes could be performed [13,49]. These studies found that genes involved in activation of the WNT pathway were generally decreased in the bulge, in line with evidence that WNT activation induces proliferation and cell differentiation and is a hallmark of anagen onset [16]. Microarray analyses of the human hair follicle bulge showed many similarities to the mouse studies, including expression of Keratin15 (K15), thus validating the mouse as a useful model for studying human hair growth [17]. However, one important difference was that CD34, a mouse bulge cell marker, is not expressed by human hair follicle bulge cells.

Among other gene candidates important for the stem cell phenotype, functional studies demonstrate that Rac-1 plays an important role in the self-renewal of the epidermis and hair follicle [18,19]. Loss of Rac-1 causes a burst of proliferation in epidermal keratinocytes and then synchronized differentiation and loss of proliferative capability resulting in thinning of the cutaneous epithelium. Thus, this gene suppresses proliferation and differentiation, and seems important for the switch from stem cell to TA cell.

Multipotence: If hair follicle stem cells are located in the bulge, then these cells should give rise to all of the epithelial cells in the lower hair follicle. Early evidence supporting the concept that bulge cells generate the lower follicle includes proliferation studies showing that bulge cells preferentially proliferate at anagen onset [15,20]. More convincing evidence suggesting that the lower follicle originates from bulge cells came from *in vivo* labeling studies and transplantation studies. Taylor used a double-labeling technique to trace the progeny of bulge cells in intact pelage follicles [21]. Faint labeling in a speckled pattern was observed in some cells of the lower follicle suggesting that these cells had indeed originated in the bulge. Similarly, Tumbar et al. used persistence of green fluorescent protein (GFP) label as an indication that lower epithelial cells were progeny of the bulge cells [49]. Neither study provided convincing evidence that all hair matrix keratinocytes in the bulb originated from bulge cells, and both suffered from an inability to permanently mark bulge cells and their progeny.

Oshima et al. took a different approach and transplanted bulge regions from vibrissa follicles isolated by dissection from ROSA26 mice into non-ROSA follicles that were then grafted under the kidney capsule of an immunocompromised mouse. ROSA 26 mice express *lacZ* under the control of the ubiquitous ROSA promoter, thus the fate of the transplanted cells could be followed. After several weeks, they found labeled cells in the lower follicle indicating that bulge cells or their progeny had migrated down the vibrissa follicle. At later time points, some follicles expressed *lacZ* in all epithelial cell layers of the lower follicle suggesting that bulge

cells do generate all of the cells of the lower follicle. These elegant studies were limited by several caveats including unclear origin of the marked cell population, the manipulation required for grafting, and the use of vibrissa follicles, which are markedly different than other mouse and human follicles.

More definitive evidence for bulge cell multipotency *in vivo* was reported using the K15 promoter to target these cells with an inducible Cre (CrePR1) construct [13]. CrePR1 is a fusion protein consisting of Cre-recombinase and a truncated progesterone receptor that binds the progesterone antagonist, RU486 [22]. In *K15-CrePR1* transgenic mice, CrePR1 remains inactive in the cytoplasm of the K15-positive cells except during RU486 treatment, which permits CrePR1 to enter the nucleus and catalyze recombination. We crossed the *K15-CrePR1* mice with *Rosa26R (R26R)* reporter mice [22] that express *LacZ* under the control of a ubiquitous promoter after Cre-mediated removal of an inactivating sequence. Transient treatment of adult *K15-CrePR1;R26R* mice with RU486 results in permanent expression of *LacZ* in the bulge cells and in all progeny of the labeled bulge cells. Immediately following RU486 induction, only bulge cells showed lacZ expression but after anagen onset, the entire growing lower follicle became lacZ$^+$ proving the bulge as the origin of all epithelial cell types in the lower hair follicle.

Multiple bulge populations, some with surprising features

Recent data suggest that the bulge contains at least three distinct subpopulations as defined by marker expression. All have been implicated as true hair follicle stem cells (Fig. 75.1a).

Keratin 15 (K15) expression in human bulge cells was first described by Lyle et al. [12], (Fig. 75.3). K15 mRNA and protein are reliably expressed at high levels in the bulge, but lower levels of expression can be present in the basal layers of the lower follicle ORS and interfollicular epidermis. A K15 promoter used for generation of transgenic mice possesses a pattern of activity restricted to the bulge in the adult mouse [23]. This proved to be a powerful tool for studying bulge cells, establishing K15$^+$ bulge cells as stem cells responsible for hair follicle growth and cycling (see above).

Gli1 expression defines a new population of stem cells in the upper bulge adjacent to perifollicular sensory nerve endings [24]. Remarkably, these nerve endings provide a niche environment by secreting Sonic hedgehog protein (Shh), an essential component for the maintenance of the gli1$^+$ stem cell fate. Gli1$^+$ cells can reestablish the anagen follicle and they appear to contribute to long-term epidermal wound repair which does not appear to be dependent upon Shh signaling (see below [24]).

Lgr5, an R-spondin receptor implicated in facilitation of canonical WNT signaling [24a], marks a population of hair follicle progenitors in the lower bulge and secondary hair germ during telogen, and in the lower bulge during anagen [25]. Lgr5 expression is strongest in the secondary germ but overlaps with that of K15. Lineage tracing analyses showed that Lgr5--expressing cells contribute to all lineages during hair follicle cycling. Lgr5+ cells can also reconstitute entire new follicles when injected with dermal cells under the skin of nude mice [25]. The majority of the evidence indicates that Lgr5$^+$ cells derive from a quiescent stem cell pool and thus represent progenitors rather than true stem cells (see below).

OTHER NEWLY DISCOVERED HAIR FOLLICLE STEM CELLS

Isthmus: Recently, the junctional zone between infundibulum, sebaceous gland, and bulge, also known as the isthmus, was shown to harbor unexpectedly diverse populations of epithelial cells with stem cell qualities (reviewed in Gordon and Andersen [26]). The top of the isthmus contains stem cells co-expressing transmembrane proteins Lrig1 and Plet1 [27]. These authors place Lrig$^+$ cells in the junctional zone just above the bulge and next to the

sebaceous gland. Lineage tracing experiments have shown that Lrig1$^+$ stem cells tend to be bipotent, physiologically contributing predominantly to infundibulum and sebaceous gland lineages and only occasionally to the interfollicular epidermis. Interestingly, mice lacking Lrig1 develop spontaneous epidermal hyperplasia and exhibit elevated levels of the pro-proliferative cMyc protein. Further analyses indicate that Lrig1 is a downstream target of cMyc and may act to regulate cMyc expression in epidermis in a feedback loop [27].

Lgr6$^+$ stem cells localize immediately below the Lrig1+ compartment [28]. While multipotent during embryonic development, Lgr6$^+$ stem cells undergo progressive developmental fate restriction, and in the adult they participate mainly in epidermal and sebaceous gland maintenance [28]. Interestingly, despite very close physical proximity, Gli1$^+$ stem cells in the upper bulge only marginally overlap with Lgr6$^+$ cells.

Conclusions

All of the above described 'stem cell' populations have been shown capable of reconstituting hair follicles in cell reconstitution experiments. The lower three populations (Gli1$^+$, K15$^+$ and Lgr5$^+$) contribute normally to hair follicle regrowth during cycling. The upper populations (including Gli1$^+$) generate long-lasting epidermal progeny in the wound epidermis, making their contributions a contrasting feature to that observed by K15$^+$ bulge stem cells [29].

The obvious question arises: Why are so many different stem cell populations involved in hair follicle cycling? In other well-defined systems, such as hematopoiesis, a single unique stem cell population gives rise to multiple progenitors for establishment of many types of differentiated progeny. Recent work from Petersson et al. [30] and Francis and Niemann [31] suggests this may also be true in the hair follicle. Using lineage tracing to follow K15$^+$ bulge cells, they found that K15$^+$ progeny actually transit through all other putative stem cell compartments, ultimately giving rise to all differentiated hair follicle cell types including sebaceous gland. This implicates the bulge as the true stem cell location and all other regions as harboring more differentiated progenitors (see Horsley review [32]) leaving the K15 high expressing cell as the true stem cell.

STEM CELLS OF OTHER ECTODERMAL APPENDAGES

Sebaceous glands

Each hair follicle is closely associated with the sebaceous gland and, together, they constitute what is known as the pilosebaceous unit. The predominant cells in the sebaceous gland, sebocytes, secrete lipid-rich products into the infundibular opening of the adjacent hair follicle. Cells expressing the transcriptional repressor Blimp1 comprise unipotent sebocyte progenitors [33]. Horsley et al. [33] found that Blimp1$^+$ progenitors give rise to terminally differentiated Pparγ^+ sebocytes via transient amplifying progenitors, yet they do not contribute progeny toward interfollicular epidermis or hair follicles. Mechanistically, Blimp1 represses cMyc transcription, likely limiting the input of proliferative progenitors towards the gland from the multipotent stem cell populations of the isthmus [28,31] and the bulge [13,30]. The key rate-limiting role of Blimp1 in sebaceous gland homeostasis was revealed following epithelial *Blimp1* deletion, which led to sebaceous gland hypertrophy and oily hair coat phenotype [33].

Sweat glands

Acral skin (palms and soles in human and ventral paw in mouse) is hairless, but contains sweat glands, a secretory type of ectodermal appendage. Unlike hair follicles, sweat glands are relatively quiescent and, until recently, very little was known about their regenerative potential. A recent study by Lu C et al. [34] revealed that despite homeostatic quiescence, sweat glands feature several distinct progenitor types, whose regenerative potential can be stimulated by

injury. Sweat glands have relatively simple organization, consisting of a secretory glandular portion and duct leading to the skin surface. Basal myoepithelial cells and suprabasal luminal cells comprise the glandular portion of the sweat gland. Curiously, sweat-producing luminal cells are maintained by suprabasal unipotent progenitors, largely independent of the basal myoepithelial cells. Neither myoepithelial nor luminal glandular cells contribute progeny toward the duct which, in turn, is maintained by its own basal unipotent progenitors. Unlike glandular cells, ductal progenitor cells become activated upon wounding and help to restore ductal openings onto the skin surface. While ductal progenitors preferentially regenerate the duct itself, they can also regenerate interfollicular epidermis immediately surrounding the sweat gland opening [34]. In this respect, ductal progenitors of the sweat gland share characteristics with the hair follicle isthmus stem cells, which can also generate permanent epidermal progeny following injury [24,27,28]. Rittie recently showed that eccrine ductal cells also contribute to wound healing in human skin [35].

HAIR FOLLICLE STEM CELLS IN SKIN HOMEOSTASIS, WOUND HEALING AND HAIR REGENERATION

Homeostasis

In addition to the role of bulge cells in hair follicle cycling, their contribution to the maintenance of interfollicular epidermis and sebaceous glands has also been considered. Historically, one view was that bulge cells continuously provide progeny for repopulation of these skin regions [21,36]. Tracking of injected bulge keratinocytes *in vivo* showed only transient contribution to the interfollicular epidermi [37]. More direct fate mapping studies have shown that $K15^+$ cells do not contribute to homoeostatic maintenance of the epidermis. Using the K15CrePR;R26R transgenic mouse model, bulge cells labeled in three week old mice and followed for 6 months to determine their potential movements out of the hair follicle were never observed in interfollicular epidermis [13]. Furthermore, genetic ablation of $K15^+$ bulge cells does not result in interfollicular epidermal deficiency. Thus, $K15^+$ bulge stem cells can shift their fate towards the epidermal lineage, but only in response to wound-induced signaling (see below [29]). The other bulge populations ($Gli1^+$ and $Lgr5^+$) also appear to have no role in skin homeostasis [24,25].

Early evidence suggested that follicular cells from the infundibulum (upper follicle) might move into the epidermis. Recent work showed that both $Lgr6^+$ and $Lrig^+$ cells in the hair follicle can migrate into interfollicular epidermis and contribute to all epidermal strata during homeostasis [27,28]. However, their relative importance to skin maintenance compared with that of interfollicular basal cells remains unknown and is likely insignificant.

Wound healing

The contribution of the hair follicle to wound healing of the epidermis has been noted for decades by investigators working with mice and rabbits [38]. Clinicians are also well aware that keratinocytes emerge from the follicle to repopulate wounds. However, the role of bulge cells in wound healing has only recently been characterized.

Bulge $K15^+$ cell progeny migrate into the epidermis after different types of wounding. Using the K15CrePR;R26R transgenic mouse, $K15^+$ bulge cells were labeled in adult mice [29]. Excisional wounding with a 4 mm (punch) trephine, resulted in the migration of $K15^+$cell progeny into the healing epidermis. At least 25% of the newly formed epidermis originated from these $K15^+$ cells. They were also stimulated to move into the epidermis following incisional wounds and after tape stripping, indicating that $K15^+$ bulge cell activation plays a role in replenishing lost cells from the epidermis after wounding. Surprisingly, however, despite the presence of these cells in the basal layer of the re-epithelialized epidermis, the majority do not persist in the regenerated epidermis (Fig. 75.4).

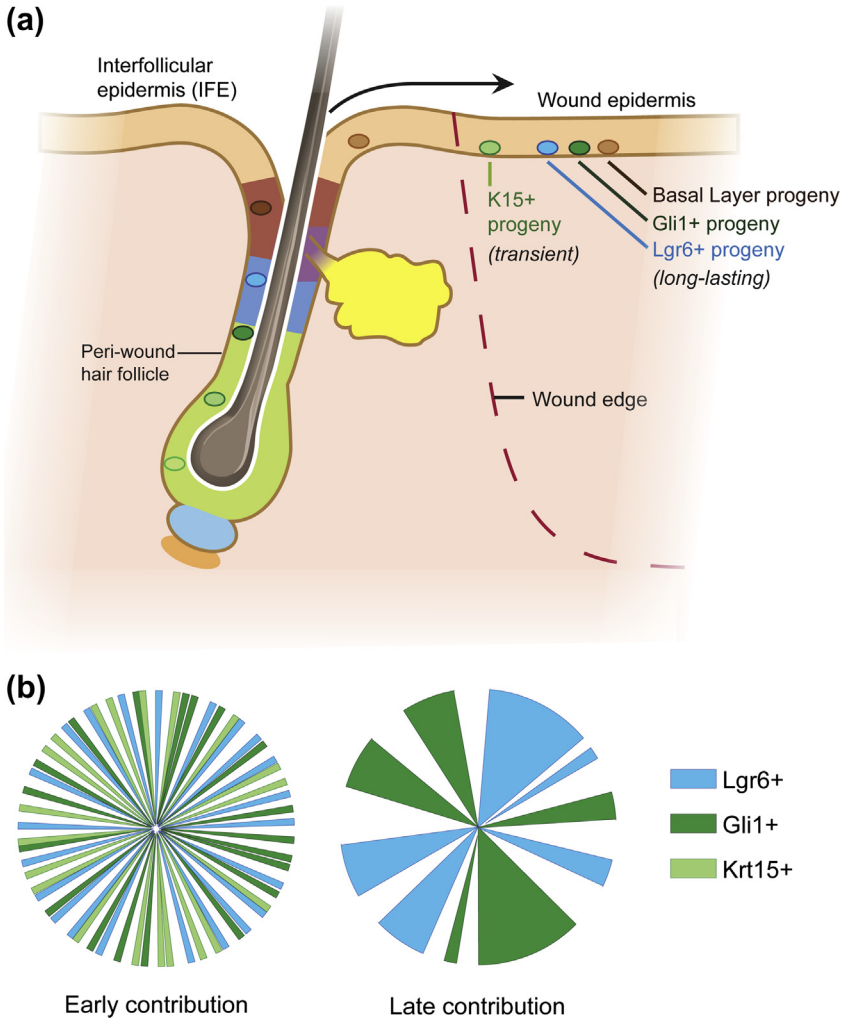

FIGURE 75.4
Contribution of epithelial stem cells to wound repair. (a) Various hair follicle and interfollicular stem cells migrate into the new epidermis for apparent contribution to re-epithelialization. (b) Although all three follicular populations are found in new epithelium during early time points, only Gli1$^+$ and Lgr6$^+$ cells exhibit permanent contribution.

Fate mapping studies of Gli1$^+$ and Lgr6$^+$ populations after wounding show that both can make long-term contributions to new epidermis [27,28]. Results suggest however that neither Gli1 nor Lgr6 progeny contributes significantly to re-epithelialization, as labeling studies indicate they make up 16% and 10% respectively of new epidermis. In contrast, interfollicular stem cells contribute substantially to skin regeneration [39]. These combined results challenge the notion that hair follicle stem cells make any significant long-term contribution to re-epithelialization following wounding.

Hair follicle neogenesis

Recently we discovered that completely new hair follicles can form in the center of large wounds via a process resembling embryonic development [40]. This hair follicle neogenesis is a relatively late event, contingent upon completion of wound re-epithelialization. Neogenic hair follicles start as bud-like invaginations of the basal layer (*aka* hair germs or placodes) and soon develop into elongated hair pegs that mature into hair shaft-producing anagen hair follicles within just a few days. Importantly, like normal hair follicles at the wound edge, neogenic hair follicles in the wound center have a prominent K15$^+$ bulge stem cell compartment and are able to undergo multiple hair follicle cycles.

Surprisingly, fate mapping approaches have shown that new hair follicles do not arise from existing bulge stem cells which have migrated into the wound during healing [40]. While currently it remains unknown what specific epidermal or isthmus stem cell types, or combination of stem cells, generate neogenic hair follicles, Snippert et al. [28] showed that

Lgr6$^+$ isthmus stem cells may be involved. Interestingly, new research suggests that neogenic hair follicles may arise from interfollicular epidermis created during wound re-epithelialization and that induction of these cells to a hair follicle fate depends upon both wound dermal and epidermal WNT activation [28a]. Thus, regenerated hair follicles may have multiple origins.

STEM CELLS AND ALOPECIA

Alopecias can be classified into scarring and non-scarring types [41]. The localization of hair follicle stem cells in the bulge may explain why some types of inflammatory alopecias cause permanent follicle loss (such as lichen planopilaris and discoid lupus erythematosis), while others (such as alopecia areata) are reversible [42]. In cicatricial alopecias, inflammation involves the superficial portion of the follicle, including the bulge area, suggesting that the stem cells necessary for follicle regeneration are damaged. The inflammatory injury of alopecia areata, however, especially in early lesions, involves the bulbar region of the hair follicle that is composed of bulge cell progeny. Because this area is immediately responsible for hair shaft production, its destruction leads to hair loss. However, the bulge area remains intact, and a new lower anagen follicle and subsequent hair shaft can be produced. Even patients with alopecia areata for many years can re-grow their hair either spontaneously or in response to immunomodulation.

The bulge may be targeted for inflammation in androgenetic alopecia (AGA, common baldness) as well. Jaworsky et al. [43] showed that in patients with early androgenetic alopecia, inflammatory cells localize to the bulge. Over time, this damage could contribute to the irreversible nature of androgenetic alopecia. To examine whether stem or progenitor cells were affected by this disease, we analyzed bald and non-bald regions from AGA patients for the presence of these cells. Facs analyses showed that true non-cycling K15$^+$ stem cells were retained in AGA bald regions. In contrast, larger, proliferative Cd200$^+$ progenitors residing in the lower bulge and secondary germ were markedly depleted. This suggested the lack of stem cell activation in balding scalp [44]. Further studies identified Prostaglandin D2 as an inhibitor of hair growth in bald scalp and a potential inhibitor of hair follicle stem cells [45].

TISSUE ENGINEERING WITH EPIDERMAL STEM CELLS

An exciting approach for the use of hair follicle stem cells in the treatment of alopecia includes tissue engineering [46]. In one scenario, isolated hair follicle stem cells could be used for generating new follicles in bald scalp. At least two groups have shown that freshly isolated bulge cells from adult mice, when combined with neonatal dermal cells, form hair follicles after injection into immunodeficient mice [13,39]. These studies provide proof of concept that isolated stem cells may be a part of tissue-engineering approaches for treating alopecia.

A goal for treating alopecia with cell-therapy approaches includes increasing the number of follicles, for example, by amplifying the number of stem cells *in vitro* prior to transplantation. Cultured keratinocytes from neonatal epidermis have been used for many years to generate hair follicles in reconstitution assays. Freshly isolated bulge cells from adult mice were shown to form hair follicles in skin reconstitution assays [13]. Importantly, cultured, individually cloned bulge cells from adult mice also formed hair follicles in skin reconstitution assays [39]. However, the ratio of new follicles formed from the number of donor follicles, and whether non-bulge keratinocytes also possessed these properties, were not analyzed.

The use of hair follicle stem cells for tissue-engineering approaches depends on isolation and characterization of human hair follicle stem cells. A major advance in this direction was reported by Ohyama et al. [17] In this work, cell surface markers, including Cd200 were identified on human bulge cells by using laser capture microdissection and microarray analysis for gene expression. A cocktail of antibodies against cell surface proteins was devised allowing for isolation of living hair follicle bulge stem cells, thus setting the stage for isolating human hair follicle stem cells for hair regeneration.

Human hair regeneration has recently been accomplished [47]. Single cell suspensions of hair bulge stem cells and dermal papilla cells have been layered into 'organ germs' *in vitro* and then transferred onto the backs of nude mice. Such germs create fully functional and pigmented human hair follicles. Analyses of comparable murine bioengineered follicles show that they cycle and maintain appropriate connections with muscle and nerve fibers. These experiments represent the first demonstration of bioengineered human hair and pave the way for exciting new advances in tissue regeneration.

Because hair follicle stem and dermal papilla cells exhibit multipotent behavior, ie the capacity to make several different cell types, they are now being exploited for use as pluripotent cells, ie those capable of generating *all* cell types (Jahoda). Elegant experiments have shown that addition of four defined transcription regulator genes (typically including oct4, nanog, cMyc) can transform fully differentiated fibroblasts into pluripotent cells, however numerous technical difficulties including the length of time required for appropriate dedifferentiation make this technology unfeasible for tissue engineering [48]. In an effort to shorten this time and to reduce the number of introduced genes, investigators have turned to multipotent stem cells for gene transfers, reasoning that these cells are closer in gene expression to pluripotent cells. Recent experiments with dermal papilla cells show that they can indeed undergo dedifferentiation but the timing and the number of transcription factors required remains the same as for fibroblasts [48a]. It remains unknown whether bulge stem cells may prove more useful for this approach.

CONCLUSION

Epithelial stem cells make essential contributions to skin and hair maintenance and repair. Balanced contributions by both interfollicular stem cells and more differentiated progenitors provide a constant supply of new cells during normal skin turnover. Interfollicular stem cells are also the primary source for new epidermis in wound healing. The hair follicle cycles throughout the life of the individual. New work suggests that the bulge is the likely source of true stem cells, giving rise to numerous progenitors throughout the follicle, each with distinct roles in hair and skin maintenance and repair. Engineered human hair can now be regenerated *in vivo* from bulge-derived stem cells, leading the way to future advances for therapeutic treatment of alopecia, chronic wound repair and with important implications for the regeneration of all tissues.

Acknowledgments

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CHAPTER 76

Wound Repair: Basic Biology to Tissue Engineering

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INTRODUCTION

The skin is the largest organ in the body whose primary function is to serve as a protective barrier against the environment. Other important functions of the skin include fluid homeostasis, thermoregulation, immune surveillance, sensory detection, and self-healing. Loss of the integrity the skin due to injury or illness compromises its protective function and when the loss is extensive may result in significant disability or even death. It is estimated that in 1996, there were 35.2 million cases of significant skin loss (US figures) that required major therapeutic intervention [1]. Of these approximately 5 million wounds become chronic.

The most common single cause of significant skin loss is thermal injury, which accounts for an estimated 1 million emergency department visits per year [2]. Other causes of skin loss include trauma and chronic ulcerations secondary to diabetes mellitus, pressure and venous stasis. Every year in the US there are approximately 2 million cases of chronic diabetic ulcers, many of which eventually necessitate amputation. Pressure ulcers and leg ulcers, including venous ulcers, affect another 3 million people in the US with treatment costs as high as $8 billion annually [3]. In 2011, a survey estimated the US market for advanced wound care products, including biological and synthetic dressings, approximately $3 billion and expected to increase significantly as the population ages, becoming more susceptible to underlying causes of chronic wounds [4]. The quality of life tolls of chronic wounds are extremely high.

Over the past two decades, extraordinary advances in cellular and molecular biology have greatly expanded our comprehension of the basic biological processes involved in acute wound healing and the pathobiology of chronic wounds [5,6]. One recombinant growth factor, platelet-derived growth factor-BB (rPDGF-BB)(Regranex, Ortho-McNeil), and several skin substitutes (e.g., dermal substitutes: Integra Matrix Wound Dressings, Integra LifeSciences; AlloDerm, LifeCell; OASIS Wound Matrix, Healthpoint; Dermagraft and TransCyte, Advanced BioHealing; and Epidermal/dermal substitutes: Apligraf, Organogenesis; Orcel, Forticell Bioscience; Tissuetech, Fidia Advanced Biopolymers) have reached the market place for second-line therapy of recalcitrant ulcers [7]. These therapeutic interventions have added to the clinicians ability to induce skin healing but they have not had the impact that was predicted. Regardless of the advanced wound care product, the ideal goal would be to regenerate tissues where both the structural and functional properties of the wounded tissue are restored to the levels prior to injury.

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In contrast to adult wounds, embryonic wounds undergo complete regeneration, terminating in a scarless repair [8,9]. Thus, investigators are now using morphogenetic cues including hair development [10,11] to develop engineered constructs capable of tissue regeneration [12,13]. Cellular response to biological stimuli depends on the mechanical strength of the extracellular matrix (ECM) [14,15]. Therefore, the therapeutic successes of tissue-engineered constructs will depend not only on their molecular and cellular activity but also on their optimal mechanical properties [16]. This chapter will begin with an overview of the basic biology of wound repair, followed by a discussion of established practices and novel approaches to engineer tissue constructs for effective wound repair.

BASIC BIOLOGY OF WOUND REPAIR

Wound repair is not a simple linear process in which growth factors released by phylogistic events activate parenchymal cell proliferation and migration, but is rather an integration of dynamic interactive processes involving soluble mediators, formed blood elements, ECM, and parenchymal cells. Unencumbered, these wound repair processes follow a specific time sequence and can be temporally categorized into three major groups: inflammation, tissue formation, and tissue remodeling. The three phases of wound repair, however, are not mutually exclusive but rather overlapping in time. The reader is referred to the text 'The Molecular and Cellular Biology of Wound Repair' [17] for a more detailed discussion of the many processes involved in wound healing.

Inflammation

Severe tissue injury causes blood vessel disruption with concomitant extravasation of blood constituents. Blood coagulation and platelet aggregation generate a fibrin-rich clot that plugs severed vessels and fills any discontinuity in the wounded tissue. While the blood clot within vessel lumen reestablishes homeostasis, the clot within wound space acts as a growth factor reservoir and provides a provisional matrix for cell migration.

The primary cell types involved in the overall process of inflammation are platelets, neutrophils and monocytes. Upon injury, successful re-establishment of homeostasis depends on platelet adhesion to interstitial connective tissue, which leads to their aggregation, coagulation and activation. Activated platelets release several adhesive proteins to facilitate their aggregation, chemotactic factors for blood leukocytes, and multiple growth factors [5,6], to promote new tissue formation.

Of the two primary phagocytotic leukocytes viz. neutrophils and monocytes, neutrophils arrive first in large numbers due to their abundance in circulation. Infiltrating neutrophils cleanse the wounded area of foreign particles, including bacteria. If excessive microorganisms or indigestible particles have lodged in the wound site, neutrophils will probably cause further tissue damage as they attempt to clear these contaminants through the release of enzymes and toxic oxygen products. When particle clearance has been completed, generation of granulocyte chemoattractants ceases and the remaining neutrophils become effete.

Transition between inflammation and repair

Whether neutrophil infiltrates resolve or persist, monocyte accumulation continues, stimulated by selective monocyte chemoattractants [17]. Besides promoting phagocytosis and debridement, adherence to ECM also stimulates monocytes to undergo metamorphosis into inflammatory or reparative macrophages. Since cultured macrophages produce and secrete the peptide growth factors interleukin-1 (IL-1), platelet-derived growth factor-BB (PDGF-BB), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF), presumably wound macrophages also synthesize

these protein products [18]. Although neutrophils and macrophages have a critical role in fighting infection and macrophages can contribute growth factors to the wound, it has become increasingly clear that too much of a good thing can be bad [19]. In fact, from knockout and knockdown experiments, it is evident that wounds in some situations heal faster with less inflammatory cells, especially if microorganism invasion is avoided by some other means [20].

Re-epithelialization

Re-epithelialization of a wound begins within hours after injury by the movement of epithelial cells from the surrounding epidermis over the denuded surface. Rapid re-establishment of the epidermal surface and its permeability barrier prevents excessive water loss and time of exposure to bacterial infections, which decreases the morbidity and mortality of patients who have lost a substantial amount of skin surface. If a wide expanse of the epidermis is lost, epidermal cells regenerate from stem cells in pilosebaceous follicles [21]. Migrating epithelial cells markedly alter their phenotype by retracting their intracellular filaments, dissolving most of their desmosomes, and forming peripheral actin filaments (which facilitate cell movement) [17]. These migrating cells also undergo dissolution of their hemidesmosomal links between the epidermis and the dermis. All these phenotypic alterations provide epithelial cells with the needed lateral mobility for migration over the wound site. Migrating epidermal cells possess a unique phenotype that is distinct from both, the terminally differentiated keratinocytes of normal (stratified) epidermis and the basal cells of stratified epidermis. It is now appreciated that the signals that control wound healing in the adult animal are similar to those that control epithelial fusion during embryogenesis [22].

If the basement membrane is destroyed by injury, epidermal cells migrate over a provisional matrix of fibrin(ogen), fibronectin, tenascin, and vitronectin as well as stromal type I collagen [23]. Wound keratinocytes express cell surface receptors for fibronectin, tenasin and vitronectin, which belong to the integrin superfamily (Table 76.1) [24]. In addition, α2β1 collagen receptors, which are normally disposed along the lateral sides of basal keratinocytes, redistribute to the basal membrane of wound keratinocytes as they come in contact with type 1 collagen fibers of the dermis. Whereas β1 integrins are clearly essential for normal

TABLE 76.1 Integrin superfamily

Integrins	Ligand	Integrins	Ligand
β1 Integrins		αv Integrins	
α1β1	Fibrillar collagen, laminin-1	αvβ1	Fibronectin (RGD), vitronectin
α2β1	Fibrillar collagen, laminin-1	αvβ3	Vitronectin (RGD), fibronectin, fibrinogen, von Willebrand factor, thrombospondin, denatured collagen
α3β1	Fibronectin (RGD), laminin-5, entactin, denatured collagens		
α4β1	Fibronectin (LEDV), VCAM-1	αvβ5	Fibronectin (RGD), vitronectin
α5β1	Fibronectin (RGD+PHSRN, the synergy site)	αvβ6	Fibronectin, tenascin
α6β1	Laminin		
α7β1	Laminin	β2 Integrins	
α8β1	Fibronectin, vitronectin	αMβ2	ICAM-1, iC3b, fibronogen, factor X
α9β1	Tenasin	αLβ2	ICAM-1, -2, and -3
		αXβ2	IC3b, fibrinogen
Other ECM Integrins			
αIIbβ3	Same as αvβ3		
α6β4	Laminin		

re-epithelialization [25], it is not clear which subtype is essential. It is most likely that there is a redundancy in the requirement for α5β1 and α2β1 in re-epithelialization.

The migrating wound epidermis does not simply transit over a wound eschar but rather dissects through the wound, separating the fibrin/fibronectin-rich eschar and desiccated dermis containing denatured collagen from underlying viable tissue [26]. The path of dissection appears to be determined by the array of integrins expressed on the migrating epidermal cells. Keratinocytes do not express αvβ3, the integrin receptor for fibrinogen/fibrin and denatured collagen, *in vitro* or *in vivo* [26]. Thus, keratinocytes do not have the capacity to interact with these matrix proteins. Furthermore, fibrinogen or fibrin appears to inhibit epidermal cell interactions with fibronectin, hence migrating wound epidermis avoids the fibrin/fibronectin-rich clot but rather migrates along the type 1 collagen-rich wound edge via the α2β1 collegen receptor until it meets the fibronectin-rich granulation tissue and then proceeds to migrate over this newly forming tissue via the α5β1 receptor (Fig. 76.1).

ECM degradation is clearly required for the dissection of migrating wound epidermis between the collagenous dermis and the fibrin eschar and probably depends on epidermal cell production of collagenase, plasminogen activator, and stromelysin. Plasminogen activator activates collagenase (MMP-1) as well as plasminogen and therefore facilitates the degradation of interstitial collagen as well as provisional matrix proteins. Interestingly, keratinocytes in direct contact with collagen greatly increase the amount of MMP-1 they produce compared to that produced when they reside on laminin-rich basement membrane or purified laminin [27]. The migrating epidermis of superficial skin ulcers and burn wounds, in fact, express high levels of MMP-1 mRNA in areas where it presumably comes in direct contact with dermal collagen [28].

One to two days after injury, epithelial cells at the wound margin begin to proliferate. Although the exact mechanism is still not clear, both proliferation and migration of epithelial cells may be triggered by the absence of neighboring cells at the wound margin (the 'free edge effect'). The 'free edge effect' in the wound epidermis may be secondary to modulation of cadherins junctions as described for V-cadherins during angiogenesis [29]. In fact, studies indicate that epidermal desmosomes loose their hyperadhesiveness and cadherins switch from E-cadherins to P-cadherins at the wound edge [30]. Other possibilities, not exclusive of the former, are release of autocrine or paracrine growth factors that induce epidermal migration and proliferation and/or increased expression of growth factor receptors. Although some growth factors, such as insulin-like growth factor (IGF), may come from the circulation and thereby act as a hormone, other growth factors, such as heparin-binding epidermal growth factor (HB-EGF) and fibroblast growth factor 7 (FGF7, also called keratinocyte growth factor,

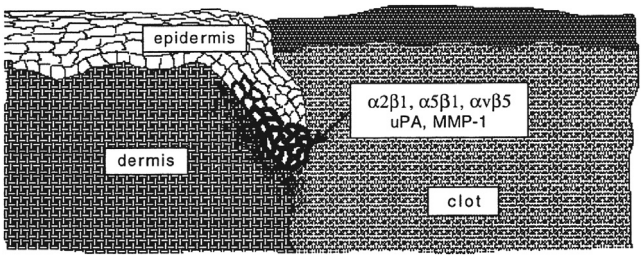

FIGURE 76.1
Integrins in early (three-dimensional) re-epithelialization. The migrating epidermis in contact with type I dermal collagen expresses a gene set including the collagen receptor α2β1, urokinase plasminogen activator (uPA), and interstitial collagenase (MMP-1). With this armamentarium, the epidermis can dissect a path between viable dermis and nonviable clot and denature stroma, ultimately leading to slough of the eschar.

KGF), are secreted from macrophages and dermal parenchymal cells, respectively, and act on epidermal cells through a paracrine pathway [31]. In contrast, transforming growth factor (TGF)-α and -β originate from keratinocytes themselves and act directly on the producer cell or adjacent epidermal cells in an autocrine or juxtacrine fashion. Many of these growth factors have been shown to stimulate re-epithelialization in animal models [5]. Furthermore, lack of growth factors or their receptors in knockout mice support the hypothesis that growth factor activation of keratinocytes is required for optimal epidermal migration and/or proliferation during normal wound healing [32]. In fact, it has been demonstrated that C-Jun N-terminal kinase (JNK) is a key signal transduction factor responsible for 're-setting' the epidermal program from differentiation to proliferation, and possibly migration [33].

As re-epithelialization progresses, basement membrane proteins reappear in a very ordered sequence from the margin of the wound inward in a zipper-like fashion [5]. Epidermal cells revert to their normal phenotype, once again firmly attaching to reestablished basement membrane through hemidesmosomal proteins, α6β4 integrin and 180 kDa bullous pemphigoid antigen [34], and to the underlying neodermis through type VII collagen fibrils [35].

Granulation tissue

New stroma, often called granulation tissue, begins to form approximately four days after injury. The name derives from the granular appearance of newly forming tissue when it is incized and visually examined. Numerous new capillaries endow the neostroma with its granular appearance. Macrophages, fibroblasts and blood vessels move into the wound space as a unit that correlates well with the biologic interdependence of these cells during tissue repair. Macrophages and ingrowing parenchymal cells provide a continuing source of cytokines necessary to stimulate fibroplasia and angiogenesis, fibroblasts construct new ECM necessary to support cell ingrowth, and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism. Recently the importance of oxygenation has been reemphasized [36]. The quantity and quality of granulation tissue depends on biologic modifiers present, the activity level of target cells, and the ECM environment. As mentioned in the section on Inflammation the arrival of peripheral blood monocytes and their activation to macrophages, establish conditions for continual synthesis and release of growth factors. In addition and perhaps more importantly, injured and activated parenchymal cells can synthesize and secrete growth factors. For example, migrating wound epidermal cells produce vascular endothelial cell growth factor (VEGF), TGF-β and PDGF-BB, to which endothelial cells and fibroblasts respond, respectively. The provisional ECM also promotes granulation tissue formation by positive feedback regulation of integrin ECM receptor expression [37]. Once fibroblasts and endothelial express the appropriate integrin receptors, they invade the fibrin/fibronectin-rich wound space (Fig. 76.2). Although it has been recognized for many years that ECM modulates cell differentiation by signal transduction from ligation of ECM receptors, more recently it has become evident that the force and geometry of the ECM influences cell behavior and differentiation [15,38,39].

FIBROPLASIA

Components of granulation tissue derived from fibroblasts including the cells themselves and the ECM are collectively known as fibroplasia. Growth factors, especially PDGF and TGF-β, in concert with the provisional matrix molecules [37], presumably stimulate fibroblasts of the periwound tissue to proliferate, express appropriate integrin receptors and migrate into the wound space. Many of these growth factors are released from macrophages or other tissue cells [5,6]; however, fibroblasts themselves can produce growth factors to which they respond in an autocrine fashion [40]. Multiple complex interactive biologic phenomena occur within fibroblasts as they respond to wound cytokines including the induction of additional cytokines and modulation of cytokine receptor number or affinity. *In vivo* studies support the

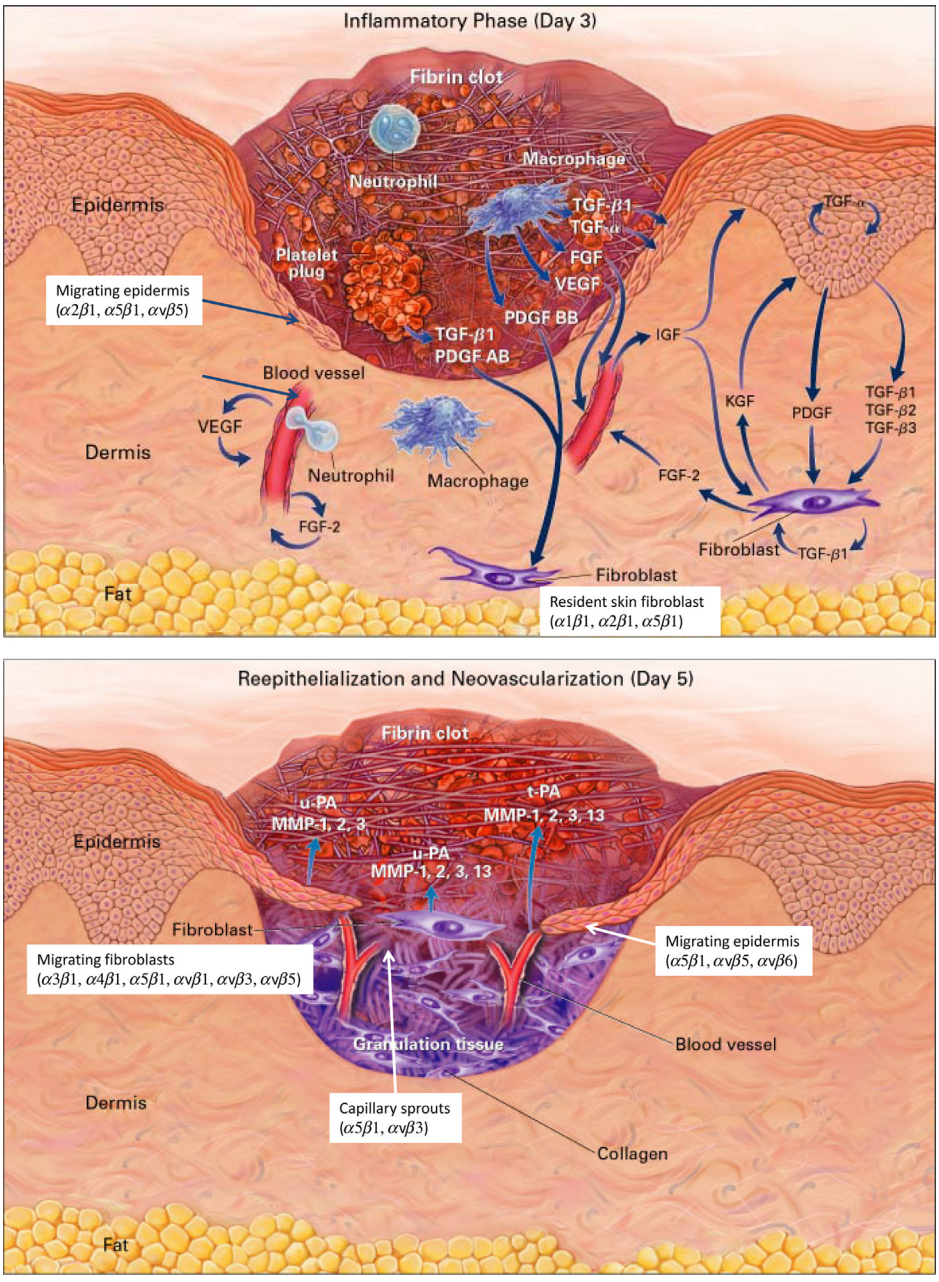

FIGURE 76.2
After injury, the platelets, inflammatory cells and the tissue cells (viz. fibroblasts, endothelial and epidermal cells) secrete abundant quantities of multiple growth factors thought to be necessary for cell movement into the wound. However, it is the provisional matrix integrin expression that acts as the rate-limiting step in granulation tissue induction. Once the appropriate integrins are expressed on periwound endothelial cells and fibroblasts on day 3, the cells invade the wound space shortly thereafter (on days 4 and 5). Fibroblasts and endothelial cells express the fibrinogen/fibrin receptor avb3 and therefore are able to invade the fibrin clot; the epidermis, however, does not express avb3 and therefore dissects under the clot. Proteinases play an important role during granulation tissue formation by clearing the path for migrating tissue cells. Ultimately, the clot that has not been transformed into granulation tissue by invading fibroblasts and endothelial cells is dissected free of the wound and sloughed as eschar. *(Modified from Singer, A.J. and Clark, R.A.F. (1999) Mechanisms of disease: cutaneous wound healing. New Eng. J. Med 341:738–46, with permission.)*

hypothesis that growth factors are active in wound repair fibroplasia. Several studies have demonstrated that PDGF, connective tissue factor (CTGF), TGF-α, TGF-β, HB-EGF and FGF family members are present at sites of tissue repair [41–44]. Furthermore, purified and recombinant-derived growth factors have been shown to stimulate wound granulation tissue in normal and compromised animals [5] and a single growth factor may work both directly, and indirectly by inducing the production of other growth factors *in situ* [45].

Structural molecules of the early ECM, coined provisional matrix [46], contribute to tissue formation by providing a scaffold or conduit for cell migration (fibronectin) [47], low impedance for cell mobility (hyaluronic acid) [48], a reservoir for cytokines [49] and direct signals to the cells through integrin receptors [24]. Fibronectin appearance in the periwound environment as well as the expression of fibronectin receptors appear to be critical rate-limiting steps in granulation tissue formation [50]. In addition, a dynamic reciprocity between fibroblasts and their surrounding ECM creates further complexity [51]. That is, fibroblasts affect the ECM through new synthesis, deposition, and remodeling of the ECM [52] while the ECM affects fibroblasts by regulating their function including their ability to synthesize, deposit, remodel and generally interact with the ECM [37,53]. Thus, the reciprocal interactions between ECM and fibroblasts dynamically evolve during granulation tissue development.

As fibroblasts migrate into the wound space, they initially penetrate the blood clot composed of fibrin and lesser amounts of fibronectin and vitronectin. Fibroblasts presumably require fibronectin *in vivo* for movement from the periwound collagenous matrix into the fibrin/fibronectin-laden wound space as they do *in vitro* for migration from a 3-dimensional collagen gel into a fibrin gel [47]. Fibroblasts bind to fibronectin through receptors of the integrin superfamily (Table 76.1). The Arg-Gly-Asp-Ser (RGDS) tetrapeptide within the cell-binding domain of these proteins is critical for binding to the integrin receptors α3β1, α5β1 αvβ1, αvβ3 and αvβ5. In addition, the CSIII domain of fibronectin provides a second binding site for human dermal fibroblasts via the α4β1 integrin receptor [54]. *In vivo* studies have shown that the RGD-dependent, fibronectin receptors α5β1 and αvβ3 are upregulated on periwound fibroblasts the day prior to granulation tissue formation and on early granulation tissue fibroblasts as they infiltrate the provisional matrix-laden wound [37]. In contrast, the non-RGD-binding α1β1 and α2β1 collagen receptors on these fibroblasts were either suppressed or did not appear to change appreciably [37,55].

Both PDGF and TGF-β can stimulate fibroblasts to migrate and can upregulate integrin receptors. Therefore, these growth factors appear to be responsible for inducing a migrating fibroblast phenotype. However, PDGF increases α5β1 and α3β1 while decreasing α1β1 in cultured human dermal fibroblasts surrounded by fibronectin [55]. Furthermore, fibronectin or fibrin-rich environments promote the ability of PDGF to increase α5β1 and α3β1, but not α2β1 by increasing mRNA stability and steady-state levels [37]. These data strongly suggest that the type of integrin increased by PDGF stimulation appears to depend on the ECM context and suggests a positive feedback between ECM and ECM receptors.

Movement into a crosslinked fibrin blood clot or any tightly woven ECM may also necessitate active proteolysis to cleave a path for migration. A variety of fibroblast-derived enzymes in conjunction with serum-derived plasmin are potential candidates for this task, including plasminogen activator, interstitial collagenase-1 and -3 (MMP-1 and MMP-13, respectively), the 72 kDa gelatinase A (MMP-2), and stromelysin (MMP-3). In fact, high levels of immunoreactive MMP-1 has been localized to fibroblasts at the interface of granulation tissue with eschar in burn wounds [28] and many stromal cells stain for MMP-1 and MMP-13 in chronic ulcers [56]. While TGF-β downregulates proteinase activity, PDGF stimulates the production and secretion of these proteinases [57]. From elegant knockout mouse studies it is clear that the plasminogen activating system is critical for clearing the wound clot [58]. While MMP-9 deficient mice demonstrate altered fracture repair [59], only single knockout MMP-8 (also called collagenase-2) adversely effected cutaneous wound repair [60]. However, double

knockout of MMP-13 (also called collagenase-3) and the plasminogen activating system in mice created more delay of healing compared to knockout of only the plasminogen activating system [61]. It was also shown that Galardin, a broad-spectrum MMP inhibitor, slowed cutaneous healing. Thus, although there is great overlap in MMP function in tissue repair, their activity is clearly necessary for proper healing of cutaneous wounds.

When fibroblasts have completed their migration into the wound site, they switch their major function to protein synthesis [52]. Thus, the migratory phenotype is completely supplanted by a profibrotic phenotype characterized by decreased α3β1 and α5β1 provisional matrix receptor expression, increased α2β1 collagen receptor expression, and abundant rough endoplasmic recticulum and Golgi apparatus filled with new collagen protein [37,52]. The fibronectin-rich provisional matrix is gradually supplanted with a collagenous matrix [52,53]. Under these conditions, PDGF, which is still abundant in these wounds [62], stimulates extremely high levels of α2β1 collagen receptor, but not α3β1 or α5β1 provisional matrix receptors, supporting the contention that the ECM provides a positive feedback for integrin expression [37]. TGF-β observed in wound fibroblasts at this time [53] can induce fibroblasts to produce great quantities of collagen [63]. IL-4 also can induce a modest increase in types I and III collagen production [64]. Since IL-4 producing mast cells are present in healing wounds, as well as fibrotic tissue, these cells may contribute to collagen matrix accumulation at these sites.

Once an abundant collagen matrix is deposited in the wound, fibroblasts cease collagen production despite the continuing presence of TGF-β [53]. The stimuli responsible for fibroblast proliferation and matrix synthesis during wound repair were originally extrapolated from many *in vitro* investigations and then confirmed by *in vivo* manipulation of wounds [17]. Less attention was directed toward elucidating the signals responsible for downregulating fibroblast proliferation and matrix synthesis until more recently. Both *in vitro* and *in vivo* studies suggest that gamma-interferon may be one such factor [65]. In addition, collagen matrix can suppress both fibroblast proliferation and fibroblast collagen synthesis [53,66]. In contrast, a fibrin or fibronectin matrix has little or no suppressive effect on the mitogenic or synthetic potential of fibroblasts [53,67].

Although the attenuated fibroblast activity in collagen gels is not associated with cell death, many fibroblasts in day 10 healing wounds develop pyknotic nuclei [68], a cytological marker for apoptosis or programmed cell death, as well as other signs of apoptosis. These results in cutaneous wounds and other results in lungs and kidney suggest that apoptosis is the mechanism responsible for the transition from a fibroblast-rich granulation tissue to a relatively acellular scar [69]. The signal(s) for wound fibroblast apoptosis have not been elucidated. Thus fibroplasia in wound repair is tightly regulated, whereas, in fibrotic diseases such as keloid formation, morphea, and scleroderma these processes become dysregulated. Recent evidence suggests that the fibroblast apoptotic signals in keloids are disrupted either directly or indirectly [70].

NEOVASCULARIZATION

Fibroplasia would halt if neovascularization failed to accompany the newly forming complex of fibroblasts and extracellular matrix. The process of new blood vessel formation is called angiogenesis [71]. Many soluble factors that stimulate angiogenesis in wound repair have been elucidated [72]. Angiogenic activity can be recovered from activated macrophages as well as epidermal cells, fibroblast, endothelial cells and numerous tumor cells [73]. Most biologically important angiogenic molecules have probably been identified and include VEGF, FGF-1 and FGF-2, TGF-α, TGF-β, TNF-α, platelet factor-4 (PF-4), angiogenin, angiotropin, angiopoietin, interleukin-8, PDGF, and low molecular weight substances including bioactive peptides, low oxygen tension, biogenic amines, lactic acid, and nitric oxide (NO) [5,6]. Some of these factors, however, are intermediaries in a single angiogenesis pathway, for example TNF-α induces PF-4 that stimulated angiogenesis through NO [74]. Even more important, low oxygen tension stabilizes hypoxia inducible factor-1α (HIF-1α) that induces increased expression of

VEGF [75]. To further complicate matters, not all growth factors within a family stimulate angiogenesis equally. For example, of four VEGF isoforms (VEGF-A, -B, -C, and -D) and three receptors (VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, and VEGFR3), VEGF-A does not interact with VEGFR1 and VEGFR2 equally and the signal transduction stimulated in not the same [76]. Furthermore, VEGF-C and -D stimulate lymphangiogenesis, rather than angiogenesis, through VEGFR3. Another complexity is that different growth factors effect blood vessel development at different stages. For example, VEGF-A stimulates nascent sprout angiogenesis, while angiopoietin induces blood vessel maturation [77].

Angiogenesis cannot be directly related to proliferation of cultured endothelial cells since endothelial cell migration is also required. In fact, Folkman [78] postulated that endothelial cell migration can induce proliferation. If true, endothelial cell chemotactic factors may be critical for angiogenesis. Some factors, however, have both proliferative (mitogenic) and chemotactic (motogenic) activities, e.g., PDGF [79] and EGF [80] are both motogenic and mitogenic for dermal fibroblasts while VEGF is both motogenic and mitogenic for endothelial cells [81].

Besides growth factors and chemotactic factors, an appropriate ECM is also necessary for angiogenesis. Three-dimensional ECM proteins gels provide a more natural environment for cultured endothelial cells than monolayer protein coats [72] as is true for many other cultured cells [82]. Not surprisingly, different ECM proteins induce differential cell responses. For example, rat epididymal microvascular cells cultured in type I collagen gels with TGF-β produce capillary-like structures within one week [71]. Omission of TGF-β markedly reduces the effect. In contrast, laminin-containing gels in the absence of growth factors induce human umbilical vein and dermal microvascular cells to produce capillary-like structures within 24 hours of plating [83]. Matrix bound thrombospondin also promotes angiogenesis [84] possibly through its ability to activate TGF-β [85]. Although type 1 collagen does not induce angiogenesis without other contributing factors, it can protect newly formed blood vessels from apoptotic effects of angiostatic agents [86]. Together, these studies support the hypothesis that the ECM plays an important role in angiogenesis. Consonant with this hypothesis, angiogenesis in the chick chorioallantoic membrane is dependent on the expression of αvβ3, an integrin that recognizes fibrin and fibronectin, as well as vitronectin [87]. Furthermore, in porcine cutaneous wounds αvβ3 is only expressed on capillary sprouts as they invade the fibrin clot [18]. *In vitro* studies in fact demonstrate that v can promote endothelial cell migration on provisional matrix proteins [88].

Given the information outlined above, a series of events leading to angiogenesis can be hypothesized. Substantial injury causes tissue-cell destruction and hypoxia. Potent angiogenesis factors such as FGF-1 and FGF-2 are released secondary to cell disruption [89] while VEGF is induced by hypoxia. Proteolytic enzymes released into the connective tissue degrade extracellular matrix proteins. Specific fragments from collagen, fibronectin and elastin, as well as many phylogistic agents, recruit peripheral blood monocytes to the injured site where these cells become activated macrophages that release more angiogenesis factors. Certain angiogenic factors, such as FGF-2, stimulate endothelial cells to release plasminogen activator and procollagenase. Plasminogen activator converts plasminogen to plasmin and procollagenase to active collagenase and in concert these two proteases digest basement membrane constituents.

The fragmentation of the basement membrane allows endothelial cells to migrate into the injured site in response to FGF, fibronectin fragments, heparin released from disrupted mast cells, and other endothelial cell chemoattractants. To migrate into the fibrin/fibronectin-rich wound, endothelial cells express αvβ3 [87], as well as αvβ5 integrin [90]. Newly forming blood vessels first deposit a provisional matrix containing fibronectin and proteoglycans but ultimately form basement membrane. TGF-β may induce endothelial cells to produce the fibronectin and proteoglycan provisional matrix as well as assume the correct phenotype for

capillary tube formation. FGF, and other mitogens such as VEGF, stimulate endothelial cell proliferation, resulting in a continual supply of endothelial cells for capillary extension. Capillary sprouts eventually branch at their tips and join to form capillary loops through which blood flow begins. New sprouts then extend from these loops to form a capillary plexus. Angiopoientin [91] and the recruitment of perienodthelial cells (pericytes) [92] are important for maturation and stabilization of the newly formed capillaries and pericytes are possibly important in providing a stem cell niche or being stem cells themselves [93].

Within a day or two after removal of angiogenic stimuli, capillaries undergo regression as characterized by: mitochondria swelling in the endothelial cells at the distal tips of the capillaries; platelet adherence to degenerating endothelial cells; vascular stasis; endothelial cell necrosis; and ingestion of the effete capillaries by macrophages. Although αvβ3 can regulate apoptosis of endothelial cells in culture and in tumors [87], αvβ3 is not present on wound endothelial cells as they undergo programmed cell death indicating that their absence may be critical or that other integrins are involved. It is fairly clear that thrombospondin [94] or other ECM molecules are good candidate ligands for controlling endothelial cell apoptosis [95].

Wound contraction and extracellular matrix organization

During the second and third week of healing, fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments disposed along the cytoplasmic face of the plasma membrane and the establishment of cell-cell and cell-matrix linkages [52,96]. In some [68], but not all [52], wound situations myofibroblasts express smooth muscle actin. Importantly, TGF-β can induce cultured human fibroblasts to express smooth muscle actin and may also be responsible for its expression *in vivo* [97].

The appearance of the myofibroblasts corresponds to the commencement of connective tissue compaction and the contraction of the wound. Fibroblasts link to the extracellular fibronectin matrix through α5β1 [52]; to collagen matrix through α1β1 and α2β1 collagen receptors [98]; and to each other through direct adherens junctions [52]. Fibroblast α2β1 receptors are markedly upregulated in seven day wounds [37], a time when new collagenous matrix is accumulating and fibroblasts are beginning to align with collagenous fibrils through cell-matrix connections [52]. New collagen bundles in turn have the capacity to join end-to-end with collagen bundles at the wound edge and to ultimately form covalent crosslinks among themselves and with the collagen bundles of the adjacent dermis [99]. These cell-cell, cell-matrix, and matrix-matrix links provide a network across the wound whereby the traction of myofibroblasts on their pericellular matrix can be transmitted across the wound [100].

Cultured fibroblasts dispersed within a hydrated collagen gel provide a reasonable functional *in vitro* model of wound contraction [101]. When serum is added to the admixture, contraction of the collagen matrix occurs over the course of a few days. When observed with time-lapse microphotography, collagen condensation appears to result from a 'collection of collagen bundles' executed by fibroblasts as they extend and retract pseudopodia attached to collagen fibers [102]. More recent elegant studies have further defined fibroblast-collagen interactions [103]. The transmission of these traction forces across the *in vitro* collagen matrix depends on two linkage events: fibroblast attachment to the collagen matrix through the α2β1 integrin receptors [104] and crosslinks between the individual collagen bundles [105]. This linkage system probably plays a significant role in the *in vivo* situation of wound contraction as well. In addition, cell-cell adhesions appear to provide an additional means by which the traction forces of the myofibroblast may be transmitted across the wound matrix [96]. In addition, gap junctions between wound fibroblasts probably provide the mechanism for contraction control across the cell population [106].

F-actin bundle arrays, cell-cell and cell-matrix linkages, and collagen crosslinks are all facets of the biomechanics of extracellular matrix contraction. The contraction process, however, needs

a cytokine signal. For example, cultured fibroblasts mixed in a collagen gel contract the collagen matrix in the presence of serum, PDGF, or TGF-β. Since TGF-β factor, but not PDGF, persists in dermal wounds during the time of tissue contraction, it is the most likely candidate for the stimulus of contraction [5]. Nevertheless, it is possible that both PDGF and TGF-β signal wound contraction: one more example of the many redundancies observed in the critical processes of wound healing. In summary, wound contraction represents a complex and masterfully orchestrated interaction of cells, ECM and cytokines.

Collagen remodeling during the transition from granulation tissue to scar is dependent on continued collagen synthesis and collagen catabolism. The degradation of wound collagen is controlled by a variety of collagenase enzymes from macrophages, epidermal cells, and fibroblasts. These collagenases are specific for particular types of collagens but most cells probably contain two or more different types of these enzymes [107]. Three MMPs have been described that have the ability to cleave native collagen: MMP-1 or classic interstitial collagenase which cleaves types I, II, III, XIII, and X collagens; neutrophil collagenase (MMP-8); and a novel collagenase produced by breast carcinomas that is prominent in chronic wounds (MMP-13) [56]. Currently it is not clear which interstitial collagenases are critical in the remodeling stage of human wound repair. For example, no wound healing defect was observed in mice deficient of MMP-13 [108], however, a double knockout of MMP-13 and the plasminogen activating system created an additional delay of healing compared to knockout of only the plasminogen activating system [61]. These findings are likely attributable to the redundancy of nature.

Cytokines such as TGF-β, PDGF, and IL-1 and the ECM itself clearly play an important role in the modulation of collagenase and Tissue Inhibitor of Metalloproteinase (TIMP) expression *in vivo*. Interestingly, type 1 collagen induces MMP-1 expression through the α2β1 collagen receptor while suppressing collagen synthesis through the α1β1 collagen receptor [109]. Type 1 collagen also induces expression of α2β1 receptors [37], thus collagen can induce the receptor that signals a collagen degradation-remodeling phenotype. Such dynamic, reciprocal cell-matrix interactions appears to occur generally during tissue formation and remodeling processes such as morphogenesis, tumor growth, and wound healing to name a few [82].

Wounds gain only about 20% of their final strength by the third week, during which time fibrillar collagen has accumulated relatively rapidly and has been remodeled by myofibroblast contraction of the wound. Thereafter the rate at which wounds gain tensile strength is slow, reflecting a much slower rate of collagen accumulation. In fact, the gradual gain in tensile strength has less to do with new collagen deposition than further collagen remodeling with formation of larger collagen bundles and an accumulation of intermolecular crosslinks. Nevertheless, wounds fail to attain the same breaking strength as uninjured skin. At maximum strength, a scar is only 70% as strong as intact skin.

CHRONIC WOUNDS

Acute wounds are those that heal through the routine processes of inflammation, tissue formation and remodeling, which occur in a timely fashion. As discussed earlier, these processes may overlap temporally. However, prolonged continuance of any of these reparative processes may result in the formation of a chronic wound. Chronic wounds are often associated with underlying pathological conditions that contribute to an impaired healing. Venous leg ulcers and diabetic foot ulcers are common examples of chronic wounds caused or accentuated by an underlying disorder. While the former is being induced by insufficient venous flow that results in increased blood pressure in the lower limb and, therefore, increased vascular permeability, the latter is caused by peripheral neuropathy that leads to abnormal load distribution on the foot surface and decreased sensation [110]. Subsequently, these

abnormalities cause a loss of tissue viability, sub-optimal local tissue permeability and an elevated and sustained inflammatory response.

TISSUE-ENGINEERED THERAPY: ESTABLISHED PRACTICE

Initial attempts to speed up wound repair and improve the quality of healing in chronic or burn wounds involved the use of synthetic, composite synthetic or biological dressings [111]. Although temporarily effective, these dressings did not offer any permanent treatment since eventually, an autograft had to be implanted to achieve complete healing. The advent of tissue-engineered constructs has, however, benefited the wound healing care [112]. These constructs could be classified into two main categories: cellular and acellular. In both cases, the basic building blocks are a biomimetic and a scaffolding material. While the biomimetic functions to stimulate cell recruitment and the desired cellular functions, the scaffold typically provides a mechanical support for the cells. Scaffolds prepared from naturally occurring biopolymers tend to provide the correct biological stimuli to support cell function and tissue formation [113]. Nevertheless, whether the scaffold is natural or synthetic, the goal is to promote faster healing which results in the development of a new tissue that bears structural and functional resemblance to the uninjured, host tissue. A comprehesive list of current cell therapeutics, biologic dressings, and skin substitutes for acute and chronic skin wounds has been recently published [7].

Engineered epidermal constructs

Immediate wound coverage, whether permanent or temporary, is one of the cornerstones of wound management. Engineered epidermal constructs with attributes similar to those of autologous epidermis have been used to facilitate repair of partial-thickness wounds where the major damage is to the epidermis.

Cultured autologous keratinocyte grafts were first used in humans by O'Conner et al [114]. Subsequently there has been extensive experience with cultured epidermal grafts for the treatment of burns as well as other acute and chronic wounds [115,116]. Epicel™ (Genzyme Tissue Repair) is an example of an engineered epidermal autograft. The potential advantage of this technique is the ability to provide autologous grafts capable of covering large areas with reasonable cosmetic results. Another significant advantage of autologous grafts is their ability to serve as permanent wound coverage since the host does not reject them. However, major disadvantages are the several week lag between host skin harvest and the availability of sufficient quantities of keratinocytes, the need for an invasive procedure to obtain autologous donor cells, and the large costs incurred. Furthermore, graft take is widely variable based on wound status, patient age, general host status, and operator experience.

Cultured keratinocyte allografts were developed to help overcome the need for biopsy and separate cultivation for each patient to produce autologous grafts and the long lag period between epidermal harvest and graft product. Cultured epidermal cells from both cadavers and unrelated adult donors have been used for the treatment of burns [117] and chronic leg ulcers [118]. Although allografts made from neonatal foreskin keratinocytes were more responsive to mitogens than adult (cadaver) cells and, therefore, initially preferred for cultured allografts, a later investigation revealed that such allografts were more immunogenic than the regular culture skin substitutes and thus potentially problematic [119].

To facilitate mass allograft production and wide availability, cryopreserved allografts have been developed and were fairly comparable to fresh allografts [120]. In spite of these advances, the culture epidermal grafts have failed to produce satisfactory response. The primary reasons include the lack of mechanical strength and graft site susceptibility to wound contractures. As an alternative, keratinocyte delivery systems were developed where the cells were delivered to the injury site via a biodegradable scaffold. For example, Laser skin, produced by FIDIA

Advanced Biopolymer, Italy, is used to deliver keratinocytes via a chemically modified hyaluronan membrane, which is perforated with micron-sized holes that allow cells to grow to confluence. Another approach has been to isolate fresh keratinocytes from the patients and then spray them onto the wound site [121,122]. Spray therapy of cultured keratinocytes is currently approved for use for partial-thickness burns in Australia and undergoing clinical trials in the US (Recell, Avita Medical, Australia). In addition, spray therapy of freshly isolated keratinocytes is being commercialized by the Gown Institute at the University of Pittsburgh.

Engineered dermal constructs

While the use of cultured keratinocytes to enhance wound healing has met with modest success, it lacks a dermal component that, if present, would provide greater mechanical stability and possibly prevent wound contraction. Allografts containing dermis, e.g., pig skin or cadaver skin, have been used for many years as temporary coverage but they tend to induce an inflammatory response [123]. However, such skin can be chemically treated to remove the antigenic cellular elements (Alloderm, Life Cell Corporation, Woodlans, TX) and used alone or in combination with cultured autologous keratinocytes for closure of various chronic wounds and burns [124]. In spite of these modifications, allogenic grafts, when compared with autologous grafts, have been shown to promote lower percent re-epithelialization and excessive wound contraction [125]. Furthermore, the overall therapeutic outcome of a skin graft depends also on the site from which the cells were isolated (during biopsy) [126].

In 1981, Burke et al. [127] developed an acellular composite skin graft made of a collagen-based dermal lattice (containing bovine collagen and chondroitin-6-sulfate) with an outer silicone covering. After placement on the wound, the acellular dermal component recruits the host dermal fibroblasts while simultaneously undergoing degradation. About 2−3 weeks later, the silicone sheet is removed and covered with an autograft. This composite graft has been used successfully to treat burns [128] and has received Federal Drug Administration (FDA) approval for this indication as well as for reconstructive surgery (Integra, Integra Life Sciences Corporation, Plainsboro, NJ) [129]. However, these constructs cannot be used in patients who are allergic to bovine products.

Another rendition of a dermal substitute is TransCyte (Dermagraft-TC) (Advanced BioHealing, LaJolla, CA). This product consists of an inner nylon mesh in which human fibroblasts are embedded together with an outer silicone layer to limit evaporation. The fibroblasts are lysed in the final product by freeze-thawing. Prior to that time, the fibroblasts had manufactured collagen, matrix proteins and cytokines all of which promote wound healing by the host. TransCyte has been used successfully as a temporary wound coverage after excision of burn wounds [130], and has been approved by the FDA for this indication. Dermagraft is a modification of this product in which a biodegradable polyglactin mesh is used instead of a silicone layer and the fibroblast remain viable [131].

Engineered skin substitutes

Full thickness wounds involve the loss of both the epidermal and dermal layers of the skin. To treat such extensive wounds, Bell et al. first described a bilayered skin composite consisting of a collagen lattice with dermal fibroblasts that was covered with epidermal cells [132]. Modification of this composite consisting of type I bovine collagen and live allogeneic human skin fibroblasts and keratinocytes has been developed (Apligraf, Organogenesis, Canton, MA). It has been used successfully in surgical wounds, venous leg ulcers, and diabetic foot ulcers [133], despite the fact that it is not a permanent skin replacement [134]. In a large multicenter trial this product resulted in accelerated healing of chronic non-healing venous stasis ulcers when compared to standard compressive therapy [135], Both Apligraf (for venous stasis ulcers) and Dermagraft (for diabetic neurotrophic ulcerations) have been available in Canada since 1997 and more recently in the US. Several other composite skin substitutes combining

dermal and epidermal elements have been developed. Composite cultured skin (CCS, Ortec International Inc, New York, NY) is composed of both neonatal keratinocytes and fibroblasts embedded in distinct layers of bovine type I collagen. This product is currently being evaluated in clinical trials for the treatment of burns and in patients with epidermolysis bullosa. More extensive comparative data on the various biologic dressings and available tissue-engineered products has been published elsewhere [7,136].

TISSUE-ENGINEERED THERAPY: NEW APPROACHES

Although the increased healing rates observed with the use of these engineered constructs show promise for the treatment of burn and/or chronic wounds, they have several intrinsic disadvantages that limit their use:

1) The epidermal grafts are very fragile and therefore difficult to handle,
2) It is difficult to quality control the large scale production of any cell-populated matrix, and
3) While autografts require skin biopsy, allografts may experience early rejection.

Moreover, these constructs in general are only about 25% efficient, implying that they must be applied on at least four patients before their effect can be seen. These limitations suggest that further improvements be made so that tissue-engineered constructs are not only more effective but also less complex.

Two approaches emerge from the foregoing discussions. The first tissue-engineering design utilizes cells that resist rejection and/or provide a more robust clinical outcome that makes their use cost-effective despite the absolute costs required to produce them. Alternatively, second-generation, acellular tissue-engineered products are being developed that more effectively recruit host tissue cells into the wound and then provide biological signals for the accumulated tissue cells to induce tissue regeneration rather than scar.

To address the first approach many groups are trying to isolate the 'right' stem cells to stimulate wound repair or preferably tissue regeneration. For the skin, both epidermal stem cells [137,138] and bone marrow- or adipose-derived meschencymal stem cells (MSCs) have been isolated by numerous methods and delivered by many different methods [139,140]. New and better methods for isolating and standarding MSCs are greatly needed and several investigators are assiduously tackling this problem. Better understanding of the niche where stem cells normally reside will help elucidate the microenvironment that the cells need to self-renew versus differentiate along the appropriate path [141,142].

For the acellular 'smart' matrix tissue-engineering approach, clues learned from embryogenesis, morphogenesis and wound repair should be implemented to engineer revolutionary constructs that facilitate and synchronize tissue repair. The transformative acellular product must be conductive to rapidly recruit the host tissue cells and inductive to stimulate the invading cells to proliferate, synthesize new ECM and then differentiate appropriately to regenerate the lost tissue.

Various engineered skin constructs have used collagen as the preferred scaffolding material for cell seeding [143]. The huge popularity of collagen can be attributed to its abundance in skin and, therefore, its recognition by cell surface receptors, its multiple biological roles during both cutaneous homeostasis and wound repair, and its ability to crosslink and thereby impart mechanical strength to the final construct [136]. However, during wound repair, collagen appears only during the later stages after the invading and proliferating fibroblasts have filled the wound space. Therefore, collagen may not be optimal for initial cell migration. Since fibronectin and hyaluronan are present during early stages of embryogenesis, morphogenesis and wound healing [17], we have favored using these biomaterials or their derivatives to design 'smart' matrix for cutaneous wound repair.

Hyaluronan is a non-sulfated glycosaminoglycan that is present in most human tissues. During wound repair, it serves multiple important functions, ranging from regulating inflammation to promoting fibroblast migration and proliferation [144]. Interestingly, hyaluronan has been implicated in the scarless or minimally scarred repair of fetal wounds, perhaps owing to its role in regulating the inflammatory response [145] and collagen deposition [146]. Furthermore, similar to synthetic polymers, hyaluronan can be chemically modified to obtain a variety of stable derivatives [147]. Therefore, by offering the advantages of both natural and synthetic materials, hyaluronan promises to be a more suitable scaffolding material for acellular matrices as compared to collagen. Indeed, chemically modified hyaluronan scaffolds have been successfully used for various tissue-engineering applications, including wound repair [148].

Since fibroblast migration is the rate-limiting step in granulation tissue formation [50], a biomaterial ± biomimetic must support maximal fibroblast migration. Fibronectin is a favorable candidate since:

1) It appears together with hyaluronan at times of cell migration during embryogenesis, morphogenesis and wound repair [17,48],
2) Fibroblast migration on hyaluronan/fibronectin gels is far greater (~four-fold) when compared to that on fibrin/fibronectin gels (Greiling and Clark, unpublished observation),
3) Fibronectin has been shown to be required for fibroblast transmigration from a collagen gel to a fibrin gel [47], and
4) Fibronectin is absent in chronic wounds, where it is produced normally [149] but eliminated rapidly by the abundant proteases present in chronic wound fluid [150,151].

Although fibronectin appears to be an ideal biomimetic for use in hyaluronan scaffolds, its stability in the proteolytic environment of chronic wounds is a major concern.

Alternatively, the proteolytically stable arginine-glycine-asparatic acid (RGD) peptide sequence, the smallest cell recognition sequence in the tenth module of type III repeat of fibronectin, can be used to support key cell functions [152]. RGD has been widely used to promote cell attachment and spreading in various tissue engineering applications in general [153], and wound healing applications in particular [154]. This is perhaps because the RGD sequence is found in a variety of ECM molecules and, therefore, recognized by the transmembrane integrin receptors of multiple cell types, including dermal and epidermal tissue cells [155]. Our previous study has shown that hyaluronan hydrogels decorated with RGDS (arg-gly-asp-ser) support NIH 3T3 fibroblast functions *in vitro* [156] and when seeded with 3T3 fibroblasts and implanted in nude mice, produce granulation-like tissue in four weeks. Therefore, RGD-modified hyaluronan hydrogels appeared to possess great inductive properties. However, these hydrogels neither supported optimal human adult dermal fibroblast functions nor demonstrate conductive properties required of any acellular scaffold [156].

To impart our construct with both inductive and conductive properties, we selected, as the biomimetics, three fibronectin (FN) functional domains viz. $FNIII_{(8–11)}$, $FNIII_{(12–15)}$, and $FNIII_{(12\text{-}V\text{-}15)}$, that are necessary and sufficient for optimal dermal fibroblast migration *in vitro* [157]. Indeed, these hydrogels were successful in supporting dermal fibroblast functions *in vitro* and promoting wound repair *in vivo* [158]. Since these hydrogels can be formulated at room temperature and physiological pH, they are compatible with both cells and the incorporated biological molecules. In addition, their rapid gelation (<10 min) advocates their possible injectable use. However, manufacturing an tissue-engineered implant containing three recombinant proteins raises concerns related to cost, quality control, sterilization and shelf-like of the product. To solve this issue we are currently working to develop fibronectin-derived peptides that substitute for the three domains previously used.

With a similar objective, several other groups have also developed 'intelligent' scaffolds for tissue repair [159]. These approaches commonly employ synthetic materials to build scaffolds since they allow great flexibility during formulation. To impart bioactivity, these scaffolds contain potent biomimetics that can be recognized by tissue cells. However, as discussed earlier, cell invasion during granulation tissue formation occurs concurrently with matrix degradation, which can be typically observed when using naturally-derived materials. To elicit a similar response in synthetic materials, protease-sensitive sequences are incorporated within the scaffold, which are cleaved upon contact with the cell-secreted proteolytic enzymes [160,161]. Therefore, these scaffolds combine the advantages of both natural and synthetic biomaterials to facilitate wound repair.

Traditionally, the structural component of the tissue-engineered constructs has been viewed as providing only a passive mechanical structure. This design reflects our common understanding that cells primarily respond to biological signals. However, over the past decade it has become increasing clear that mechanical forces alone can govern cell and tissue phenotype in ways similar to biological stimuli [162]. Further studies have revealed that cells use an active tactile sensing mechanism to feel and respond to substrate mechanics [15,38] and that stem cells use clues from this mechanical sensing to select pathways for differentiation [163]. The latter observation was not too surprising since it had already been demonstrated that dermal fibroblasts respond to substrate mechanics by regulating levels of gene transcription that eventually lead to differential ECM synthesis and their transformation into myofibroblasts [100]. Since these processes are critical during wound repair, effective tissue-engineering approaches for wound repair would require optimization of both biological and mechanical effectors.

The acellular tissue-engineered constructs discussed so far utilize scaffolding materials to provide mechanical support for tissue ingrowth and biomimetics to induce key cell functions. The primary goal of these novel approaches is to mimic the attributes of fibrin clot for parenchymal cell migration. However, a fibrin clot is not only composed of a fibrin/fibronectin scaffold and an array of clotting and fibrinolytic enzymes, but also a plethora of growth factors that had been released during platelet aggregation [164]. Growth factors play a crucial role in the overall healing response where they function to stimulate cell migration, proliferation, differentiation and angiogenesis. Furthermore, growth factor deficiency often leads to impaired wound repair [165]. As a result, several groups have investigated the use of tissue engineered constructs for local growth factor delivery, where the release of appropriate growth factors produced an increase in angiogenic activity [166,167].

It is interesting to note that in spite of the release of growth factors immediately after wounding, there remains a three day lag before granulation tissue is formed. This suggests that the growth factors may be retained and functional within the clot. This form of 'solid-state' biochemistry may be unconventional but is backed by data from recent studies, which have shown that IGF and VEGF bound to specific molecular domains of FN retain or accentuate their bioactivity [168,169]. In fact, we have recently demonstrated that two FN domains used in our hyaluronan gel PDGF-BB [170] as well as other growth factors important in soft tissue healing. Therefore, by incorporating the appropriate growth factor-binding sequences/domains, tissue-engineered construct can be used as a growth factor repository, causing an increase in local concentration that may ultimately accentuate cell functions.

In conclusion, wound healing is a dynamic and fine-tuned cellular response aimed at reinstating tissue homeostasis after an insult. Vigorous cellular activities observed during wound repair are similar to those occurring during embryogenesis and morphogenesis, which indicates the enormous complexity of this physiological reparative process. That may also explain why despite over two decades of intense research and development, we have still not identified an 'ideal' therapy. However, novel tissue-engineering approaches are showing tremendous promise and aiming to push the limits of human expectations of wound therapy.

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CHAPTER 77

Bioengineered Skin Constructs

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INTRODUCTION

Cutaneous wounds normally heal by formation of epithelialized scar tissue rather than regeneration of full-thickness skin. Consequently, strategies for the clinical management of wound healing have depended historically on providing a passive cover to the site of the wound while allowing the reparative mechanisms of wound healing, including re-epithelialization, remodeling of granulation tissue, and formation of scar tissue, to occur, and therapy could do little more than facilitate these processes. However, advances in our understanding of wound healing, wound assessment, the concerted action of several growth factors, the role of the extracellular matrix in regulating the healing process, and the demonstrated ability of bioengineered constructs to promote wound healing highlights the potential for intervening therapeutically in tissue repair by providing lost epithelium, stimulating dermal regeneration, and reconstituting full-thickness skin.

Bioengineered skin substitutes can be classified as both cell-based constructs that actively stimulate wound healing, and acellular constructs that provide a substrate or covering to facilitate wound healing. Cell-based constructs include autologous epidermal cell sheets (Epicel®, Genzyme, Cambridge, MA), allogeneic dermal substrates (Dermagraft™, Shire Regenerative Medicine, San Diego, CA), and human skin equivalents (HSE) composed of both dermal and epidermal components (Apligraf® [1], Organogenesis Inc., Canton, MA; Orcel®, Forticell Bioscience Inc., Englewood Cliffs, NJ; StrataGraft®, Stratatech, Madison, WI) and acellular products (Transcyte®, Shire Regenerative Medicine, San Diego, CA; Integra® Dermal Regeneration Template, Integra Life Sciences, Plainsboro, NJ; Biobrane®, UDL, Rockford, IL). Another construct, comprising cultured keratinocytes and fibroblasts in bovine collagen, is the living cellular sheet, GINTUIT™ (Organogenesis Inc., Canton, MA), indicated for topical application to a surgically created vascular wound bed in the treatment of mucogingival conditions. Although not all of these products are still commercially available, they represented the first of their kind, and are the result of basic research in the biology of skin and wound healing and clinical experience with skin grafts, cultured keratinocyte grafts, acellular collagen matrices, cellular matrices, and cultured composite grafts [1–4].

[1] Apligraf is a registered trademark of Novartis

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SKIN STRUCTURE AND FUNCTION

Skin has several distinct functions. As the interface between the environment and the body, skin provides a protective barrier from microbes, toxins, ultraviolet radiation and abrasion while also preventing water loss. Additionally, skin enables the body's ability to sense heat and cold, pressure, vibration and pain and regulate body temperature through perspiration and blood flow. The passive and active functions of skin are carried out by specialized cells and structures located in its two main layers: the epidermis and the dermis (Fig. 77.1). Complex functional relationships between these two anatomic structures of skin maintain its normal properties. Tissue-engineering applications in skin depend upon an understanding of the structural components of skin, their spatial organization, and their functional relationships.

The epidermis

As stated above, skin is a physical barrier between the body and the external environment. The outermost layer of skin, the epidermis, is the layer that is impermeable to toxic substances and harmful organisms. It is also the layer that controls the loss of water from the body to the relatively drier external environment.

The epidermis is composed primarily of keratinocytes, which form a stratified squamous epithelium (Fig. 77.1). Proliferating cells in the basal layer of the epidermis anchor the epidermis to the dermis and replenish the terminally differentiated epithelial cells lost through normal sloughing from the surface of the skin. These basal cells stop proliferating and terminally differentiate into squamous keratinocytes as they move from the basal layer through the suprabasal layers to the surface of the epidermis. Keratin filaments and desmosomes contribute physical strength in the living layers and maintain the integrity of the epidermis. The cornified envelopes serve as the bricks and the lipids as mortar.

The most superficial keratinocytes in the epidermis form the stratum corneum, the dead outermost structure that provides the physical barrier of the skin. In the last stages of differentiation, epithelial cells extrude lipids into the intercellular space to form the permeability barrier. The cells break down their nuclei and other organelles and form a highly crosslinked protein envelope immediately beneath their cell membranes. The physically- and chemically-resilient protein envelope connects to a dense network of intracellular keratin filaments to provide further physical strength to the epidermis.

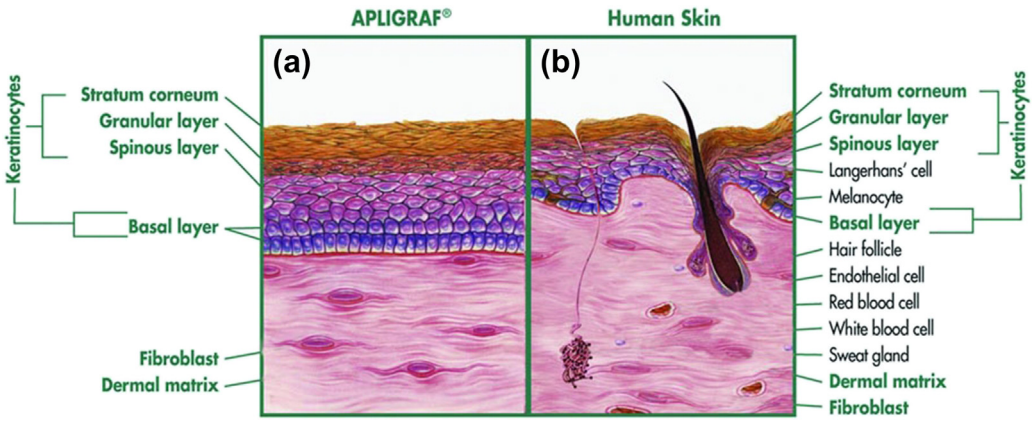

FIGURE 77.1

The basic components of skin and engineered skin equivalent. (a) Diagram showing the major cell types of skin and their organization. Note that stratified keratinocytes make up the epidermis and display distinct morphological phenotypes. (b) A histological section of Apligraf HSE, (hematoxylin and eosin,142 X). Italics indicate cell types present in real skin but not in the engineered skin equivalent.

Additional cells and structures in the epidermis perform specialized functions (Fig.77.1). Skin plays a major role in alerting the immune system to potential environmental dangers. The interacting cells in skin comprise a dynamic network capable of sensing a variety of perturbations (trauma, ultraviolet radiation, toxic chemicals, and pathogenic organisms) in the cutaneous environment, and rapidly sending appropriate signals that alert and recruit other branches of the immune system [5,6]. To restore homeostasis in the skin immune system, the multiple proinflammatory signals generated by skin cells must eventually be counterbalanced by mechanisms capable of promoting resolution of a cutaneous inflammatory response. Dendritic cells of the immune system (Langerhans cells) reside in the epidermis and form a network of dendrites through which they interact with adjacent keratinocytes and nerves [7]. Melanocytes distribute melanin to keratinocytes in the form of melanosomes. Melanin protects the epidermis and underlying dermis from ultraviolet radiation. Sweat glands help to regulate body temperature through evaporation of sweat secreted onto the skin surface. Sebaceous glands associated with hair follicles secrete sebum, an oily substance that lubricates and moisturizes hair and epidermis. Hair keeps the body warm in many mammals, although maintaining body temperature is not an important role for hair in humans. Hair follicles, however, are an important source of proliferating keratinocytes during re-epithelialization after wounding.

The dermis

The dermis underlies the epidermis (Fig. 77.1). The dermis is divided into two regions: the papillary dermis, which lies immediately beneath the epidermis, and the deeper reticular dermis. The papillary dermis is composed of loose connective tissue and form papillae that intertwine with the rete ridges of the epidermis. The reticular dermis is more acellular and has a denser meshwork of thicker collagen and elastic fibers than the papillary dermis. The reticular dermis provides skin with most of its strength, flexibility, and elasticity. Loss of reticular dermis can often lead to excessive scarring and wound contraction.

The dermis provides physical strength and flexibility to skin as well as the connective tissue scaffolding that supports the extensive vasculature, lymphatic system, and nerve bundles. The dermis is relatively acellular, being composed predominantly of an extracellular matrix of interwoven collagen fibrils. Interspersed among the collagen fibrils are elastic fibers, proteoglycans, and glycoproteins.

Fibroblasts, the major cell type of the dermis, produce and maintain most of the extracellular matrix (Fig. 77.1). Endothelial cells line the blood vessels and play a critical role in the skin immune system by controlling the extravasation of leukocytes. Cells of hematopoietic origin in the dermis (e.g., macrophages, lymphocytes) contribute to a surveillance function. A network of nerve fibers extends throughout the dermis, which serves the sensory role in the skin (and, to a more limited extent, a motor function). These nerve fibers also secrete neuropeptides that influence immune and inflammatory responses in skin through their effects on endothelial cells, leukocytes, and keratinocytes [8].

The process of wound healing

Wound healing progresses through three distinct phases, namely inflammation, proliferation, and remodeling. The immediate tissue response to wounding is clot formation to stop bleeding. Simultaneously, there is a release of inflammatory cytokines that regulate blood flow to the area, and recruitment of lymphocytes and macrophages to fight infection. This initial inflammatory phase stimulates angiogenesis, fibroblast proliferation, and collagen deposition [8], which marks the proliferation phase. Fibroblasts rich in smooth muscle actin, called myofibroblasts [9], are recruited through the action of factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). These latter processes result in the formation of granulation tissue, a highly vascularized and cellular wound

connective tissue. In the remodeling phase of healing, granulation tissue is gradually replaced by scar tissue through the action of the myofibroblasts and factors such as TGF-β. Keratinocytes are stimulated to proliferate and to migrate into the wound bed to restore epidermal coverage.

Impaired healing and its mechanisms

Although there have been many recent advances in our understanding of the scientific basis of tissue repair, the treatment of chronic wounds and intervening in situations associated with impaired healing have been very challenging. The healing of chronic wounds is impaired for a number of reasons, and in general these types of wounds remain challenging because of our inability to completely correct their fundamental pathophysiological abnormalities [10]. Preparatory steps before the use of advanced treatments seem to be required; this preparatory phase may not have received proper attention in the past. This situation is slowly being corrected, but much more needs to be implemented in preparing the wound for the optimal success of advanced wound healing factors and bioengineered constructs. Several years ago, we proposed the notion of 'wound bed preparation' as a series of steps to improve the wound before advanced products are used [11]. This concept has gained acceptance (see below).

ACUTE VERSUS CHRONIC WOUND HEALING

One of the basic differences between acute and chronic wounds is that in the former the sequence of steps and phases involved (clot formation, inflammation, migration and proliferation, and remodeling) occur in a very orderly and linear fashion. Such is not the case in chronic wounds, where there is a fundamental asynchrony of the healing process. Within the chronic wound, the various phases of wound repair may be occurring at the same time, or not in the appropriate sequence. Wound bed preparation is a way to get the chronic wound to behave more like an acute wound. Often, surgical debridement is all that is required. At other times, treatment of bacterial infection, removal of edema, etc., are essential additional steps [11].

BACTERIAL COLONIZATION

Bacterial and, in some cases, fungal colonization or infection is a fundamental problem with non-healing wounds. Some of the causes that foster this colonization and the development of occult infection have already been addressed. These factors include absent epithelium and its barrier properties, the constant wound exudate resulting from bacterial products and inflammation, and poor blood flow and hypoxia [10]. It is well established that wounds have a 'bacterial burden' that interferes with healing. Thus, there is evidence that, regardless of the type of bacteria present, a level greater than or equal to 10^6 organisms per gram of tissue is associated with serious healing impairment [12]. The configuration of the bacterial sheets or colonies is also important. Therefore, there is a great deal of interest at this time in the role played by biofilms, which represent bacterial colonies surrounded by a protective coat of polysaccharides. Biofilms develop mechanisms for antibiotic resistance [13].

GROWTH FACTOR IMBALANCES

The poor healing response in chronic wounds has also been attributed to an imbalance of one or more growth factors [14]. Identification of putative wound healing factors has led to several attempts to speed wound healing by local application of one or more factors that promote cell attachment and migration. However, most if not all, factor-based approaches have had marginal success. TGF-β, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and PDGF have been candidates for this purpose [15–19]. Of these, only Platelet-Derived Growth Factor (PDGF) has shown efficacy in clinical trials and has FDA approval for clinical use (Regranex®, HealthPoint Biotherapeutics Ltd., Ft. Worth, TX).

The arginine-glycine-aspartic acid (RGD) matrix peptide sequence has been found to promote the migration of connective tissue cells, and thus stimulate production of a dermal scaffold within

the wound bed. This approach has been shown to accelerate healing of sickle-cell leg ulcers [20] and diabetic ulcers [21], compared with placebo, but not when compared with standard care.

Complex cell extracts have been used in hopes of providing the appropriate mixture of elements. These include the use of platelet extract to provide primarily PDGF [22], and the use of conditioned growth medium to provide a complex mixture of elements derived from rapidly-growing keratinocytes [23] again, with marginal effect, in part due to the complex nature of the wound healing response [24]. In addition, the use of factors is not a sufficient approach, in and of itself, in situations where there is severe or massive loss of skin tissue.

MATRIX METALLOPROTEASE ACTIVITY

The non-healing wound microenvironment can best be described as hostile. Wound exudate in general, the vast abnormalities in the release, activation, and persistence of matrix metalloproteases (MMPs), lack of cell adhesion to substrates within the wound bed, all may render the growth factors and cytokines unavailable to the healing process. A concept that takes into account these various components has been called the 'trap hypothesis' [25]. It has been hypothesized that non-healing wounds, particularly in response to bacterial antigens, are characterized by chronic leakage of macromolecules into the wound. These macromolecules may impair healing by 'trapping' cytokines and growth factors. The trap hypothesis suggests that, in spite of achieving adequate levels and even the orchestrated release of these growth factors, the polypeptides are quickly bound and unavailable to the healing process. Common macromolecules that might be involved in trapping include albumin, fibrinogen, and α-2-macroglobulin. The latter is particularly important because it is an established scavenger for growth factors. Fibrinogen can bind to fibronectin, providing a mechanism for the trapping of TGF-β1 [26].

MOIST WOUND HEALING IN CHRONIC WOUNDS

Moist wound healing has been shown experimentally to accelerate re-epithelialization of acute wounds [27], and these observations have led to a number of moisture retentive dressings [28]. For chronic wounds, moist wound healing has not been clearly shown to improve epidermal healing, but we do know that moist wound healing helps in the formation of granulation tissue and in relieving pain. Painless debridement, too, is another important property of moisture retentive dressings [28]. The properties of moist wound healing are important in the field of bioengineered skin constructs, because such constructs lead to increased moisture in the wound bed. Proposed advantages of moist wound healing include retention of cytokines within the wound, facilitation of keratinocyte migration, prevention of bacterial entry, and even poorly understood but favorable electrical gradients. For example, acute wound fluid stimulates the *in vitro* proliferation of fibroblasts, keratinocytes, and endothelial cells. However, fluid and exudate from chronic wounds appear to have a definite adverse effect on cellular proliferation, and contain excessive amounts of matrix metalloproteinases (MMPs), which can break down key matrix proteins critical to cell migration, such as fibronectin and vitronectin [10]. There is a great deal of information we still need about MMPs and their inappropriate activation in chronic wounds. Some of the information is often contradictory. For example, interstitial collagenase (MMP-1) is essential for keratinocyte migration. However, other enzymes (MMP-2, MMP-9) may prevent or interfere with healing [10].

ISCHEMIA

An ultimate goal for tissue-engineering constructs would be to offset very fundamental abnormalities that lead to impaired or slow healing. We have been discussing components of impaired healing that in some way or another can be approached or at least partially corrected by already available means. However, one important component of impaired healing is ischemia, due to poor arterial supply because of narrowing of blood vessels (i.e., atherosclerosis) or, indirectly, because of pressure upon those blood vessels (i.e., pressure ulcers, diabetic neuropathic ulcers). The ischemia, of course, has important consequences for the other

components of impaired healing we have discussed, such as bacterial colonization and infection. A challenge is how to use available tissue-engineering products or modify and develop new ones to correct the problem of ischemia. There are some interesting possibilities that one can use as proof of principle. An example is the role of oxygen tension. Thus, there is no debate over the fact that long-term hypoxia is detrimental to the healing process. This has been readily shown with diabetic ulcers, where low levels of transcutaneous oxygen tension ($TcPO_2$) correlate with inability to heal [29]. However, and this makes sense even from a teleological point of view, short-term hypoxia actually stimulates healing. It has been shown that hypoxia can increase fibroblast proliferation, fibroblast clonal growth, and the synthesis of several growth factors, including PDGF, TGF-β, and VEGF, among others [10]. Therefore, modulation of the oxygen environment within the wound may offer the possibility of accelerating the healing process.

ABNORMALITIES AT THE CELLULAR LEVEL

A very important mechanism for impaired healing is the phenotypic make-up of wound cells. This has critical implications for the use of bioengineered skin constructs, in that these constructs may offset cellular phenotypic abnormalities. There is increasing evidence that the resident cells of chronic wounds have developed phenotypic changes that interfere with their response to growth factors and cytokines. Such abnormalities may affect cellular proliferation, locomotion, and the overall capacity to heal [10]. Also, the signaling mechanisms, which are so critical to the action of cytokines, may be impaired. For example, at least in venous ulcers, there is decreased phosphorylation of Smad2/3 and MAPK p44/42 [30], and a complete deregulation of keratinocyte differentiation and activation and therefore wound closure [31], a result of impaired TGF-β signaling [32].

ENGINEERING SKIN TISSUE

Although the epidermis has an enormous capacity to heal, there are situations in which it is necessary to replace large areas of epidermis, or in which normal regeneration is deficient. The dermis has very little capacity to regenerate. The scar tissue that forms in the absence of dermis lacks the elasticity, flexibility, and strength of normal dermis. Consequently, scar tissue limits movement, causes pain, and is cosmetically undesirable. Engineered tissues that not only close wounds but also stimulate the regeneration of dermis would provide a significant benefit in wound healing.

Bioengineered skin equivalents should incorporate as many of these factors as possible:

1) An extracellular matrix,
2) Dermal fibroblasts,
3) An epidermis containing keratinocytes, and
4) A naturally occurring semi-permeable membrane, the stratum corneum to provide barrier function (Fig. 77.2).

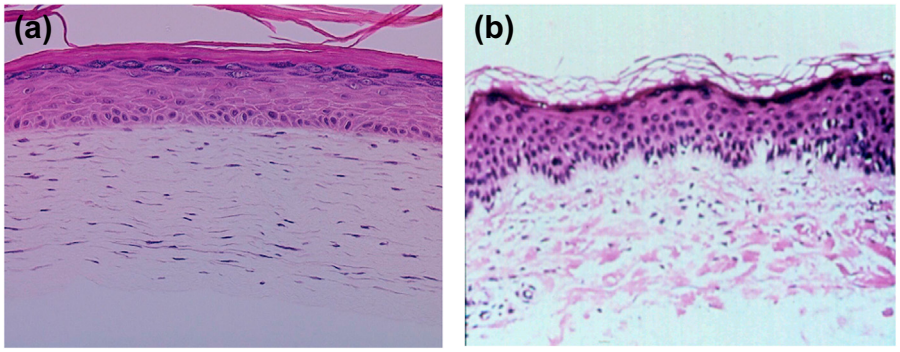

FIGURE 77.2
Histological appearance of Apligraf.
(a) compared to normal human skin. (b) A well-defined epidermal and dermal layer is observed in both. *(Copyright V. Falanga, 2006.)*

These components may act alone, but more importantly, they act in concert as part of a fully integrated tissue to protect the underlying tissues of a wound bed and to direct healing of the wound [33]. Dermis containing fibroblasts may be necessary for the maintenance of the epidermal cell population [34]. In turn, the epidermis is necessary for the formation of the so-called neodermis, in the absence of a dermal layer [35] and can dramatically influence underlying connective tissue response. The formation of the epidermal barrier also likely influences these processes through control of water loss and its influence on epidermal physiology [36].

Design considerations

Tissue engineering has not focused on regenerating certain skin structures, like hair follicles or sebaceous glands, whose loss is clinically less significant than the loss of dermis and epidermis needed to cover and protect the underlying tissues. Despite some early evidence for rudimentary eccrine tubules within the HSE [37], the development of functioning adnexal structures is likely to be years away. There has also been little need to extraneously stimulate regeneration of other dermal components (e.g., blood vessels and cells of the immune system) through tissue-engineering methods because these components have the ability to repopulate quickly and to normalize the area of a wound. Langerhans cells, for example, have been shown to migrate and repopulate effectively within months [9]. Control of vascularization is dependent on the make-up of the extracellular matrix and the degree of inflammation present in the wound. Whether modification of vascularization through the use of exogenous factors will be of additional benefit for certain wounds remains to be determined. While pigmentation is not critical for wound healing, clinical studies using Apligraf, which lacks melanocytes, have shown repigmentation of the grafted areas through repopulation of the area with host melanocytes, resulting in normal skin color for each individual. The constructs should have sufficient mechanical strength to allow for clinical manipulations (Fig. 77.3). The approach skin tissue engineering has taken has been to focus primarily on providing or imitating structural and biological characteristics of dermis, epidermis, or both.

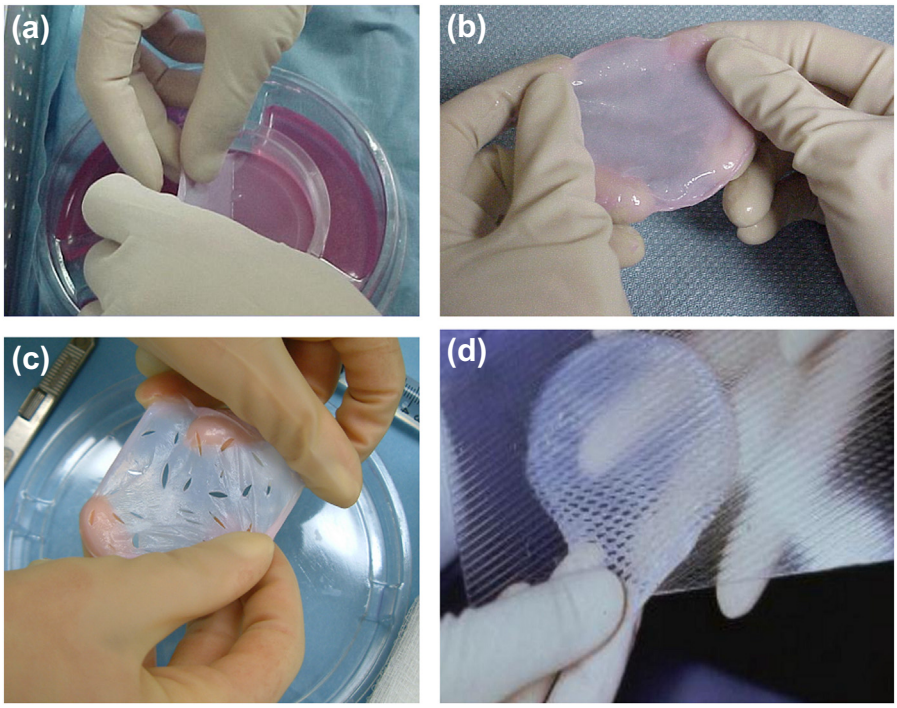

FIGURE 77.3
Appearance of Apligraf. (a) Being removed from its pink nutrient agar. (b) Being held just prior to fenestration or meshing. (c) After fenestration with a scalpel. (d) After meshing at a 1.5:1 ratio. *(Copyright V. Falanga, 2006.)*

The key features to be replicated in an engineered skin construct are:

- A dermal element capable of aiding appropriate dermal repair and epidermal support.
- An epidermis capable of easily achieving biologic wound closure.
- An epidermis capable of rapid re-establishment of barrier properties.
- A permissive milieu for the components of the immune system, nervous system, and vasculature.
- A tissue capable of achieving normalization of structure and additional functions such as reduction of long-term scarring and re-establishment of pigmentation.
- Active cellular component(s) capable of responding to different wound types and conditions.
- Sufficient mechanical strength to allow for clinical manipulation.
- Persistence of cells in the wound for multiple weeks to stimulate the healing process through delivery of cytokines and matrix proteins.

Commercial considerations

Engineered skin constructs, by virtue of being the first bioengineered products to be commercialized, have been at the forefront of science, industry, and regulation. In recent years more attention has been given to the subject of commercialization. In 2012 Jaklenec et al., published a review of the tissue engineering and stem cell industry [38]. The authors reported that there was approximately a three-fold increase in commercial sales, generating $3.5 billion from 2008 to 2011. During that time, the number of tissue-engineering companies increased from 171 in 2007 to 202 in 2011, with 62% of them based in the United States. These companies are spending $3.6 billion and employ 13,810 people. The findings indicate that the tissue engineering and stem cell industries are stabilizing.

Early industry leaders, Organogenesis, Advanced BioHealing (now Shire Regenerative Medicine) and Forticell Biosciences, initially struggled with the task of commercializing their technologies. The focus at Organogenesis, in the case of Apligraf, has been on understanding the cost of manufacturing and working to reduce those costs while increasing efficiency and maintaining high quality. Organogenesis has also built robust sales and marketing, customer service, reimbursement, and distribution functions to drive Apligraf sales. Having all of these functions within the same organization is proving to be of benefit by allowing for a more integrated, strategic company.

Process considerations

Consideration should be given to the design of the manufacturing process early in the product concept stage. Elements such as critical process parameters, quality control assays, production components and materials, process equipment, production facility requirements, product shelf life and distribution methods need to be developed in detail. From this design the cost of manufacturing and strategies for scale-up and automation can be developed. The requirements for the manufacturing facility are directly related to the design of the manufacturing process. For example, a manual process generally requires a large highly-specialized staff and a relatively large manufacturing floor space, whereas an automated process requires less specialized staff and potentially less manufacturing floor space. An aseptic process where the culture vessels are periodically opened to facilitate feeding and other manipulations requires tighter environmental controls to mitigate risk from contaminants on work surfaces or in the air, whereas developing a closed process that eliminates aseptic manipulations reduces the need for tight environmental controls, thus reducing facility costs. All of these elements impact the cost of manufacturing and the potential for scale-up.

Process components that will pose significant barriers to scale-up or automation can be identified and addressed while the product is still in research and development. This is critical when you consider that the further you advance in the development process the more

committed you become to the process design. The cost of making a significant process change increases the closer you get to manufacturing, and becomes exponentially more expensive and time consuming post product launch. Along these same lines, materials used in the process that are ill-defined, cell-based, single sources or of limited supply can contribute to a manufacturing process that is difficult to scale-up and control from a quality standpoint. These factors will influence the yield from the manufacturing process or, stated another way, the scrap rate for the product. High scrap rates increase manufacturing costs and create issues for inventory management. The pioneering lessons learned by early industry leaders in process scale-up apply to the broader field of tissue engineering. Ultimately, strategies for scale-up and automation can be evaluated and planned for.

Regulatory considerations

The Center for Devices and Radiological Health (CDRH) at FDA requires products to be developed following design control procedures (21 CFR 820.30). These design guidelines are in place to ensure that the products that are developed will be safe, effective and have a market research based justification for the product design. Establishing a Design Control system early will help refine the business model throughout the development life cycle. Design Control is not only a regulatory requirement, but it makes good business sense. HSEs have historically been classified and regulated as medical devices, though the trend at FDA is to regulate products having a cellular component as biologics. A case in point is the recent approval of GINTUIT™, a fibroblast and keratinocyte-containing construct, as a combination product (containing biologic and devices components) by the Center for Biologics Evaluation and Research (CBER) [39]. Finally, agencies like the US Center for Medicare and Medicaid Services (CMS) and foreign government reimbursement agencies (e.g., U.K National Institute for Health & Clinical Excellence, NICE) are increasingly requiring convincing data on the value and effectiveness of therapy as compared to standard of care or competing technologies [40].

Immunological considerations

The ability to utilize allogeneic cells rather than autologous cells facilitates the reproducible, large-scale commercial manufacture of an HSE [37], because large cell banks can be created, allowing manufacture of thousands of units of product from one cell strain and enabling a more accurate forecast of manufacturing demands. However, the problem of rejection needs to be taken into consideration when using living allogeneic cells in wound healing applications.

The first stage in the induction of a primary immune response after skin allografting is the presentation of antigen by donor dendritic cells (Langerhans cells with dermal dendritic cells), the professional antigen-presenting cells (APCs) in skin. These cells migrate out of the skin to the draining lymph node, where they can activate T cells directly through the presentation of MHC-class II antigens and co-stimulatory molecules, thereby eliciting both cell-mediated and humoral (antibody-mediated) immune responses to the grafted skin. Although cell-mediated cytotoxicity is a component of rejection, the primary mode of skin rejection is likely mediated via an attack on the vasculature present in a normal skin graft by recipient antibodies [41,42].

Bioengineered skin constructs are fabricated from highly purified banks of fibroblasts, keratinocytes or both, that are either poor or deficient in dendritic and other APCs found in skin. This has important implications for the use of allogeneic cells in the treatment of acute and chronic wounds. In the absence of APCs, fibroblasts and keratinocytes are the only cells capable of presenting donor antigen to the recipient. Under normal conditions, keratinocytes and fibroblasts do not express MHC-class II antigens. They can be induced by interferon-γ to express MHC-class II molecules and thereby acquire the ability to present antigen to T cells, however keratinocytes and fibroblasts do not express co-stimulatory molecules [43,44], so antigen presentation by keratinocytes and fibroblasts does not result in T cell activation. Instead, this antigen presentation can result in T cell non-responsiveness [45,46] or T cell anergy [47].

Autologous HSEs would avoid issues of immunogenicity, of course, but autologous grafts have significant limitations. Growing graft tissues from biopsy takes several weeks, the donor site creates another wound, and in some patients (e.g., severe burn patients) there may be no appropriate donor site. Reproducibly making complex HSE constructs to order from autologous cells would be time consuming, and very costly. Therefore, the ability to effectively use allogeneic human cells is a key element in the commercial success of engineered skin therapies.

Summary: engineering skin tissue

In summary, good science alone is not enough to ensure success. There are critical considerations to commercialization along the entire continuum from product concept, development, clinical evaluation, FDA and other regulatory agency approval, product launch, reimbursement and commercial scale manufacturing. Developing a comprehensive business model that takes all of these factors into account will increase the likelihood for a profit-generating product. Understanding the challenges and working to incorporate process designs that are forward-looking, will be amenable to scale-up, address regulatory hurdles and are supported by a viable business model are necessary for companies to be successful and the industry to grow.

EPIDERMAL REGENERATION

Re-epithelialization of the wound is a paramount concern. Without epithelial coverage, no defense exists against contamination of the exposed underlying tissue or loss of fluid. The approaches to re-establishing epidermis are numerous, ranging from the use of cell suspensions to full-thickness skin equivalents possessing a differentiated epidermis. Silicone membranes have been used as temporary coverings in conjunction with dermal templates [48], Regardless of approach, living epidermal keratinocytes are necessary to achieve permanent, biological wound closure.

Green et al., [49,50] developed techniques for growing human epidermal keratinocytes from small patient biopsy samples using co-culture methods [51]. The mouse 3T3 fibroblast feeder cell system allows substantial expansion of epidermal keratinocytes and can be used to generate enough thin, multi-layered epidermal sheets to resurface the body of a severely burned patient [52]. Once transplanted, the epidermal sheets quickly form epidermis and re-establish epidermal coverage [35]. With time, the cultured epithelial autograft (CEA) stimulates formation of new connective tissue ('neodermis') immediately beneath the epidermis [53], but scarring and wound contraction remain significant problems [54]. Studies have shown that grafting of a CEA onto pre-grafted cadaver dermis greatly improves graft take [55]. CEAs (Epicel®, Genzyme, Cambridge, MA) have been available since the late 1980s.

DERMAL REPLACEMENT

Dermal replacement is an essential first step in restoring normal skin structure following injury and leads to better cosmesis in full-thickness soft tissue defects. In one of the earliest tissue-engineering approaches to improving dermal healing, Yannas et al. [56] designed a collagen-glycosaminoglycan sponge to serve as a scaffold or template for dermal extracellular matrix. The goal was to promote fibroblast repopulation in a controlled way that would decrease scarring and wound contraction. A commercial version of this material composed of bovine collagen and chondroitin sulfate, with a silicone membrane covering (Integra™, Integra Life Sciences, Plainsboro, NJ) is currently approved for use in burns [48,57]. The dermal layer is slowly resorbed, and the silicone membrane is eventually removed, to be replaced by a thin autograft [58].

Biobrane® (UDL, Rockford, IL) is a non-viable temporary covering for burns, in which a nylon mesh, coated with porcine collagen-derived peptides and layered with a non-permeable

silicone membrane serves as a platform for deposition of human matrix proteins and associated factors by host wound-derived dermal fibroblasts. After epidermalization, the construct becomes non-adherent and is removed from the wound bed. A similar product, Transcyte (Advanced BioHealing, Westport, CT) [59], consists of a porcine collagen-coated nylon mesh that is incubated with human neonatal fibroblasts, which secrete extracellular matrix proteins and growth factors. The material is then frozen to preserve the matrix and factors produced by the fibroblasts, although the fibroblasts themselves are non-viable when the construct is applied to the wound. The temporary silicone covering is removed to allow epidermalization or prior to application of an autograft.

Although matrix scaffolds have shown some improvement in scar morphology, no acellular matrix has yet been shown to lead to true dermal regeneration. This may be due in part to limits in cell repopulation, the type of fibroblast repopulating the graft, and control of the inflammatory and remodeling processes (i.e., the ability of the cells to degrade old matrix while synthesizing new matrix).

The inflammatory response must be controlled in dermal repair in order to avoid the formation of scar tissue. Therefore, dermal scaffolds must not be inflammatory and must not stimulate a foreign body reaction. This has been a problem in the past for some glutaraldehyde cross-linked collagen substrates [60]. The ability of the matrix to persist long enough to redirect tissue formation must be balanced with effects of the matrix on inflammatory processes. One way to achieve this is to form a biological tissue that is recognized as living tissue, not a foreign substance.

There have been advances in the design of *in vitro* grown dermal tissues using living human neonatal fibroblasts grown on rectangular sheets of biodegradable vicryl mesh (Dermagraft, Shire Regenerative Medicine, San Diego, CA). The fibroblasts propagate among the fibers of the mesh, producing extracellular matrix in its interstices [61]. Like Transcyte, the final Dermagraft construct is then frozen, but in a manner that preserves approximately 60% fibroblast viability upon thawing and placement on the wound [62].

BIOENGINEERED LIVING SKIN EQUIVALENTS

HSEs have been generated by seeding cultured keratinocytes onto the surface of a variety of scaffolds, including artificial membranes, de-epidermalized dermis, collagen gels, chitosan-GAG sponges or fibroblast-populated nylon meshes [63]. One of the first attempts to replicate a full-thickness skin graft was by Bell et al. [64] who described a bilayered skin equivalent, which was the predecessor to Apligraf. The dermal component consisted of a lattice of type I collagen contracted by tractional forces of rat dermal fibroblasts trapped within the gelled collagen. This contracted lattice was then used alone or as a substrate for rat epidermal keratinocytes. These primitive skin equivalents were further demonstrated to take as skin grafts in rats. This technology has now advanced to enable the production of large amounts of Apligraf from a single donor [37].

Boyce et al. modified the approach first proposed by Yannas et al. to form a bilayered composite skin made using a modified collagen-glycosaminoglycan substrate seeded with fibroblasts and overlaid with epidermal keratinocytes [65]. An autologous form of this composite skin construct was used to treat severe burns with some success [66]. An allogeneic form of the construct showed improved healing in a pilot study in chronic wounds [67]. A similar technology has been studied in the treatment of patients with genetic blistering diseases [68]. Using methods of organotypic culture that provides a three-dimensional culture environment that is permissive for proper tissue differentiation, the resulting HSE develops many of the structural, biochemical, and functional properties of human skin [65,69–71], apart from the aforementioned lack of adnexal structures.

The process for formation of HSEs has been covered in detail many times [37,65,72] and will not be detailed here. However, there are points to be made about the approach to these procedures. The culture of an HSE proceeds best with minimal intervention. Normal keratinocyte populations seem to have an intrinsic ability to re-express their differentiation program *in vitro*. A medium that supplies adequate amounts of nutrients, lipid precursors, vitamins, and minerals may be all that is required [37]. This has now been achieved with both normal human keratinocytes (Apligraf, Orcel) and with immortalized human keratinocytes (StrataGraft) [73]. Another element is the environmental stimulus provided by culture at the air-liquid interface which promotes differentiation and formation of the epidermal barrier [65,69]. In the case of StrataGraft, the trisomy that caused immortalization of the cell line has apparently not affected the capacity of the cells to undergo differentiation in organotypic culture [73].

Recently, L'Heureux et al. were able to take advantage of the fact that mesenchymal cells secrete their own extracellular matrix (ECM) when cultured in the presence of ascorbic acid, to generate sheets of ECM, in the absence of a bovine collagen scaffold, from cultured fibroblasts and smooth muscle cells (SMCs). Initially, these ECM tissues were used to generate the first tissue engineered blood vessels [74]. Subsequently, the same technology was used to generate a scaffold-free HSE [75], in which the dermal layer normally constructed with bovine collagen was replaced with a completely fibroblast-derived ECM. The epidermis of the HSE was generated by seeding keratinocytes onto the self-assembled ECM and incubating at the air-liquid interface to promote stratification and differentiation. After 4 weeks of culture, a fully stratified and differentiated epithelium developed, which expressed the normal markers of differentiation. The tissue also included an organized basement membrane, including laminin and collagens IV and VII. Since this early work, a few groups have investigated the physical and biochemical properties of self-assembled ECMs [75,77,78] and have used these self-assembled constructs as *in vitro* models of percutaneous absorption [75] and hypertrophic scarrin [79,80]. However, self-assembled HSEs have yet to become a clinical and commercial reality.

BIOENGINEERED SKIN: FDA APPROVED INDICATIONS

Cutaneous indications

As discussed above, the rather formidable obstacles to healing chronic wounds have made it difficult for the simple topical application of growth factors and cytokines to have a successful outcome. It can be argued that tissue engineering, particularly with living cells, has an advantage in that cells may be capable of responding to the microenvironment and thus behave in a 'smart' way from an engineering point of view. Because of these considerations, cell therapy with bioengineered skin has been tested in difficult to heal wounds. Figs. 77.2 and 77.3 show Apligraf before and after meshing and fenestration, as well as its histological appearance. Venous leg ulcers and neuropathic diabetic foot ulcers have received the greatest attention. Two main types of living bioengineered skin have been proven to be effective in diabetic neuropathic foot ulcers and have received regulatory approval from the FDA. In a randomized 12 week trial of 208 patients with neuropathic ulcers, Apligraf led to complete wound closure in 56% of patients, compared to 38% in the control group [81]. The Kaplan-Meier median time to complete wound closure was 65 days for Apligraf and 90 days for control. Fig. 77.4 shows a diabetic neuropathic foot ulcer successfully treated with Apligraf. In an evaluation of the clinical trial adverse events, Apligraf treated patients had significantly fewer amputations/resections of the study limb and less incidence of osteomyelitis. These results on key complications of diabetic foot ulcers may have been due to faster healing. Another living cell product, Dermagraft, was shown in a 12 week randomized study to heal neuropathic foot ulcers, with an incidence of closure of 30% and 18% for the active and control arm, respectively [82].

In addition to its indication for diabetic ulcers, Apligraf also remains the only approved bioengineered product for venous ulcers. In a pivotal multicenter randomized study of 293

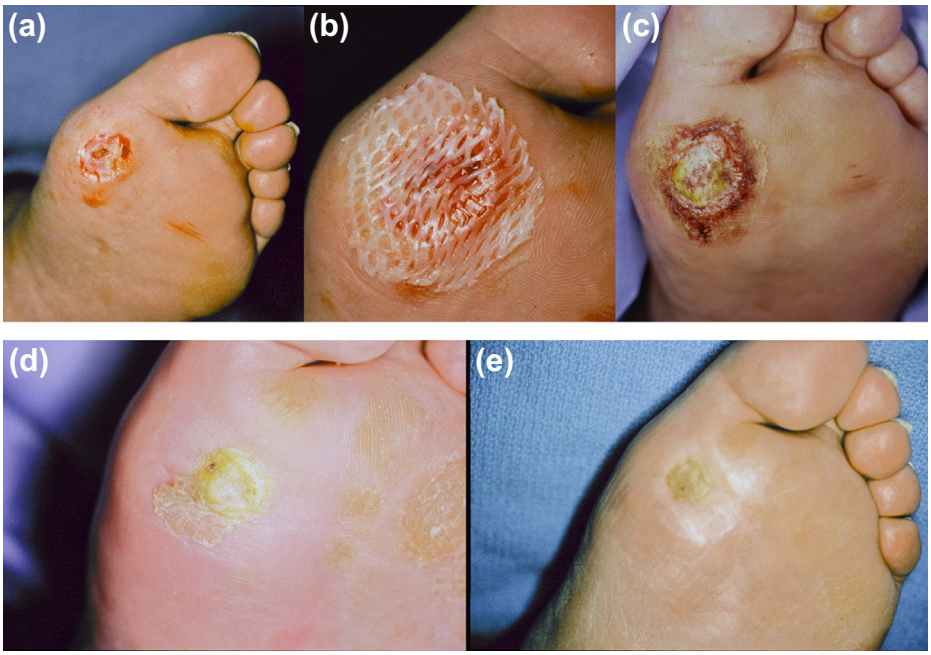

FIGURE 77.4
Diabetic neuropathic foot ulcer successfully treated with Apligraf. (a) Just after surgical debridement to remove the necrotic wound bed and surrounding callus. (b) Wound covered with meshed Apligraf. (c) Appearance of construct a week later. (d) At 3 weeks. (e) Complete wound closure at 5 weeks. *(Copyright V. Falanga, 2006.)*

patients with venous leg ulcers, Apligraf was more effective than leg compression alone (control) in the percentage of patients who healed by six months (63% vs. 49%) [83]. That study also showed that Apligraf healed ulcers that were larger and deeper than those in the control group. No evidence of sensitization was observed with the bioengineered skin product. Interestingly, a subsequent re-analysis of the data from this pivotal trial, limited to 120 patients having venous ulcers for over one year, showed that Apligraf healed 47% of patients, compared to 19% in the control group [84]. This observation, taken together with the mechanisms underlying impaired healing, suggests that cell therapy might have a greater effect when there are more substantial abnormalities leading to a prolonged failure to heal.

Oral indications

Like skin, soft tissues in the oral cavity need to be replaced or repaired due to trauma, disease or congenital defects. Treatment of soft tissue defects in the oral cavity has been done by either secondary intention healing (removing the diseased tissue and allowing it to heal on its own), by the use of flaps (a graft that is advanced over a defect and contains its own blood supply), or by the use of free grafts (tissue that lacks its own blood supply). Depending on the procedure, auto- or allografts may be used. Autografts are generally harvested from the palate, and are associated with significant donor site morbidity. However, some groups have had success with autologous epithelial cells, and with injected autologous mucosal fibroblasts [85].

Two types of allogeneic, tissue-engineered constructs have been used in the oral cavity. Dermagraft has been used in the oral cavity to treat gingival recessions, in which the amount of attached gingiva was insufficient. As with its Diabetic Foot Ulcer (DFU) indication, it is thought that the Dermagraft construct provides a scaffold for mucosal keratinocyte migration, and that it secretes growth factors and cytokines that promote secondary intention healing. Dermagraft has been the subject of a controlled within-patient clinical trial in the oral cavity, in which its efficacy was compared to that of a gingival autograft for the treatment of gingival recessions [86,87]. Treatment with Dermagraft resulted in an increase in the amount of attached gingiva, although the amount of keratinized tissue was inferior to that generated by the autograft, as expected from a construct that promotes secondary intention healing rather than immediate coverage. At the time of publication, Shire Regenerative Medicine is no longer pursuing an oral indication for Dermagraft.

GINTUIT, a product derived using a similar technology platform as Apligraf, has also been evaluated for its ability to replace a connective tissue autograft in the treatment of gingival recessions in a free gingival graft procedure [88]. Like Apligraf in the DFU and Venous Leg Ulcer (VLU) indications, GINTUIT did not persist long-term in the oral cavity. Like Dermagraft, GINTUIT was found to be superior to a gingival autograft in terms of color and texture match to surrounding tissue. In the pilot and pivotal studies, treatment with GINTUIT resulted in regeneration of ≥ 2 mm of keratinized gingiva in 81.8% and 95.3% of patients, respectively, which is clinically accepted as a level important for periodontal health. As of 2012, GINTUIT, is the first living cell-based therapy that has FDA approval for the treatment of oral soft tissue defects [39]. Although derived from the same technology platform as Apligraf, GINTUIT was approved as a combination product (containing biologic and devices components) by the Center for Biologics Evaluation and Research (CBER) [39] and is therefore subjected to additional product release criteria compared to Apligraf. Interestingly, the platform that gives rise to Apligraf and GINTUIT is able to regenerate tissue in a site-appropriate manner, in that it promotes secondary intention healing of difficult to heal cutaneous soft tissue defects, and also promotes regeneration of keratinized gingiva site-appropriately in the oral cavity.

APLIGRAF AND DERMAGRAFT: OFF-LABEL USES

Off-label use is the practice of using products for purposes not approved by the FDA. Physicians can prescribe and use products as they see fit because they are not regulated and are recognized as experts in their fields. Manufacturers of the products are not permitted to promote off-label use; however, data generated by off-label use can be very valuable and lead to new insights into product function and potentially to clinical trials for new indications, therefore the following discussion has been included in this chapter. It must be emphasized, however, that the determination of off-label use is not always clear-cut when it comes to chronic wound etiology. In some cases applicable to Apligraf, for example a scalp or hand wound, one is clearly using the product off-label. However, for many lower extremity wounds, the situation is much more complex. Thus, a patient may have a mixed etiology for the ulcer (for example, lymphatic and venous insufficiency) and will therefore not heal until the venous component is properly addressed. In addition to the use of standard care for venous insufficiency, such as compression bandages and wound dressings, one could make the argument that Apligraf is indicated in that clinical setting because the construct has indeed been proven to improve venous ulcers. It would be difficult, perhaps impossible, to conduct clinical studies that address ulcers of mixed etiology in a scientifically sound way. An even more complex clinical setting is when one treats patients who appear to have inflammatory ulcers (i.e., pyoderma gangrenosum, ulcers involving vasculitis, ulcers associated with cryoprotenemias, etc.) on a lower extremity that also exhibits definite signs and findings of venous insufficiency. The question then centers upon the predominant etiology of the ulceration. From a clinical standpoint, it can become very difficult to make that determination. Yet, treatment of that ulcer with Apligraf to improve the venous component can lead to complete wound closure (V. Falanga., personal observation as of 2012). These are challenging situations but, in our experience and exchanges with colleagues, these types of ulcers are probably not uncommon. Still, while being able to use certain constructs off-label, the clinician must keep in mind the appropriate approved indications for that product and not be swayed in his or her clinical judgment as to what is indeed the primary cause of the ulcer.

From both a therapeutic and purely scientific standpoint, of critical importance in the context of the clinical trials for venous leg and diabetic foot ulcers detailed is that, for the very first time, one has been able to show a beneficial effect of bioengineered products in situations of impaired healing. Understandably, clinicians have also used Apligraf off-label to accelerate healing in wounds not due to venous disease or diabetes [89]. Wounds that have been treated off-label with Apligraf include acute wounds after extensive skin surgery, donor sites after split-thickness skin

harvesting, traumatic wounds, burns, inflammatory ulcers such as pyoderma gangrenosum and vasculitis, scleroderma digital ulcers, wounds after keloid removal, genetic conditions such as epidermolysis bullosa, and a variety of situations that defy proper diagnosis because of their complexity. In these cases, bioengineered skin treatment with Apligraf seems to offer a viable alternative to stimulate wound healing and relieve unacceptable pain and suffering. For example, pyoderma gangrenosum is an inflammatory ulcer often associated with rheumatoid arthritis, inflammatory bowel disease, IgA gammopathy, among other predisposing factors. A peculiar feature of pyoderma gangrenosum is that it worsens with surgical manipulation and even develops at distant sites that are traumatized; thus, autologous grafting is a contraindication and bioengineered skin is an attractive therapeutic modality [90]. In a patient with multiple myeloma undergoing conditioning for bone marrow transplantation, extravasation of the chemotherapeutic drug adriamycin, occurred in his chest and created a very large, deep and painful wound. Apligraf played a major role in healing the full-thickness wound (Fig. 77.5), and led to a dramatic relief in pain. Pressure ulcers, too, have been treated with Apligraf. This is of particular interest because, based on the engineering aspects of the construct, one would have expected that very deep wounds with loss of a great deal of tissue would not respond to Apligraf. Yet, it appears that in such cases Apligraf is able to stimulate tissue re-growth in deep ulcers, and not just re-epithelialization from the edges of the wound (Fig. 77.6). Scleroderma digital ulcers are ischemic in nature, and have no known accepted treatment. Yet, Apligraf appears promising in those wounds (Fig. 77.7). There is no evidence that Apligraf stimulates malignancy within the wound bed, possibly due to its stimulatory action. An interesting case in point is shown in Fig. 77.8, where multiple and ulcerated basal cell carcinomas in the foot of a patient with the hereditary form of basal cell nevus syndrome were treated with Apligraf.

Epidermolysis Bullosa (EB) is a genetic skin disorder characterized by blistering of the skin and mucosae following mild mechanical trauma [91,92]. Falabella et al. performed an open-label uncontrolled study of 15 patents with EB treated with Apligraf [92]. The conclusion was that Apligraf induced rapid healing; the wounds remained healed for some weeks and there was no acute rejection reaction or other adverse effects related to the treatment. Currently there is no specific treatment for EB, and the nature of the condition is that these wounds tend

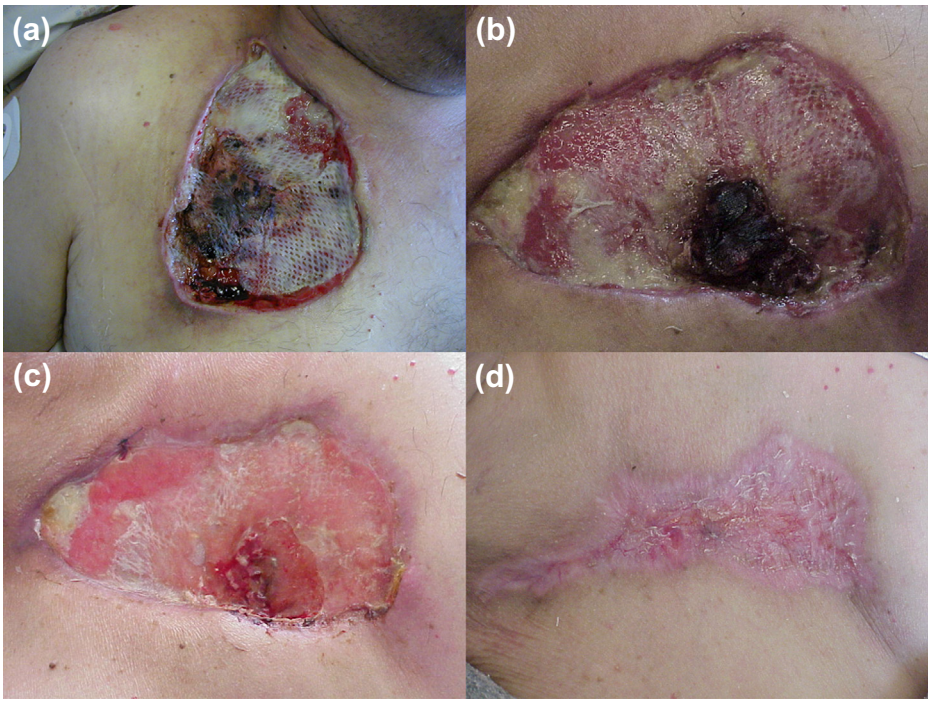

FIGURE 77.5
Large and deep wound from extravasation of adriamycin into the chest wall during treatment for multiple myeloma and bone marrow conditioning. (a) Wound still partially covered with meshed Apligraf, two weeks after treatment with construct. (b) Wound at week 3. (c) Wound at 8 weeks. (d) Complete closure after 10 weeks. *(Copyright V. Falanga, 2006.)*

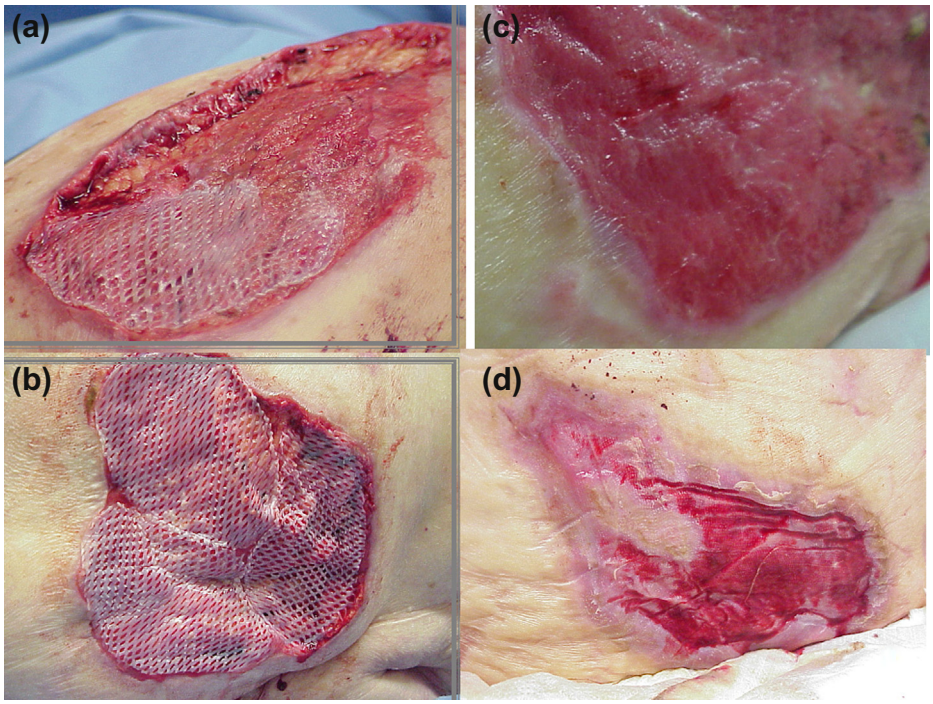

FIGURE 77.6
Large sacral pressure ulcer. (a) Immediate after extensive surgical debridement and beginning of Apligraf application. (b) Wound fully covered with meshed Apligraf right after surgical debridement. (c) After three weeks the wound bed is now flush with the surrounding skin. (d) At week 5 the wound is largely epithelialized. The edges of the wound are rapidly advancing toward the center of the wound. *(Copyright V. Falanga, 2006.)*

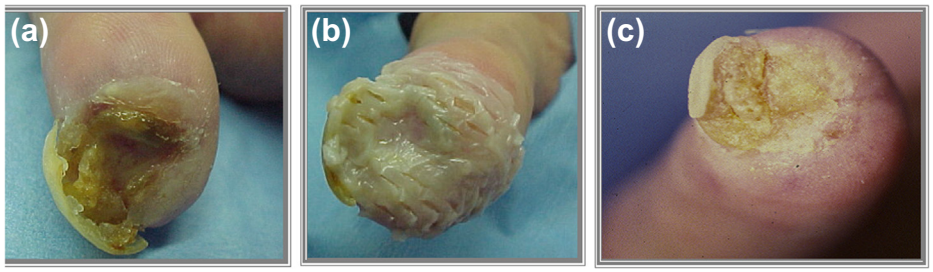

FIGURE 77.7
Systemic sclerosis (scleroderma) digital ulcer treated with Apligraf. (a) Baseline ulcer with fibrinous wound bed. (b) Wound covered with meshed Apligraf. (c) Wound closure four weeks later. *(Copyright V. Falanga, 2006.)*

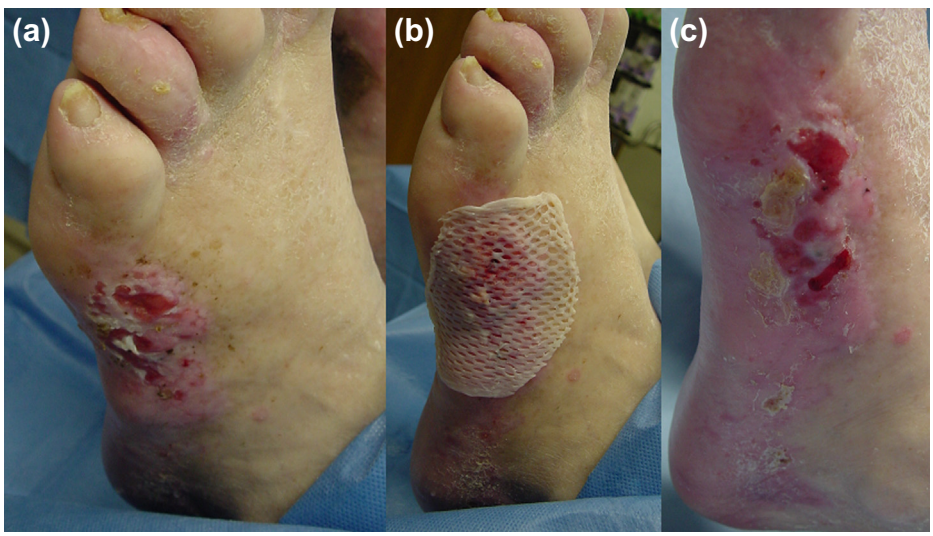

FIGURE 77.8
Patient with a hereditary form of basal cell carcinoma (the basal cells nevus syndrome). His entire body was covered with these skin cancers. (a) Non-operative ulcerated basal cell carcinomas (proven histologically) on the lateral side of the foot. (b) Ulcerated cancers treated with Apligraf. (c) Appearance of same site several months after Apligraf application and without obvious clinical evidence of extension of the skin cancers. *(Copyright V. Falanga, 2006.)*

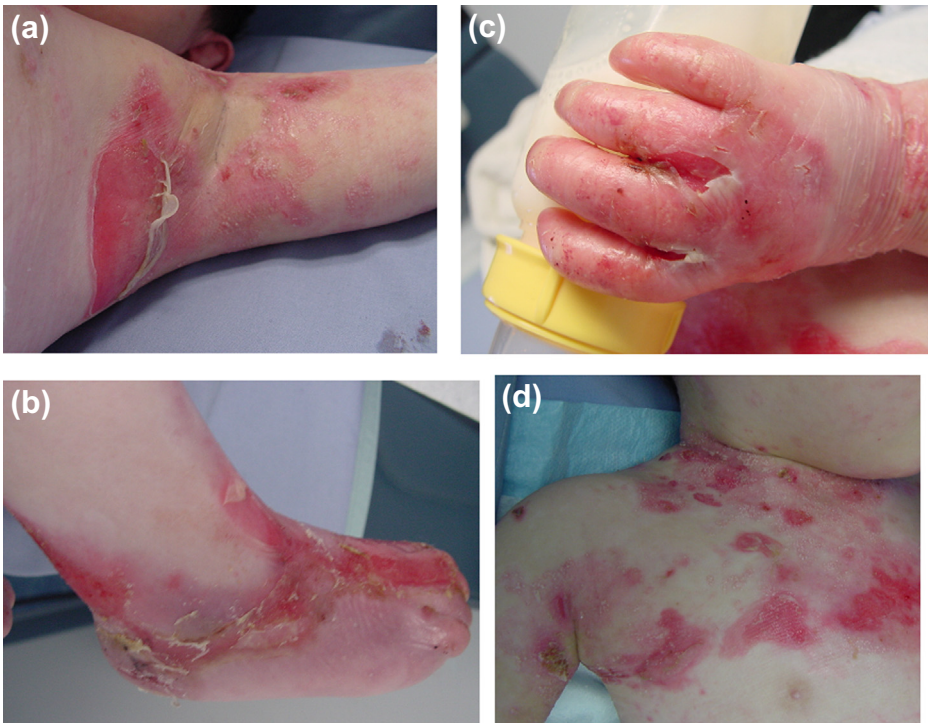

FIGURE 77.9
Epidermolysis bullosa (dystrophic type). (a) Denuded area in axilla, with milia formation from repeated breakdown and healing. (b) Foot that has experienced many episodes of wounding, resulting in scarring and loss of toe webs. (c) Mitten deformity of the hand well under way due to constant injury and attempts at repair. (d) Several denuded areas on the chest, showing evidence of active epithelialization. The child had been treated with Apligraf. *(Copyright V. Falanga, 2006.)*

to heal slowly and in some instances fail to heal, becoming chronic wounds. Fig. 77.9 shows an example of a pediatric EB patient that was treated with Apligraf.

A case of vasculitis treated with Dermagraft is another good example of the use of a tissue-engineered construct to promote closure of a difficult to heal wound (Fig. 77.10). The patient (48 years old) had a severe course of cutaneous polyarteritis nodosa, which did not involve her kidneys. She was always in severe pain and could not be healed by conventional means. Dermagraft proved useful in terms of alleviating pain and promoting epidermalization. Stimulation of epidermal migration was observed by Day 14 after application of the construct. After the wound healed, the patient was placed on an anti-TNF-α biological agent to prevent recurrence and she remains healed.

THE IMPORTANCE OF WOUND BED PREPARATION (WBP)

Shortly after the FDA approval of two major products for the treatment of non-healing chronic wounds (topical PDGF for diabetic neuropathic foot ulcers and Apligraf for venous ulcers) it became apparent that many clinicians were either not well-versed in the treatment of chronic wounds, or could not easily incorporate these novel and advanced therapeutic technologies into their management strategies. The initial clues to this problem came from obviously sub-optimal results when compared to those obtained in the clinical trials, or the apparent use of these technologies at inappropriate times during the healing or non-healing trajectory of the wounds. In an editorial we published at that time [11] we laid down what we thought to be the reasons for these observations, and focused on a new term; Wound Bed Preparation (WBP), which we described as a global approach to the wound to improve its wound bed at multiple levels (optimizing the granulation tissue, decreasing the bacterial burden, removing phenotypically abnormal cells, enhancing epidermal migration) and which would then lead to a better outcome upon treatment with Apligraf and other advanced therapeutic agents. Since then, WBP has dominated the chronic wound field and has provided a more consistent way of approaching chronic wounds. Other publications on this subject have followed [93,94].

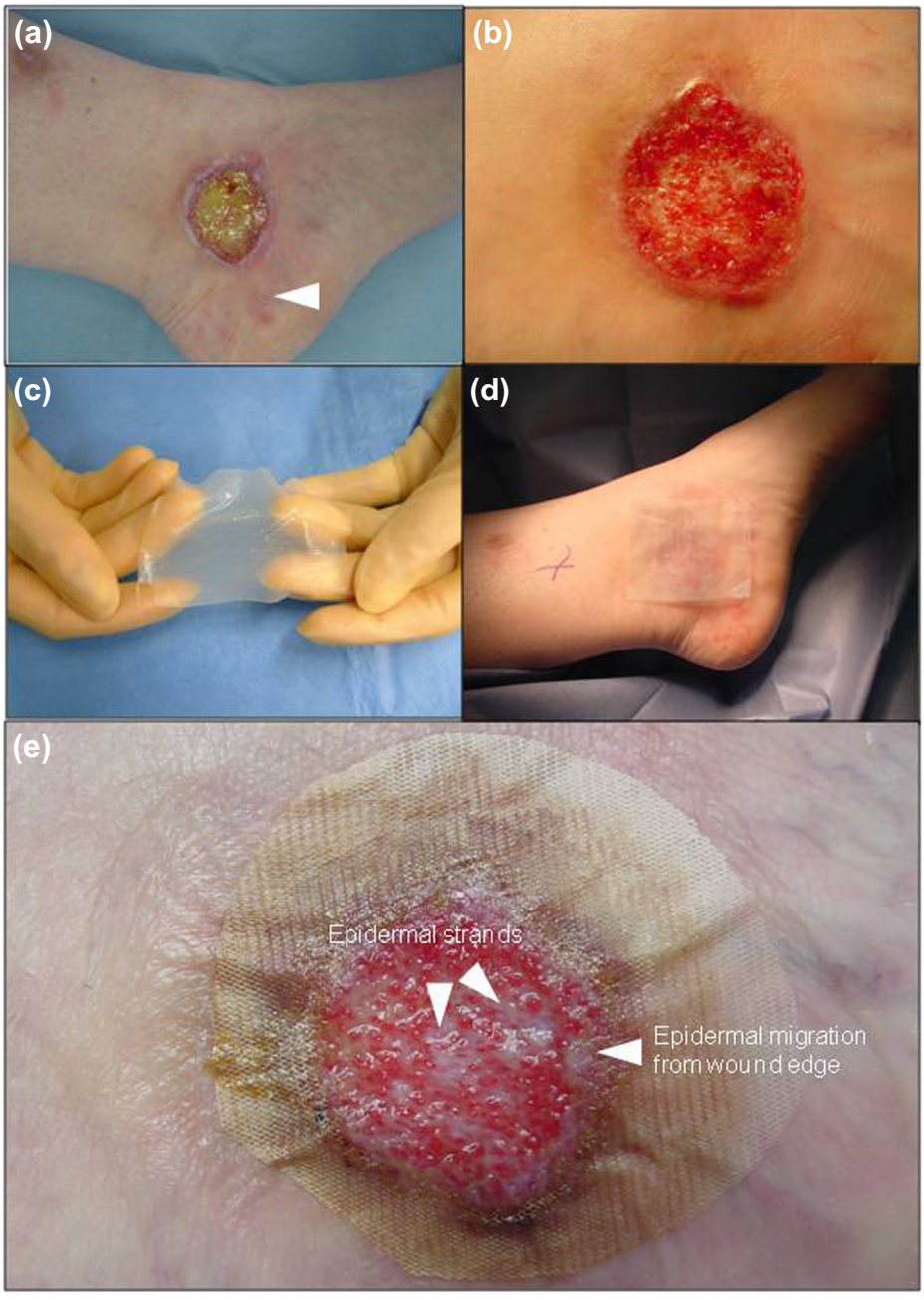

FIGURE 77.10
Treatment of vasculitis with Dermagraft. (a) Vasculitis at baseline. Arrowhead indicates livido pattern suggestive of vasculitis. (b) Appearance of wound after surgical debridement. (c) Dermagraft prepared for application. (d) Dermagraft applied to wound. (e) Wound appearance 14 days after applying Dermagraft. Granulation tissue has developed and there is evidence of epidermal migration (arrowheads). *(Copyright V. Falanga, 2012.)*

There has been some struggle to define exactly what constitutes optimal wound bed preparation but we have prepared a working scoring system to begin to investigate this issue [95]. Figure 77.11, taken from that publication, summarizes the scoring system. The Wound Bed Score (WBS) gives each of eight clinical parameters, assessable at the bedside) a score of 0, or 1, or 2. The maximum and best score is 16. Our experience with the WBS indicates that it is a useful starting point for deciding when to use a complex and advanced biological therapy.

PROPOSED MECHANISMS OF ACTION (MOA) OF BIOENGINEERED SKIN

As stated in the previous section, there are a number of bioengineered skin products that have been designed and in all cases, the mechanisms of action by which these bioengineered skin

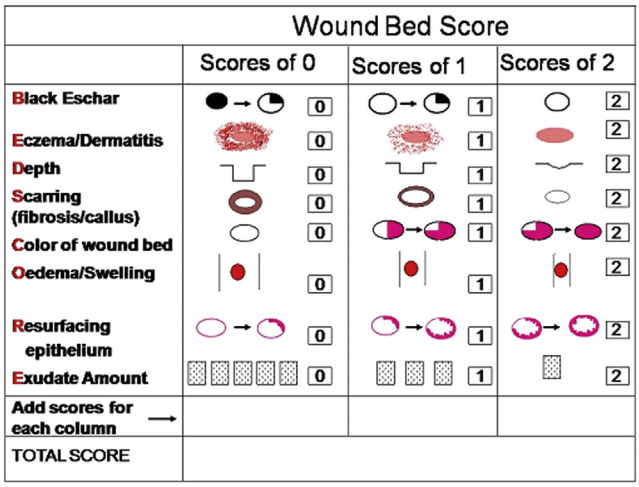

FIGURE 77.11
Wound bed scoring scheme. *(Copyright V. Falanga, 2007.)*

remains unknown. However, despite this, there is some strong evidence that supports plausible explanations of MOA. It has been stated that the delivery of living cells is associated with the release of growth factors and cytokines [62,96]. For example, it has previously been shown that, when wounded *in vitro*, Apligraf undergoes a staged expression of inflammatory cytokines and, later, growth factors, that is very much reminiscent of the normal process of wound healing [96]. Furthermore, a host of cytokines are produced by Apligraf, and there is evidence that its epidermal and dermal components work in concert to produce these mediators that would not normally be detected with the epidermal and dermal component alone [95].

Another interesting property of Apligraf, which is due to its unique bilayered configuration, is that it undergoes epiboly *in vitro* [97]. This ability to migrate over its own dermis suggests a very viable epidermal component, capable of allowing the construct to heal itself after injury [96].

The available evidence indicates that the cells from allogeneic constructs, including Apligraf, do not persist in wounds [98,99]. This has been shown in acute wounds as well as in venous ulcers, where the DNA from donor allogeneic cells is not detectable at 4–6 weeks, based on PCR-based detection of donor cells. Thus, there is strong evidence that true engraftment or prolonged persistence of cells from these allogeneic constructs does not occur. Apligraf may possibly persist longer when applied to patients with certain genetic conditions, as suggested by reports that Apligraf specific DNA may be detectable many more weeks or even months when the construct is applied in the wounds of young patients with EB, a condition characterized by a variety of genetic defects in molecules responsible for anchoring the epidermis to the dermis (i.e., laminin 5, type VII collagen) [100,101]. Still, in spite of lack of longevity of the construct, the clinical trial results indicate that even a few days or weeks of exposure of the wound to Apligraf have beneficial effects. Possible mechanisms include a more orderly and orchestrated release of cytokines, deposition of extracellular matrix material important for early migration of mesenchymal cells and keratinocytes, and even in the attraction of progenitor or stem cells from deep in the tissue or from the circulation [10].

Although it is tempting to think of Apligraf as a 'graft', available evidence indicates that this construct promotes secondary intention healing in an otherwise stalled wound. We have already discussed the issue of allogeneic cell longevity, which in itself speaks against the idea of Apligraf being a graft. Moreover, there is no evidence that wound bed blood vessels grow into Apligraf, as is commonly observed with autologous grafts. Perhaps most telling is the fact that, uniformly, Apligraf seems to stimulate the edges of this wound to migrate toward the center. This 'edge effect', which has also been reported with the application of living keratinocyte sheets, is the predominant observation with successful Apligraf treatment (Fig. 77.12). The edge effect strongly suggests that Apligraf does not act as a tissue replacement but,

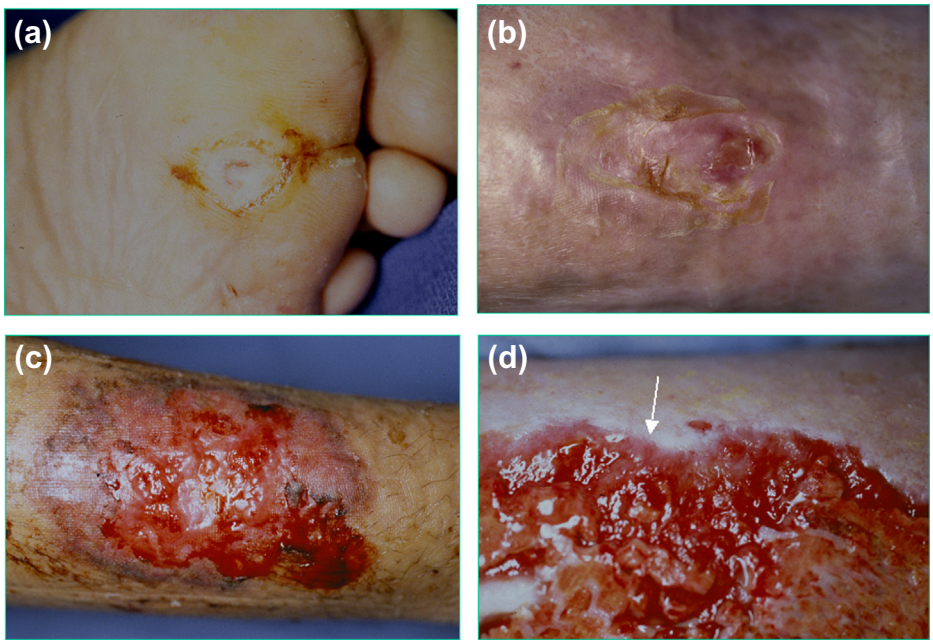

FIGURE 77.12
Different clinical appearances of wounds after Apligraf application. (a) Diabetic foot ulcer with white center representing degraded Apligraf remnants three week after application. (b) Venous ulcer six weeks post application. (c) Pyoderma gangrenosum only two weeks after Apligraf application and with thin layer of epidermis completely covering the wound bed. (d) Classical 'edge effect' (arrow) at the margins of a venous ulcer treated with Apligraf. The yellow material at the bottom of the pictures represent what is left of meshed Apligraf applied three weeks earlier. *(Copyright V. Falanga, 2006.)*

rather, stimulates secondary intention healing. Indeed, the rate at which the edges of a wound migrate toward the center has been used to determine whether eventual wound closure will occur, and Apligraf appears to accelerate wound edge migration [102].

CONSTRUCT PRIMING AND A NEW DIDACTIC PARADIGM FOR CONSTRUCTS

Two new concepts, priming and use of the construct in conjunction with stem cells, have emerged in our understanding of how constructs could potentially be used in different ways, even without modifying the initial manufacturing process of the product. These concepts are also mentioned by one of the authors (V. Falanga) in one of his recent publications on

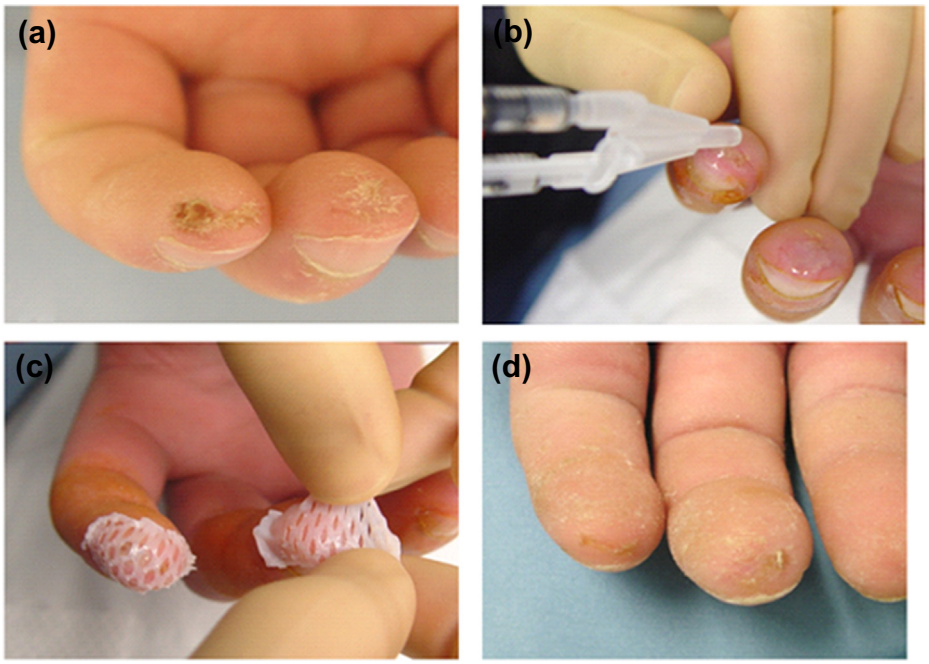

FIGURE 77.13
(a) Digital ulcers in a patient with systemic sclerosis (scleroderma). (b) autologous cultured bone marrow-derived mesenchymal stem cells being applied within a fibrin delivery system. (c) The digital ulcer was dressed with bioengineered skin (Apligraf). (d) Results observed one month later. *(Copyright V. Falanga, 2011.)*

bioengineered skin [102]. If priming proves to be clinically feasible, it would lead to a construct containing a richer and augmented repertoire of activated genes and proteins before it is applied to the wound. It has been demonstrated that this is possible using Apligraf as proof of principle *in vitro* and *in vivo* [103]. Indeed simple incubation of the construct for 24 hours in Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS leads to increased transcription (up to 200-fold) of hundreds of genes related to cellular proliferation and migration (V. Falanga, unpublished). The results obtained by microarray suggest that IL-6 is the most stimulated gene after using this priming step, and the results have been confirmed by western blot and by immunohistochemistry.

Another potentially useful concept, especially with the current interest in stem cells, is that bioengineered skin could be used in conjunction with stem cells to provide them with a didactic component and guidance for their differentiation. The wound bed can be treated with autologous bone marrow-derived cultured mesenchymal stem cells, and bioengineered skin can then be placed over the stem cells. We have successfully used this approach with scleroderma digital ulcers (V. Falanga, unpublished), and by delivering the stem cells with a modified fibrin spray. However, more work needs to be done to confirm these findings and to conduct appropriate clinical studies. Fig. 77.13 illustrates this type of approach [103].

CONCLUSION

Bioengineered skin constructs have played a lead role in the tissue-engineering field, particularly in terms of commercial successes. Like the natural tissue these constructs are designed to mimic, the engineered tissues are proving to be dynamic in the way they respond to environmental stimuli. The understanding of how these products induce healing is evolving. Greater understanding into how engineered tissues promote wound healing is being achieved through the clinical research, presented above, being done with Apligraf in different wound types. Initially the action was believed to be similar to skin grafts where the patient's native tissue was replaced with the graft tissue. It is now believed that the mode of action is more complex. Stimulation of healing by secondary intention appears to be the primary mode of action however there may be other mechanisms at work as well. The work being done in this area is very exciting and researchers have only begun to scratch the surface in terms of understanding the mechanisms and environmental stimuli involved.

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PART 20

Tissue-Engineered Food

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CHAPTER 78

Principles of Tissue Engineering for Food

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INTRODUCTION

Most techniques in tissue engineering were developed for medical applications. The potential benefits of tissue engineering and regenerative medicine for the repair of non-regenerative organs in the human body have not really been questioned. It is generally accepted that these technologies offer therapeutic opportunities where very limited alternatives are at hand to improve quality of life. Therefore, a tremendous amount of government funded research and business R&D has been and continues to be devoted to tissue engineering. Still, 25 years after its introduction, regenerative medicine by tissue engineering is not yet part of mainstream medical therapy [1]. This suggests that the technical challenges to generate tissues that are fully functional and can immediately replace damaged tissue are substantial.

As a spin off from this research activity, techniques in tissue engineering and regenerative medicine may be used to produce organs to produce food. This idea is not new and had in fact been proposed by Winston Churchill in his 1932 book 'Thoughts and adventures' [2] and by Alexis Carrel [3]. Although the biological principles of tissue engineering of food are very similar to the medical application there are also differences in goals, scale of production, cost-benefit ratio, ethical-psychological considerations and regulatory requirements.

In this chapter the distinctions between the challenges of tissue engineering for food production are highlighted and discussed. The focus will be mainly on tissue engineering of meat as a particularly attractive and suitable example.

WHY TISSUE ENGINEERING OF FOOD?

Growing food through domestication of grasses, followed by other crops and livestock has a 13,000 years head start. The success of economical food production likely determined the growth and sophistication of our civilization [4]. Why would we try to replace the relatively low-tech, cheap and easy natural production of food by a high-tech complicated engineering technology that is likely to be more expensive? There are two main reasons why current ways of food production need to be reconsidered.

First, with growth of the world population to 9.5 billion and an even faster growth in global economy, traditional ways of producing food, and in particular meat, may no longer suffice to feed the world [5]. Food security is already an issue for some populations, but absence of this security may spread across all civilizations due to generalized scarcity of food. Meat production through livestock for example already seems maximized by the occupation of 70% of current

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arable land surface, yet the demand for meat will double over the next four decades [6]. Without change, this will lead to scarcity and high prices. Likely, the high prices will be an incentive for intensification of meat production, which will increase the pressure of using crops for feed for livestock instead of feeding people. The arable land surface could be increased but this would occur at the expense of forests with predictable unfavorable climate consequences. Lifestyle changes that include the reduction of meat consumption per capita would also solve the problem, but historically this seems unlikely to happen. A technological alternative such as tissue engineering of meat might offer a solution. In fact, the production of meat is a good target for tissue engineering. Pigs and cows are the major sources of the meat we consume, and these animals are very inefficient in transforming vegetable proteins into edible animal proteins, with an average bioconversion rate of 15% [7]. If this efficiency can be improved through tissue engineering, this will predictably lead to less land, water and energy use for the production of meat [8], which introduces the second major reason why alternatives and more efficient meat production should be considered.

Livestock accounts for approximately 20% of total greenhouse emission [9]. Economizing meat production may therefore be an ecological imperative, improving the condition of the energy, water and food nexus.

In addition to these major issues with industrial dimensions of livestock breeding and feeding, there is an increasing public pressure to improve animal welfare and to reduce the risk of zoonoses.

Although there are other alternatives to avert an imminent meat and food scarcity, a technological solution might include tissue engineering of skeletal muscle and other tissues. It could be an excellent example of valorization of medical technologies that goes well beyond the traditional medical need and market.

SPECIFICS OF TISSUE ENGINEERING FOR MEDICAL APPLICATION

The general outline of tissue engineering (TE) for food is exactly the same as for tissues constructed for medical purposes. Somatic cells are seeded onto or printed with a temporary scaffold made of biocompatible natural or synthetic polymer. Subsequently, the cell scaffold combination is cultured under tissue specific biochemical and physical conditions to mature into the target tissue until it reaches maximum resemblance with the targeted tissue in the recipient.

However, there are differences as well that could have major implications for the technologies that need to be developed and used.

Uniqueness

To avoid immunologic or physiologic host versus graft reactions, medical tissue engineering aims to create a tissue construct that is mostly personalized and produced as a unique sample or at best in a limited volume [10]. Its uniqueness is determined by its shape and form and by its cellular components. The shape and form is adapted to the particular environment in the recipient by for instance rapid prototyping, molding or casting [11]. In some applications this might be achieved by computer assisted design linked to printing or molding equipment so that it can be automated. In other applications however, molds and casts are hand-made and therefore labor intensive and expensive.

In many examples of medical tissue engineering, cells producing the construct are of homologous origin, thus patient derived [12]. The number of tissue specific cells or even progenitor cells that can be harvested from patients is typically small and needs to be expanded before they can be used to populate a construct. The amount of cell culture that is required to reach sufficient cell numbers is a time and labor intensive process, especially when performed

for one sample of a personalized product. In many cases, the tissue still needs to undergo some form of conditioning, which again is time and labor intensive.

Thus, the production of patient-specific products is labor and time intensive and therefore extremely costly. In a medical application the cost of production is weighed against the benefit for the patient, which is usually expressed in quality adjusted life years (QALYs) that are gained through therapy. The value of a single QALY is set at approximately 40,000 USD [13], which sets the economic boundary condition of producing tissue-engineered constructs for medical application. The cost-benefit analysis will be very different between medical application and food production.

Function

Once implanted, the tissue-engineered products need to be functional and metabolically active, which means that they need to integrate with the circulation, the nervous system and the endocrine regulation. The integration is not only important to sustain and coordinate function (12); it is also crucial for homeostatic control by the recipient body. A good example is tissue-engineered myocardium to replace scar tissue in the heart that has developed after myocardial infarction. Myocardial tissue is continuously active in a highly coordinated and internally as well as external regulated. Implanted tissue-engineered constructs need to be fully integrated to maximize efficacy and minimize the risk of adverse effects (see Box 78.1).

BOX 78.1

Myocardial tissue has an extremely high metabolism to support the arduous labor of continuously alternating contraction and relaxation. To supply the tissue with sufficient nutrients and oxygen and to remove waste, the heart is highly vascularized with a microvascular density of one capillary per cardiomyocyte [14]. Without sufficient blood supply, tissue-engineered myocardial constructs would die within 30 min. The newly implanted myocardium also needs to integrate electrically with its neighboring myocardial cells. The heart shows continuous and highly integrated electrical activity as illustrated by the typical monomorphic and regular electro cardiogram (ECG). Failure to integrate through dyssynchronous electrical activation or through regional changes in conduction velocity can lead to lethal arrhythmias. Indeed in early trials using skeletal myoblasts to replace myocardial tissue, arrhythmias constituted the principle risk [15]. In addition to these already challenging forms of integration, the heart is a key effector in the homeostatic control of the body for instance during exercise, meals or abrupt changes in temperature. The heart is therefore under tight control of the autonomic nervous system, which regulates heart rate, contractility and coronary flow according to requirements imposed by the body. The implanted tissue needs to respond to these stimuli in concert with the native tissue to avoid dyssynchrony in electrical and mechanical activity during exercise for instance. An even more sensitive homeostatic mechanism that is essential to coronary flow regulation is the autoregulatory control of blood vessel diameter through organ specific myogenic responses of the vascular smooth muscle cells [16]. Organs such as the brain, heart and kidney have an important autoregulatory blood flow control that is partly dependent on these myogenic responses. At present it cannot be predicted how lack of this response would affect the function of the implanted tissue engineered myocardium, but it would be safe to optimize engineered tissues for all anatomic, histologic and physiologic characteristics.

The example of the heart illustrates the immense challenge for engineers and biologists to create a fully integrate tissue from cells of defined stages of differentiation and from biomaterials. Some of these challenges might be theoretical and will be resolved by remodeling of the construct after its implantation, but others might prove essential for safety and function.

SKELETAL MUSCLE AND FAT TISSUE ENGINEERING

To understand the specific requirements of tissue engineering of consumption meat a brief summary of the state of the art techniques to tissue-engineer skeletal muscle and fat is necessary. It is envisaged that the first tissue-engineered meat will be some form of processed meat such as in hamburgers or sausages. Producing processed meat is in that scenario translated into tissue engineering a large number of separate pieces of tissue that are later combined into a patty or a sausage.

Cells

The satellite cell is considered to be the tissue specific stem cell, responsible for *in situ* regeneration of damaged muscle although more pluripotent skeletal muscle derived stem cells have been described as well [17]. Culturing of skeletal muscle cells from satellite cells includes a proliferation phase followed by differentiation (Fig 78.1).

The goal of the proliferation phase the goal is to maximize the number of doublings. With the current isolation and culturing methods for satellite cells 30 doublings can be achieved. With proper conditions this can probably be improved to 50–70 doublings. A major improvement to keep the satellite cells in the replication phase comes from harvesting them through a combination of mild enzymatic treatment and trituration of remaining skeletal muscle fibrils according to Collins et al. [18]. Once harvested, the stem cell behavior is maintained in a niche environment [19]. The elasticity of the substrate for instance, seems important to maintain the stem-cell-ness of the satellite cells being cultured [20]. In our hands,

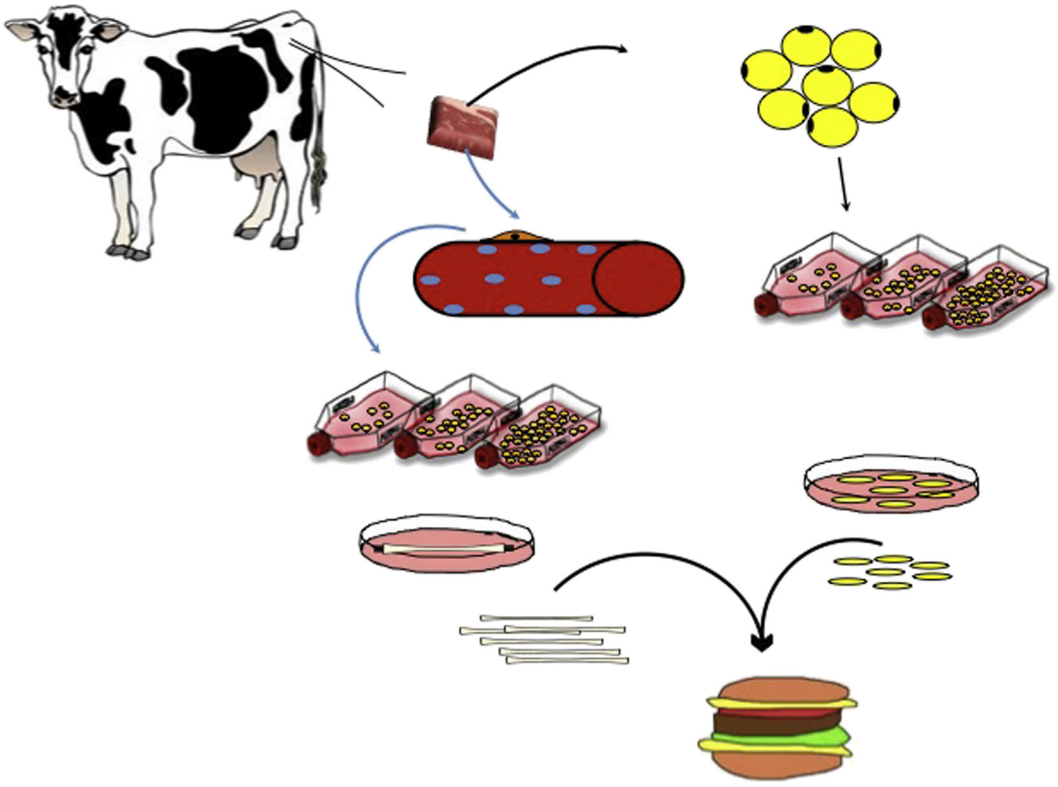

FIGURE 78.1

Tissue engineering of meat. From a biopsy of a cow, adipose tissue derived stem cells (ASCs) and satellite cells are harvested and cultured separately. The satellite cells are seeded in a gel onto a culture plate with anchor points (black pentagonal shapes). They self-organize into a bioartificial muscle. The ASCs are differentiated into adipocytes and cultured in a gel or scaffold, without the need for anchors. Minced meat, a hamburger for example, will be constructed from a mixture of bioartificial muscles and fat tissue.

the effect of physiologic substrate stiffness on myoblast cell proliferation was mild, higher than on very flaccid substrates (3kPa range) but not significantly different from stiff, plastic, surfaces [21]. Coating the culture surface with laminin and collagen IV, two major basal membrane proteins, has also some impact on the proliferation rate of satellite cells [22]. In addition, there are many biochemical stimuli for such as TGFβ1, Pax7, Notch and Wnt [23]. These mechanisms can be targeted with specifically designed agonists or with known biological modulators in order to delay differentiation.

Scaffold and seeding

Differentiation is primarily initiated by reducing the serum concentration in the medium. Cells are typically cast in a collagen gel with or without Matrigel™ supplement or they are seeded into a biodegradable polymer structure. In case of the casting in the gel it is important to provide anchor points in the culture dish. Typical for mesenchymal cells, differentiating satellite cells will organize the gel to the smallest structure possible. With the anchors provided the smallest configuration is a thin tissue strip in between the anchors. This will later form the myooid or bioartificial muscle (BAM, Fig 78.2) [24]. Differentiation leads to merging of satellite cells into primitive skeletal muscle cells, called myotubes. At this time, the cells will start to express early stage skeletal muscle markers such as MyoD, myogenin and embryonic isoforms of muscle myosin heavy chain [24a]. The cues for subsequent hypertrophy are of metabolic, biochemical and mechanical nature. After organization into a BAM and with subsequent differentiation, the muscle will develop increasing tension between the anchor points. This tension development is major trigger for protein production [25], the optimization of which is the final goal of tissue engineering of food. Several other mechanical stimuli such as passive cyclic stretch and electrical stimulation have been investigated. Interestingly, cyclic stretch did not further improve protein synthesis but had in fact a slight negative effect [26,27]. This result is somewhat controversial, as others have observed positive effects of cyclic stretch on muscle maturation [28]. In addition to passive stretch and tension, we and others have investigated the effect of electrical stimulation to further stimulate protein production and force generation [29]. In combination with specific coatings, electrical stimulation did lead to earlier maturation of the skeletal muscle fibers and more protein production. A more extensive review on the modes and efficacy of mechanical stimulation for muscle differentiation can be found at [30].

With the above described techniques it is feasible to generate BAMs of small dimensions, limited by the dependence on an adequate nutrient and oxygen supply through diffusion. No attempts

FIGURE 78.2
A bioartificial muscle from bovine satellite cells, three weeks after loading the gel onto the culture dish with anchors (black).

have been made yet to create large BAMs with a built-in blood vessel or channel system conducting a continuous flow of oxygenized, nutrient-rich medium. However, printing and biomaterial technologies have been described that would make this possible and certainly testable [31,32].

Although contractile proteins comprise the bulk of protein content and quality of muscle tissue, there are other proteins that are important for texture, color and taste of the muscle tissue. One particularly important protein is myoglobin. As a heme-carrying protein it is in part responsible for the pink color of meat and since it is a major carrier of iron, its presence will likely determine taste as well. The transcriptional regulation of myoglobin is reasonably well understood and involves the transcriptional activators MEF2 and NFAT/calcineurin [33] and co-activator PGC-1α. It appears that contractile activation of muscle in the setting of hypoxia will stimulate myoglobin maximally. It seems therefore feasible to increase the myoglobin content using stimuli that are compatible with tissue engineering of products that eventually should be consumed.

In summary, the effective culture of skeletal muscle is possible with current technology. There are numerous options for refinement and extension suggesting that it will take time and effort to optimize the product. Current progress however suggests that an acceptable mimic of meat tissue can eventually be generated.

Tissue engineering of fat

CELLS

The tissue engineering of fat tissue is less advanced than that of skeletal muscle because the medical need seemed less pressing. Indeed for the purpose of plastic surgery, tissue engineering of fat might be applicable in the clinic and scant efforts have therefore been made to create adipose tissue constructs [33a]. Also, differentiation of mesenchymal stem cells (MSCs) into adipocytes is one of the hallmarks of MSC phenotyping and as a result the various steps towards differentiation have been described in great detail.

BIOCHEMISTRY OF ADIPOCYTE DIFFERENTIATION

Pre-adipocytes are precursors of fat cells and determined by the expression of PREF-1 (pre-adipocyte factor 1 aka Dlk-1 or delta-like 1 homologue), CD105 (endoglin) and the absence of CD45 and CD31. These cells are typically fat tissue derived and therefore often referred to as Adipose tissue derived Stem Cells (ASCs). These collagenase-extracted, plastic, adherent cells initially comprise a heterogeneous population [34] that can differentiate into adipocytes, chondrocytes, and osteocytes. Unfortunately, these cells undergo limited proliferation and after a couple of passages they lose their capacity to differentiate [35] and are therefore not suitable for the tissue engineering of food. In contrast, human multipotent adipose derived stem cells (hMADS) that are derived from infants continue to proliferate and retain their differentiation capacity [35]. It remains to be shown if this is a consistent finding across species. If so, MADS can be harvested from young animals and these cells can then be used for tissue engineering of edible fat. An alternative cell source for production of fat tissue has been obtained by so-called ceiling culture of digested human adipose tissue [36]. In this technique two types of fibroblast like cells can be distinguished that both have a strong capacity to proliferate while retaining their ability to differentiate into adipocytes. It is likely that these cells can be harvested from bovine adipose tissue, as it has already been successful in humans, rats and sheep [36]. Differentiation of pre-adipocytes is typically initiated by starvation and a temporary cocktail of isobutylmethylxanthine (IBMX), dexamethasone and insulin. In some protocols, triiodothyronine [37] and or the PPARγ ligand Rosiglitazone [38] are added to the differentiation stimulus. It remains to be shown how much and if any of these stimulators are still present upon harvesting of the tissue after 3–4 weeks of differentiation, replacement of these agents by approved food additives is advisable.

IBMX is a very potent methyl-xanthine and a non-selective phosphodiesterase inhibitor that is not approved as a drug. IBMX induces the activation of PPAR$_\gamma$, which is a critical transcription factor required for adipocyte differentiation [39]. It probably acts through induction of intracellular cAMP. If so, other agents that act through induction of that second messenger and that are approved for drug or food purposes can replace IBMX. Dexamethasone is a glucocorticosteroid that acts through the intracellular glucocorticosteroid receptor (GR) through a number of intermediate products that eventually upregulate another major transcription factor, which in turn promotes adipocyte differentiation: C/EBPα (CCAAT enhancer binding protein). The steroid stimulated GR interaction with promotor elements in the intermediate products is required for its function; therefore it will be difficult to find a suitable substitute. The third component is insulin, which is typically short-lived and is therefore less likely to be a regulatory issue.

SCAFFOLD AND CELL SEEDING

The fat cells can be captured in a scaffold to form fat tissue. The same considerations apply that were presented for skeletal muscle tissue engineering, yet the specific biochemical and physical cues needed to mature and maintain fat cells are likely to be different from satellite cells. It is evident from the experience from many investigators that mature fat cells are fragile, prone to apoptosis and are terminally differentiated so they no longer can expand. For that reason, most cell seeding on scaffolds is performed in the pre-adipocyte stage [40–43], or is even performed with cell lines such as the mouse 3T3L1. In the vast majority of studies the constructs were implanted at an early stage and *in vivo* adipogenesis was allowed to develop for a couple of weeks after which the state of differentiation was assessed. This suggests that it is difficult or time consuming to reach full adipocyte differentiation and mature fat tissue by *in vitro* conditioning alone. A good summary of material and cell combinations that have been studied can be found at [43]. The specific requirements for the biomaterial component of fat tissue-engineered constructs for food purposes are:

1) Non-toxic biomaterial, degradation products or biochemical additives
2) Non-allergenic biomaterial, degradation products or biochemical additives
3) Residual biomaterial should be digestible
4) The scaffold should support full maturation of fat tissue *in vitro*

Any of these characteristics is unchartered territory and the present information on scaffold-adipocyte combinations should be re-evaluated in light of these requirements.

It is likely that non-toxicity of ingested material correlates with biocompatibility in current cell-based assays. If a particular biomaterial is not cytotoxic in direct exposure to cells it probably is not toxic after ingestion either. This reasonable hypothesis however, should be formally tested for each biomaterial as it is conceivable that rapid mechanic and enzymatic degradation by the gastrointestinal system release other or highly concentrated cytotoxic metabolites. The biomaterials that have been tested with adipocytes all conform to the requirement of being non-cytotoxic.

Non-allergenicity is less obvious. Most proteins and ingestible materials do not lead to an allergic reaction, but some foods, such as eggs, milk, shellfish and peanuts are particularly allergenic [44]. Bio-based materials that have been used for engineering adipose tissue include collagen [45], hyaluronic acid [46], fibrin [47], and chitosan [48]. Except for the latter, we already have bio-based materials in our present diet, so it is not to be expected that tissue-engineered food with these material will augment the risk of food allergy.

Of the synthetic or semi-synthetic biomaterials, poly(lactid-co-glycolic acid) (PLGA) [49] and esterified hyaluronic acid [46,50] are the most promising in terms of *in vivo* differentiation of adipocytes. In some studies, the addition of FGF-2 seemed to facilitate adipose differentiation. Confirming the results of others, but using bovine ASCs, we observed little development of fat tissue in electrospun PLGA scaffolds and fibrin sheets (preliminary data).

As for skeletal muscle engineering and mesenchymal stem cell differentiation, the stiffness of the matrix may be a particularly interesting parameter to affect specification and maturation of ASCs *in vitro* [51]. Human bone marrow derived MSCs assume quiescence when placed on a 250 Pa surface, which represents the natural elasticity of fat tissue. In this quiescent state they cease to proliferate, enter differentiation and develop into adipocytes that adipose tissue [52].

Recently the use of decellularized extracellular matrix has been gaining attention in regenerative medicine [53]. It is obvious that this technology will not be applicable in tissue engineering of food, since you first need to harvest the original tissues and the animals would still have to be bred as donors of these tissues. While the volume of tissue generated during culture cannot be drastically increased, such a practice would not result in higher efficiency of meat production or a reduction in livestock.

In summary, the optimal scaffold and biomaterial conditions to grow edible pieces of fat through tissue engineering will be a combination of chemical, biochemical and physical factors. The goals are in part different from fat tissue engineering in, for instance, reconstructive surgery and many variables have not been optimized yet.

SPECIFICS OF FOOD TISSUE ENGINEERING

The two major advantages of tissue engineering of food are that the eventual product can be made in large series, and also that it requires no functional integration. Instead, food tissue engineering faces at least three new challenges.

The first is the opposite of small-scale individualized production of medical tissues. To generate sufficient tissue volume to feed large populations, the scale of cell culture and tissue culture and/or conditioning will be several orders of magnitude higher than needed for medical application. The focus of tissue engineering for food will therefore shift from individual construct production to mass production. This shift has major implications for bioreactor design, biomaterial selection and production, culture medium optimization, optimization of tissue conditioning and quality control of for instance the genetic stability of the cells.

Second, food production by tissue engineering can only be a viable alternative to conventional livestock meat production if the bioconversion of vegetable proteins to edible animal proteins is more efficient than in the common domestic animals such as cows and pigs.

The third challenge is that tissue-engineered food needs to be very similar if not exactly the same as the conventionally produced food it intends to replace. Meat for example, has been replaced with very limited success by products made of vegetable proteins. The typical texture, color and taste of meat are apparently still very difficult to mimic through current innovations in food technology. In some cases, the science of these characteristics is not far enough advanced to design rational strategies for synthesis. For instance, the chemistry of the taste of meat is very complex and incompletely understood and therefore mainly relies on subjective analysis by taste panels [54].

Scale

The volume of global meat production is 293 million tons/year [55]. Given that each gram of muscle tissue contains approximately 5×10 [6] cells, this amounts to 1.5×10 [21] cells/year, provided that all muscle progenitor cells differentiate into mature skeletal muscle cells and get incorporated into skeletal muscle. This number may be an order of magnitude off, due to inaccuracies with respect to muscle type, fat content etc., but it provides an estimate of the rough scale at which cell production needs to be industrialized.

Currently, large bioreactors have a capacity of up to 25,000 liters or more, with a maximum cell load of 7×10 [6] cells/mL; i.e., 35×10 [9] cells/bioreactor [56,57], provided that the cells are cultured in suspension. Mesenchymal cells such as satellite cells need to adhere to surfaces to

sustain proliferation and survival. Several microcarriers have been designed to grow large numbers of adherent cells in large volume bioreactors [58]. This would require a stage in the process where cells are again detached from the carriers, which could be as simple as just changing temperature [59]. The limits on bioreactor size and number of cells per mL further depend on a number of factors such as oxygen transfer, nutrient delivery, and washout of waste. These factors are all related to unequal mixing of the fluid. Stirring or otherwise agitating the cell mixture might solve most of the problems, but there is a limit to the agitation that mammalian cells can handle in the absence of a firm cell wall that withstands high shear stresses [60].

As a result, the cells may suffer from insufficient and inhomogeneous transfer of oxygen and nutrients [60]. Inadequate mixing of bulk fluids also becomes more difficult as the volume of the bioreactors increases. Simulations in smaller tanks show that gradients of pH and nutrients reduce cell growth and maturation [61]. Another important factor in scaling-up cell culture in large bioreactors is adequate removal of CO_2. Carbon dioxide is typically removed from the medium through a combination of agitation and sparging: controlled air bubble inlet to retrieve CO_2 from liquid to the gas phase. Accumulation of CO_2 presents an important limit to mammalian cell culture due to the relatively low agitation and air sparging rates that are utilized [62]. The optimal dissolved carbon dioxide (dCO_2) level at which cells thrive varies between cell types, but relatively high dCO_2 levels universally inhibit cell growth and protein production [63,64].

Even when the technical limitations of large-scale cell culture are overcome, the sheer amount of cells needed to produce sufficient meat to feed the world population in 2050, would still require a mind-boggling amount of bioreactor space. Assuming that each bioreactor has a four week turnover of its cell content and would therefore have 13 operation cycles per year, one would still need 600 million bioreactors of 25,000 L, roughly one for every 10 humans. This already staggering figure does not even take into account that a seed train is needed to get sufficient cells for the initial start of a large volume bioreactor culture [57]. In addition, for skeletal muscle production to produce protein rich and firm muscle tissue, the proliferation phase should be followed by differentiation and conditioning of the muscle progenitor cells, which also requires bioreactor space.

For this volume of bioreactor space one needs equivalent volumes of culture medium. The production and storage of these amounts of medium will be a logistic challenge unless powder formulations are being developed that can be easily reconstituted within the same production line as the cell culture. In making powder formulations of medium, the addition sequence and the milling equipment seem to be important parameters [57].

Directly related with the scale of cell culture is the quality control of the progenitor cells. Cells may undergo differentiation towards undesirable phenotypes, they may be epigenetically modified or they can accumulate karyotypic abnormalities over time. For safety and efficacy reasons these undesirable outcomes should be detected and controlled [65,66]. Current technologies such as polymer chain reaction (PCR) and fluorescent *in situ* hybridization (FISH) are not designed for large sample sizes or for in-line monitoring of quality. Several other techniques based on surrogate physical parameters such as electric conductivity have been developed that are capable of in-line monitoring of the cell culture process, which will hopefully be sufficiently robust to guarantee quality of the cell culture [67].

From these considerations it may be clear that scaling-up mammalian cell culture to the level that is needed for safe and high quality production of one of our basic foods is a non-trivial enterprise and requires substantial innovation through chemical and mechanical engineering in close collaboration with cell biologists.

Efficiency

Although cell culture of mammalian cells has been evolving over the last five decades, relatively little attention has been paid to optimization of the culture process in terms of efficiency.

In such an optimization strategy, hundreds of variables can be – and have to be – controlled to make the process reliable and efficient. These variables include all individual components of medium and serum, but also physical culture conditions. The vast number of variables that likely interact and therefore need to be approached in their contexts creates challenges as well as opportunities. The level of each variable (e.g., feed component, biochemical and biophysical culture conditions), and the possible interactions with other variables need to be established. Current culture protocols have largely developed through trial and error, leading to a gradual optimization, whereas it clearly requires a more systematic approach. For bacteria and simple eukaryotic organisms such as yeast a systems biology approach is starting to be developed [68–70]. Either a biological systems strategy should be developed for more complex mammalian cells as well or a large-scale, high-throughput analysis should be set up to optimize culture media. This is true for the synthetic part of the culture medium as well as the serum part; together, a formidable task. Eventually, culture media should be completely synthetic and devoid of serum products. A limited number of such products have been developed for medical purposes and it is to be expected that more of these will become available [71,72]. In our hands, serum-based media are still superior to synthetic ones (unpublished data).

Opportunities to increase the efficiency of skeletal muscle cell culture are also numerous. In the production phase, recycling mechanisms and combining culture with nutrient supplying systems through for instance photosynthesis would create substantial benefit and value [8]. Given the scale of cell culture, these recycling mechanisms need to be an integral part of the production chain.

Taste, texture, juiciness

A large number of small peptides and fat dissolved aromatic substances have been described that contribute to the taste of meat [54,73–76]. However, the relative importance of each component is unknown. The reaction between certain sugars or glycosylated peptides and fatty acids probably interact especially during heating of the tissue to create the specific taste of meat in the so-called Maillard reaction [77,78]. Taste is a subjective sensation and blinded panels in fact still primarily judge the taste of meat. To add to the complexity, in addition to the chemistry of the meat, the cultural background of the panelists appears to weigh in their evaluation of meat products [54]. It has become clear that the taste of meat can be affected by feed and not so much by the strain of cattle [75], suggesting that the feed conditions of the cultured skeletal muscle is important for taste development. Even postmortem conditions determine the taste of meat, as a result of oxidation of proteins, sugars and fatty acids during controlled decay of the muscle [74]. Thus, in the tissue engineering of meat, taste is an extremely important feature to be investigated and taken into account. On the other hand, culturing of meat while controlling various conditions very precisely will greatly contribute to meat science in general and in particular to the effectors of taste.

The texture of meat is mainly determined by the presence of intramuscular connective tissue and its perimysial distribution [79] and the amount and distribution of fat [80]. Fat is also the main determinant of juiciness, although the percentage of fat in various meats shows modest variability between 0.5% to 8.0% with a 3% average [81]. As intramuscular connective tissue and content can be affected by feed, these characteristics can likely also be optimized in cell culture and should be primary targets for producing high quality meat.

It is not obvious that tissue-engineering food is a cheaper proposition than other alternatives such as vegetable protein meat replacers. Therefore, the outcome should be indistinguishable from livestock grown meat in terms of taste, color, texture, and juiciness. The quality should also be constant and preferable more predictable than for the natural product.

ENHANCED MEAT

Tissue engineering of meat enables the creation of novel products. All vertebrate animals have satellite cells in their muscles and currently there is reason to believe that skeletal muscle can be tissue engineered from these species. We have found that very small adjustments in protocols were needed to transfer satellite cells from mice, rats, pigs or cows into mature skeletal muscle. Novel meats could be made from hitherto unused species or from combination of cells sources. It is however more important to design and produce cultured meat that is healthier than the original product.

One particularly interesting example is increasing the content of conjugated linoleic acid (CLA; octadiendecadienoic acid 18:2). CLA is naturally present in meat, and the main source of our dietary CLA is beef fat and bovine milk [82]. CLA is formed in the stomach by bacteria that conjugate linoleic acid which is subsequently taken up by the fat cells. This mechanism can be used to load tissue-engineered fat with CLA to increase it to levels that are associated with anti-carcinogenic, anti-diabetic, and anti-atherogenic effects, as well as a strengthening the immune system, bone metabolism, and improving body composition [82].

OTHER FOODS

Although the tissue engineering of meat is probably the first and best example of food production through this technology, there are other foods that could be tissue engineered. Currently, the need to create an efficient alternative to agricultural food production precludes tissue engineering of crops and of meat from animals that have a high bioconversion rate, such as fish. However, if some fish species are threatened with extinction, tissue engineering of fish meat might also be considered. In fact, the first showcase of tissue-engineered food constituted some goldfish meat to be used by astronauts on their space missions [83].

Milk production by tissue-engineered mammary glands might also be feasible in the future. If cows are no longer needed in large numbers for meat production, an alternative method to produce milk should also be found. A recent study showed that bovine mammary gland stem cells could be isolated with techniques described earlier for their human counterparts. The bovine stem cells recapitulated the organization of bovine mammary tissue, and they produced milk. The investigators conclude that there is a potential for novel engineering and transplant strategies and a variety of commercial applications, including the production of modified milk components for human consumption [84].

Lastly, organ meats such as stomach, liver, kidney, or sweet breads might be future target foods to be tissue engineered.

CONSUMER ACCEPTANCE

Tissue engineering for medical purposes usually provides treatment options in situations where very few alternatives are present. For food tissue engineering the situation is completely different. In fact, novel tissue-engineered foods are competing with highly valued products that have been around for centuries. It is to be expected that tissue-engineered meat will generate a wide societal interest and a diversity of responses.

It has been proposed by various parties that research into tissue-engineered meat represents one of the more promising and morally responsible efforts to overcome the problems of global meat production [85,86].

Positive responses often stem from considerations of animal welfare, since this technology promises to enable meat consumption that reduces harm to animals. Many people, including many meat eaters, dislike the idea of animal suffering and dying; the prospect of meat with fewer animal victims therefore inspires great moral hope [87,88]. That the production of

tissue-engineered meat may also substantially reduce burdens on land and water use, in comparison with conventional meat production, is another source of hope.

More reluctant responses are also voiced, for example in the form of an immediate adverse opinion, often indicated as 'yuck'. A pilot exploration with the help of brief interviews [89] found that this initial response was connected with two associations: the thought that such meat is increasingly muddled with the idea of genetically modified (GM) food [87,89]. Both associations are informative of the cultural context in which the meaning of tissue-engineered food is taking shape and in which moral judgment and emotion influence each other in complicated patterns of mutual causality [90].

'Muddling with meat' refers to the growing moral ambivalences that surround meat. Latent feelings of uneasiness are inherently connected with meat [90], but they have been enhanced in recent decades by perceptions of how industrial farming deals with animals and their diseases [91,92]. Though the number of vegetarians has not grown spectacularly, many meat eaters are ambivalent about the way animals, as well as meat from them, are being manipulated [93]. The idea of tissue-engineered meat generates thoughts of hot dogs [89].

Associations with GM food also should be understood in a cultural and historical context. Negative consumer responses to GM food have mainly been studied in terms of risk perception, but it is increasingly clear that risk is only part of the story. For example, in one study the great majority of people who rejected GM food were quite willing to taste an apple that they were told to be genetically modified [94]. Consumer distrust is not necessarily directed at the product; it can also be aimed at the producers, and in this respect it has not been helpful that the benefits of GM food for consumers have never been made very clear [89,95].

Initial associations and responses can be fleeting; in the case of tissue-engineered meat they often give way to wider considerations almost within the same breath, for example when people start to reflect on the possible benefits for animals and realize that these benefits come with benefits for consumers, too, in the form of moral relief. Yet, reluctance also exists on second and third levels of consideration. In particular, a widespread ideal among reflective consumers is that food should be as natural as possible. From this perspective, tissue-engineered meat represents a technological approach that may lead to further estrangement from nature [86,96]. Yet it can also be argued that tissue-engineered meat promises to improve our relations with nature, through reduction of intensive farming and of agricultural land and water use. The future development of this theme is hard to predict.

A tasty product is a basic requirement for societal acceptance, and it is probably also vital that information will be clear and transparent. Beyond that, societal framing and perception depends on many uncertain factors. But if the problems of conventional meat continue to increase and if tasty tissue-engineered meat and other tissue-engineered foods lead to an improvement of our relations with animals and with nature, this new meat may well end up as a highly valued as well as routine part of our food system.

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CHAPTER 79

Prospects for *In Vitro* Cultured Meat — A Future Harvest

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INTRODUCTION

Current meat production systems are a major source of pollution and a significant consumer of fossil fuels, land and water resources. Nutrition related diseases, food borne illnesses, resource use and pollution, and use of farm animals are some of the serious consequences associated with them. Globally, 30% of the land surface is used for livestock production, with 33% of arable land being used for growing livestock feed crops and 26% being used for grazing [1]. The livestock sector contributes 18% to anthropogenic greenhouse gas emissions, and 37% of the anthropogenic methane emissions to the atmosphere worldwide [1]. This 18% contribution of livestock sector in the current greenhouse gas (GHG) emissions, as measured in carbon dioxide equivalent, is higher than the share of GHG emissions from transportation [2]. They produce 37% of methane, which has more than 20 times the global warming potential of carbon dioxide, and they emit 65% of nitrous oxide, another powerful GHG, most of which comes from manure [2]. Livestock also contributes 68% to total ammonia emissions [2]. The animals themselves are mostly responsible for the emission of greenhouse gases [3] and the demand for meat is expected to grow worldwide as global meat production is anticipated to increase to 465 million tonnes by the year 2050, accompanied by a rise in annual greenhouse gas emissions to 19.7 gigatonnes of carbon dioxide, carbon equivalent [1]. Meat production is also responsible for emissions of nitrogen and phosphorus, pesticide contamination of water, heavy metal contamination of soil, and acid rain from ammonia emissions [4]. The water use for livestock and accompanying feed crop production has a dramatic effect on the environment, such as a decrease in the fresh water supply, erosion and subsequent habitat and biodiversity loss [5,6]. The irrigation of feed crops for cattle alone accounts for nearly 8 percent of global human water use [2].

Until 2020, meat demand is expected to increase greatly in developing countries and slightly in developed countries [7,8]. To meet these increased meat demands of modern society, animals are intensively kept and production is optimized; disregarding the well-being of the

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animals. Much of the growing demand for animal products worldwide is being met by concentrated animal feeding operations, or factory farms [2,9]. Factory farms account for 67% of poultry meat production, 50% of egg production, and 42% of pork production [10]. Globally some 56 billion animals are raised and slaughtered for food each year [11]. Because of the large number of animals being used, an efficient and cheap production system is required. Herding of animals in confined spaces in unfavorable conditions is practiced. Many of the world's 17 billion hens and meat chickens each live in an area that is less than the size of a sheet of paper [12]. Cattle in feedlots often stand knee-high in manure and arrive at slaughterhouses covered in feces [13]. The adaptability of the animals is not high enough to cope with these unnatural conditions, and high stress levels are observed, resulting in disease, abnormal behavior and death [14]. Furthermore, such operations are increasingly located in or near cities in the developing world, making urban areas the center of industrial meat production in some countries. This extraordinary proximate concentration of people and livestock poses probably one of the most serious environmental and public health challenges for the coming decades [15]. The world organization for animal health (OIE) estimates that no less than 60% of human pathogens and 75% of recently emergent diseases are zoonotic [2]. Another problem is that of animal disease epidemics, and a more serious threat is posed by chicken flu, as this can lead to possible new influenza epidemics or even pandemics, which could kill millions of people [16]. Food borne illnesses have become increasingly problematic, with a six-fold increase in gastroenteritis and food poisoning in industrialized countries in the last 20 years [17]. The most common causes of food borne diseases in EU, USA and Canada are contaminated meats and animal products [18–21]. Nutritionally related diseases, such as cardiovascular disease and diabetes, which are associated with the overconsumption of animal fats are now responsible for a third of global mortality [22]. In addition, there is the problem of antibiotics being used as growth promoters for animals kept in intensive farming. This use probably contributes to the emergence of multi-drug-resistant strains of pathogenic bacteria [23]. In the United States, livestock now consume 70% of all antimicrobial drugs [24].

Moreover, livestock competes with humans for crops and grains as they detract more from food supply than they provide. Livestock consumes 77 million tones of protein contained in foodstuffs that could potentially be used for human nutrition, whereas only 58 million tones of protein are contained in foodstuffs that livestock produce and in terms of energy the relative loss is much higher [2]. Thus it is clear that besides the use of farm animals and non-sustainable meat supply, current meat production methods have many health, environmental and other problems associated with them, like high risk of infectious animal diseases, nutrition related diseases, resource use and environmental pollution, decrease in the fresh water supply, erosion, and subsequent habitat and biodiversity loss [5,6]. Continuation of meat production without change will lead to further environmental degradation and destruction of habitats. However, solutions are within reach, many of which originate from the scientific sector. However, although these will not be immediate but will need investment in the form of time and money, and possibly changes in consumer's habits. Scientific innovations can and should come from all sectors involved and an important contribution can be made via the generation of meat alternatives using improvements of already existing concepts and products. An example of such a concept is biofabrication; i.e., production of complex living and non-living biological products. A new approach to produce meat which thereby reduces these risks is probably feasible with existing tissue-engineering techniques and has been proposed as a humane, safe and environmentally beneficial alternative to slaughtered animal flesh. The industrial potential of biofabrication technology is far beyond the traditional medically oriented tissue engineering and organ printing and, in the long term, biofabrication could contribute to the development of novel biotechnologies that can dramatically transform traditional animal-based agriculture by inventing 'animal-free' food, leather, and fur products. The techniques required to produce 'animal-free' *in vitro* meat are not beyond imagination and the basic methodology involves culturing muscle tissue in a liquid medium on a large scale but

the production of highly structured, unprocessed meat faces considerably greater technical challenges and a great deal of research is still needed to establish a sustainable *in vitro* meat culturing system on an industrial scale. In the long term, tissue-engineered meat is the inescapable future of humanity. However, in the short term the extremely high prohibitive cost of the biofabrication of tissue-engineered meat is the main potential obstacle, although large-scale production and market penetration are usually associated with a dramatic price reduction. This chapter discusses the requirements needed for increasing the feasibility of *in vitro* meat production, which include finding an appropriate stem cell source, their growth in a three-dimensional environment inside a bioreactor, providing essential cues for proliferation and differentiation and evolving new processing technologies.

NEED FOR AND ADVANTAGES OF *IN VITRO* CULTURED MEAT

1) With the advent of functional and enriched foods, consumers are more willing to try products that have been altered to have particular nutritional characteristics [25,26]. There are many possibilities through which the designer meat can be produced *in vitro*. The flavor and fatty acid composition of the cultured meat can be influenced by manipulating the composition of the culture medium. Health aspects of the meat can be enhanced by adding factors like certain types of vitamins to the culture medium which might have an advantageous effect on the health [27]. Moreover, co-culturing with other cell types might further enhance the meat quality. The ratio of saturated to poly-unsaturated fatty acids could be better controlled and the fat content can also be controlled by supplementation of fats after production.
2) The chances of meat contamination would be lower in an *in vitro* meat production system due to strict quality control rules, such as Good Manufacturing Practice, that are impossible to introduce in modern animal farms, slaughterhouses, or meat packing plants and thus the incidence of food borne diseases could be significantly reduced. In addition, the risks of exposure to pesticides, arsenic, dioxins, and hormones associated with conventional meat could be significantly reduced.
3) In theory, cells from captive rare or endangered animals (or even cells from samples of extinct animals) could be used to produce exotic meats in cultures as the global trade of meats from rare and endangered animals has reduced wild populations of many species in many countries.
4) The *in vitro* meat production system will significantly reduce the animal use as theoretically a single farm animal may be used to produce the world's meat supply.
5) As only muscle tissues will be developed in an *in vitro* meat production system and no other biological structures like bones, respiratory system, digestive system, skin, and the nervous system will be involved, it will reduce the amount of nutrients and energy needed for growth and maintenance of muscle tissue.
6) It takes several weeks instead of months (for chickens) or years (for pigs and cows) before the meat can be harvested in the current meat production systems. *In vitro* systems will need a significantly shorter time to grow the meat than does traditional meat production. This means that the time that tissue has to be maintained will be much smaller and thus, the amount of feed and labor required per kg of *in vitro* cultured meat will be much lower.
7) The nutritional costs for *in vitro* cultured meat will be significantly lower than for traditionally cultured meat, as bioreactors for *in vitro* meat production do not need extra space and can be stacked up in a fabric hall. The financial advantages are yet unclear and it might very well be that the decrease in costs of resources, labor and land is compensated by the extra costs of a stricter hygiene regime, stricter control, computer management, etc.
8) There are many situations in which it is costly to supply people with food, and in which it is more economical to produce food *in situ*. These include scientific stations in Polar

Regions, troop encampments in isolated theaters of war and bunkers designed for long-term survival of personnel following a nuclear or biological attack. Thus long-term space missions such as a settlement on the moon or a flight to Mars will likely involve some food production *in situ* within a settlement or spacecraft to reduce liftoff weight and its associated costs.

9) The need for other protein sources demands production of cultured meat. Cultured meat may be the preferred alternative because it is, unlike the other products, animal-derived and with respect to composition most like meat.
10) A definite market is available for meat substitutes. Examples are legume-based and mycoprotein-based meat substitutes.
11) A small market comprising vegetarians who do not eat meat for ethical reasons is also available.
12) Demand for meat is increasing with the growing population, and it will not be possible to produce all the meat required in an environmental and animal friendly way. Thus there is a rather conventional meat market for *in vitro* meat.
13) Being comparatively safer than conventional meat and due to the non-sustainability of traditional meat production, there is a huge market for cultured meat.
14) One of the most important reasons to produce *in vitro* meat would be consumer demand. More and more people are interested in cultured meat and it can be a very successful product.
15) Other factors like potential impact on reducing cardiovascular diseases and greenhouse gas emissions, liberation of land for nature (including wild animals), prevention of animal suffering and prevention of food scarcity that can be expected with an increasing world population will push the production of *in vitro* meat in future.

Therefore, cultured meat has the potential to greatly reduce animal suffering and make eating animals unnecessary even while satisfying all the nutritional and hedonic requirements of meat eaters [28]. Cultured meat is potentially a much more reliable alternative. In comparison with animals, a product from a bioreactor could be attractive as it does not come with all the vicissitudes of animals. Furthermore, cultured meat is not bound to soil or place, which opens up possibilities for new places of production and for alternative land use. However, from a perspective of social acceptance, the technological character of cultured meat can have a negative value, and associations with Frankenstein, cloning, transgenesis and unknown risks are close at hand.

IN VITRO MEAT

The idea of cultured meat for human consumption is not new but was predicted some time ago by Winston Churchill in the 1920s. In his essay 'Fifty Years Hence', later published in 'Thoughts and adventures' in 1932, he declared that:

> 'Fifty years hence we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing by growing these parts separately under a suitable medium.'

In 1912, Alexis Carrel managed to keep a piece of chick heart muscle alive and beating in a Petri dish. This experiment demonstrated that it was possible to keep muscle tissue alive outside the body, provided that it was nourished with suitable nutrients. It was much later, in the early 1950s, when Willem van Eelen of The Netherlands independently had the idea of using tissue culture for the generation of meat products. Since at that time the concept of stem cells and the *in vitro* culture of cells still had to emerge, it took until 1999 before van Eelen's theoretical idea was patented. Some efforts have already been put into culturing artificial meat. SymbioticA harvested muscle biopsies from frogs and kept the tissues alive and growing in culture dishes [29]. In 2002, a study involving the use of muscle tissue from the common goldfish (*Carassius auratus*) cultured in Petri dishes was published in which the possibilities of culturing animal

muscle protein for long-term space flights or habituation of space stations were explored. In this study, muscle tissue cultured with crude cell extracts showed a limited increase in cell mass and the cultured muscle explants so obtained were washed, dipped in olive oil with spices, covered in breadcrumbs and fried. A test-panel judged the processed explants and agreed that the product was acceptable as food [30]. Thus some efforts have already been put into culturing artificial meat but obviously, small biopsies will not be practical for large-scale meat production. Therefore, it is proposed to use tissue engineering to produce *in vitro* cultured meat. With the help of tissue engineering it is attempted to mimic neo-organogenesis *ex vivo* for the treatment of various diseases and surgical reconstruction. This is a powerful technique which is mainly being designated for regenerative medicine in a wide variety of tissues and organs [31,32]. In particular, tissue engineering of skeletal muscle has many applications, ranging from *in vitro* model systems for drug-screening [33], pressure sores [34] and physiology to *in vivo* transplantation to treat muscular dystrophy and muscular defects [35]. Obviously, tissue engineering could also be employed for the *in vitro* production of skeletal muscle tissue from farm animals for consumption purposes [36].

Considering the benefits of an *in vitro* meat production system, it is not surprising that a number of parties have proposed and patented the methodology for actualizing this idea [27,36,37]. As of yet none of these processes, though detailed, have been tested, this chapter introduces the techniques so far proposed. This is partly because livestock animal cell lines have not been well-established *in vitro* [38] and also because growing muscle cells *ex vivo* on a large scale is certainly a vast and unexplored undertaking. The technical demands of large-scale production are unseen in the world of medical research, where most efforts in growing tissue *ex vivo* have been directed. The nutritional composition of *ex vivo* engineered muscle tissue has not yet been paid much attention. As a result, establishment of an *in vitro* meat production system is faced with many unique challenges so far unexplored in the field of tissue engineering.

The only aim in developing an *in vitro* meat production system is the proliferation of animal muscle tissue. Meat is already cultured on small scales using a variety of basic procedures, including techniques that use scaffolds and those that rely on self-organization [36]. There are different design approaches for an *in vitro* meat production system, all of which are designed to overcome the diffusion barrier, ranging from those currently in use to the more speculative possibilities.

Scaffolding techniques

In scaffold-based techniques, embryonic myoblasts or adult skeletal muscle satellite cells are proliferated, attached to a scaffold or carrier such as a collagen meshwork or micro-carrier beads, and then perfused with a culture medium in a stationary or rotating bioreactor. By introducing a variety of environmental cues, these cells fuse into myotubes, which can then differentiate into myofibers [39]. The resulting myofibers may then be harvested, cooked, and consumed as meat (Figs. 79.1, 79.2).

Currently there are two detailed proposals based on emerging field of tissue engineering [40,41] for using cell culture for producing *in vitro* meat. Both these proposals are similar in nature and neither of the two has been tested. One of the two proposals to create an *in vitro* meat production system has been written by Vladimir Mironov for the NASA [42], while the other has been written by Willem van Eelen, who also holds a worldwide patent for this system [27]. However, Catts and Zurr [29] appear to have been the first to have actually produced meat by this method. Both of these systems work by growing myoblasts in suspension in a culture medium. The Mironov proposal uses a bioreactor in which cells are grown together with collagen spheres to provide a substrate onto which the myoblasts can attach and differentiate, whereas van Eelen's proposal uses a collagen meshwork and the culture medium is refreshed from time to time or percolated through the meshwork. Once differentiated into myofibers, the mixture of collagen and muscle cells can be harvested and used as meat. Other forms of

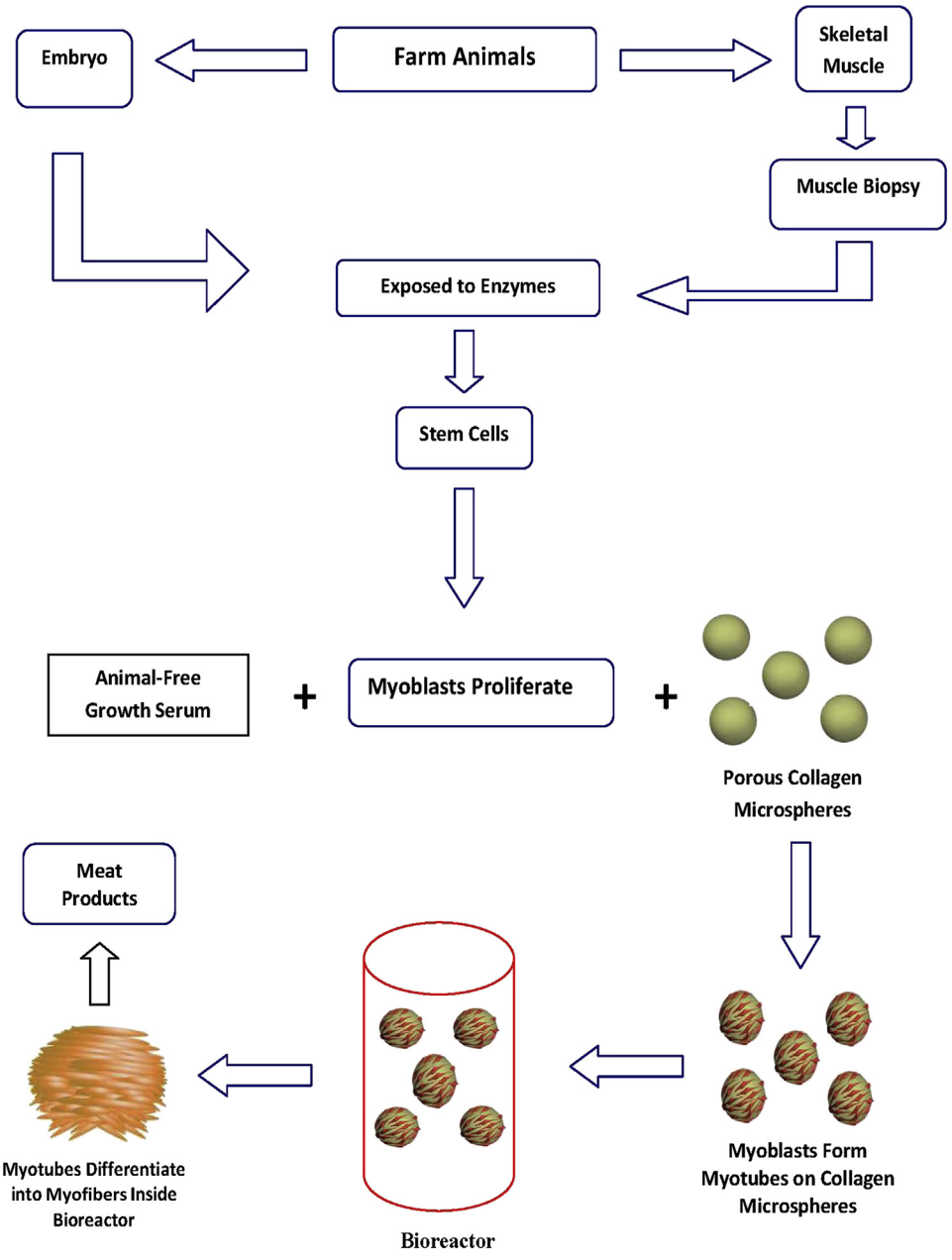

FIGURE 79.1
Scaffold-based cultured meat.

scaffolding could also be used, for example, growing muscle tissue on large sheets of edible or easily separable material. The muscle tissue could be processed after being rolled up to suitable thicknesses [36]. While these kinds of techniques work for producing ground processed (boneless) meats with soft consistency, they do not lend themselves to highly structured meats like steaks. However, cells can also be grown in substrates that allow for the development of 'self-organizing constructs' that produce more rigid structures.

Self-organizing tissue culture

To produce highly structured meats, one would need a more ambitious approach, creating structured muscle tissue as self-organizing constructs [43] or proliferating existing muscle tissue *in vitro*, like Benjaminson et al. who cultured Gold fish (*Carassius auratus*) muscle explants [30]. They took slices of goldfish tissue, minced and centrifuged them to form pellets, placed them in Petri dishes in a nutrient medium and grew them for seven days. The explanted

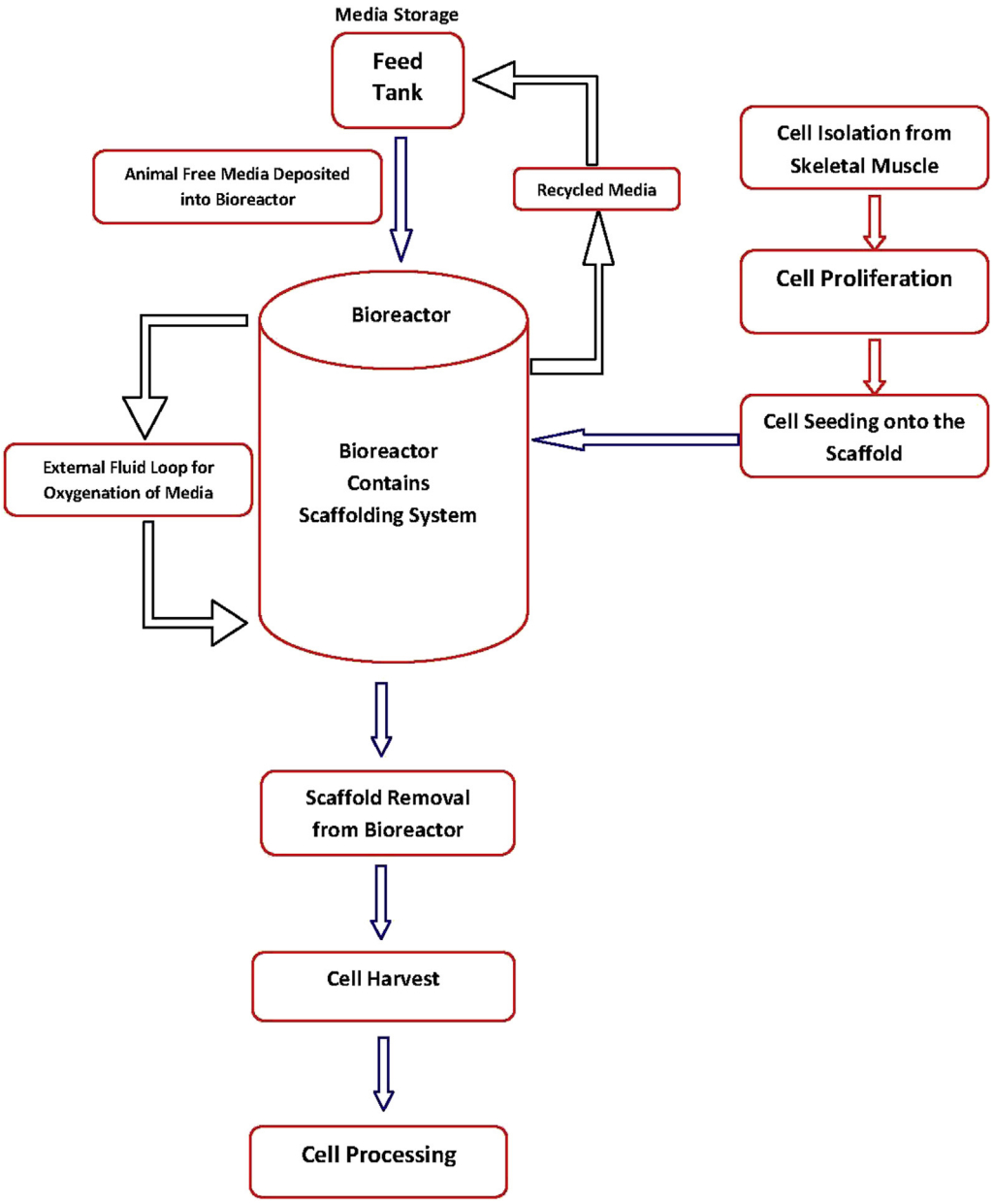

FIGURE 79.2
Possible *in vitro* meat production system.

tissue grew nearly 14% when using fetal bovine serum as the nutrient medium and over 13% when using Maitake mushroom extract. When the explants were placed in a culture containing dissociated *Carassius* skeletal muscle cells, the explant surface area grew by a surprising 79% in a week's time. After a week, the explants and their newly grown tissue, which looked like fresh fish filets, were cooked (marinated in olive oil and garlic and deep-fried) and presented to a panel for observation. The panel reported that the fish looked and smelled good enough to eat [30,44–46]. In another experiment, Benjaminson kept chicken muscles alive in a Petri dish for at most two months before it became necrosed [42].

Tissue culture techniques have the advantages that explants contain all the tissues which make up meat in the right proportions and closely mimics *in vivo* situation. However, lack of blood circulation in these explants makes substantial growth impossible, as cells become necrotic

if separated for long periods by more than 0.5 mm from a nutrient supply [43]. According to Vladimir Mironov, entirely artificial muscle can be created with tissue-engineering techniques by a branching network of edible porous polymer through which nutrients are perfused and myoblasts and other cell types can attach [42]. Such a design using the artificial capillaries for the purpose of tissue engineering has been proposed [41]. Small muscle-like organs have been demonstrated to grow from co-cultures of myoblasts and fibroblasts. These organs, termed myooids, are able to contract both spontaneously and by electrical stimulation, albeit with only a fraction of the force observed in control muscles probably because of lack of innervation [43,47,48]. The diameter of myooids is limited at most to 1 mm [48] due to the lack of perfusion; which is probably the biggest problem to overcome in designing an *in vitro* meat production system. Like the myooids, it is possible to co-culture the myoblasts with other cell types to create a more realistic muscle structure which can be organized in much the same way as real muscles [43,47,48].

Organ printing

The various problems associated with the current tissue-engineering techniques are that they cannot provide consistency, vascularization, fat marbling or other elements of workable and suitably-tasting meat that are not simply versions of ground soft meat. A potential solution to such problems comes from research on producing organs for transplantation procedures known as organ printing. Organ printing uses the principles of ordinary printing technology — the technology used by inkjet printers to produce documents. Researchers use solutions containing single cells or balls of cells and spray these cell mixtures onto gels that act as printing paper. The 'paper' can actually be removed through a simple heating technique or could potentially be automatically degradable. What happens is essentially that live cells are sprayed in layers to create any shape or structure desired. After spraying these three-dimensional structures, the cells fuse into larger structures, such as rings and tubes or sheets. As a result, researchers argue that the feasibility of producing entire organs through printing has been proved. The organs would have not only the basic cellular structure of the organ but would also include appropriate vascularization providing a blood supply to the entire product. For applications focused on producing meat, fat marbling could be added as well, providing taste and structure. Essentially, sheets and tubes of appropriate cellular components could create any sort of organ or tissue for transplantation or for consumption [28,49,50].

Biophotonics

Biophotonics is a new field that relies on the effects of lasers to move particles of matter into certain organizational structures, such as three-dimensional chessboard, or hexagonal arrays. In general, biophotonics refers to the process of using light to bind together particles of matter and the mechanisms of this field are still poorly understood. A surprising property of interacting light, this phenomenon produces so called 'optical matter' in which the crystalline form of materials (such as polystyrene beads) can be held together by nets of infrared light that will fall apart when the light is removed. This is a phenomenon a step-up from 'optical tweezers' that have been used for years to rotate or otherwise move tiny particles in laboratories. This has a binding effect among a group of particles that can lead them not only to be moved one by one to specific locations but that can coax them to form structures. Although primarily sparking interest in medical technologies, such as separating cells, or delivering medicine or other microencapsulized substances to individual cells, there is an intriguing possibility that such a technology could be used for the production of tissues, including meat [28]. Arrays of red blood cells and hamster ovaries have already been created using this technology [51]. Given the success of creating two-dimensional arrays, there is the possibility of producing tissue formations that use only light to hold the cells together, thus eliminating the need for scaffoldings [28].

Nanotechnology

The ability of optical tweezers to rotate or move tiny particles has intrigued nanotechnologists, who have inventive plans for what to do with the molecular scale-sized robots they would like to create (but so far, having few tools with which to make them). Nanotechnology (the production and alteration of materials at the level of the atom and molecule) holds out enormous possibilities and the holy grail of nanotechnology is some version of an 'assembler', a robot the size of a molecule that would allow moving matter at the atomic and molecular level. The obvious power of such a technology — given that everything is made of the same basic atoms but simply arranged in different ways — is that we would be able to construct virtually any substance we wanted from scratch by putting together exactly the molecules we wanted. Interestingly, one of the first examples given of the speculative technology of nanotechnology was that of synthesized meat. Thus technologies ranging from the actual to the speculative promise a variety of ways to create real meat without killing animals. Though still commercially infeasible at the moment or in some cases technologically infeasible for several years to come, the point here is not to be distracted by the fact that we cannot yet make use of these technologies but rather to decide whether we should support the development of these technologies [28].

CHALLENGES IN THE COMMERCIAL PRODUCTION OF *IN VITRO* MEAT

There are two different ideas regarding the concept of the cultured meat. Because people like meat and cultured meat is explicitly introduced as an alternative to the problems of normal meat, it should be as meat-like as possible in order to be a real alternative for 'traditional' meat from animals. It is therefore important that an alternative should have a similar taste and nutritional value. On the other hand, a new product needs a profile of its own; otherwise it will not be able to compete. From this perspective it is not essential for the product to resemble and should in fact be clearly distinctive from traditional meat.

Cultured meat technology is still in its infancy and the most important challenge is sufficient knowledge of the biology of the stem cell and its differentiation into muscle cells. Tissue engineering on a very large scale is the second requirement, along with the maintenance of constant conditions around all individual cells in a large-scale reactor with sophisticated instrumentation for measuring and controlling conditions. The need for cell growth and differentiation and subsequent release from support without damage upon harvesting is the third requirement, along with the need for on-site cleaning and sterilization systems in the large-scale reactors. Studies are required to determine the consumer preferences and marketing strategies. When meat from animals is available, why would a consumer prefer cultured meat and if it is all about sustainability or animal welfare issues, then eating more plant proteins and less animal protein is a good alternative. The following challenges have to be met before cultured meat can be produced on a commercial scale.

Generation of suitable stem cell lines from farm animal species

In vitro meat can be produced by culturing the cells from farm animal species in large quantities starting from a relatively small number. Culturing embryonic stem cells would be ideal for this purpose since these cells have an almost infinite self-renewal capacity and theoretically it is being said that one such cell line would be sufficient to literally feed the world. In theory, after the embryonic stem cell line is established, its unlimited regenerative potential eliminates the need to harvest more cells from embryos however; the slow accumulation of genetic mutations over time may determine a maximum proliferation period for a useful long-term ES culture [52]. While embryonic stem cells are an attractive option for their unlimited proliferative capacity, these cells must be specifically stimulated to differentiate into myoblasts and may inaccurately recapitulate myogenesis [31]. Although embryonic stem cells have been cultured for many generations but so far it has not been possible to culture cell lines with unlimited self-renewal

potential from pre-implantation embryos of farm animal species. Until now, true embryonic stem cell lines have only been generated from mouse, rhesus monkey, human and rat embryos [38] but the social resistance to cultured meat obtained from mouse, rat or rhesus monkey will be considerable and will not result in a marketable product. The culture conditions required to keep mouse and human embryonic cells undifferentiated are different from the conditions that will be required for embryonic cells of farm animal species and fundamental research on the early development of embryos of these species can provide clues.

Different efforts invested into establishing ungulate stem cell lines over the past two decades have been generally unsuccessful with difficulties arising in the recognition, isolation and differentiation of these cells [53]. According to Bach et al. myosatellite cells are the preferred source of primary myoblasts although, they have the disadvantage of being a rare muscle tissue cell type with limited regenerative potential because they recapitulate myogenesis more closely than immortal myogenic cell lines [31]. Myosatellite cells isolated from different animal species have different benefits and limitations as a cell source and that isolated from different muscles have different capabilities to proliferate, differentiate, or be regulated by growth modifiers [54]. Myosatellite cells have been isolated and characterized from the skeletal muscle tissue of cattle [55], chicken [56], fish [57], lambs [58], pigs [59], and turkeys [60]. Porcine muscle progenitor cells have the potential for multilineage differentiation into adipogenic, osteogenic and chondrogenic lineages, which may play a role in the development of co-cultures [59]. Advanced technology in tissue engineering and cell biology offer some alternate cell options having practical applications and multilineage potential allowing for co-culture development with suitability for large-scale operations.

Alternatively, we can use adult stem cells from farm animal species and myosatellite cells are one example of an adult stem cell type with multilineage potential [61]. Adult stem cells have been isolated from several different adult tissues [62] but their *in vitro* proliferation capacity is not unlimited and can proliferate *in vitro* for several months at most. These cells also have the capacity to differentiate into skeletal muscle cells, although not very efficiently but for now, these are the most promising cell type for use in the production of cultured meat. A rare population of multipotent cells found in adipose tissue known as adipose tissue-derived adult stem cells (ADSCs) is another relevant cell type for *in vitro* meat production [63] which can be obtained from subcutaneous fat and subsequently transdifferentiated to myogenic, osteogenic, chondrogenic or adipogenic cell lineages [64]. However, adult stem cells are prone to malignant transformation in long-term culture [65] that is the greatest matter of debate. It has been observed that ADSCs immortalize at high frequency and undergo spontaneous transformation in long-term (4–5 months) culturing [66], while evidence of adult stem cells remaining untransformed have also been reported [67]. To minimize the risk of spontaneous transformation, re-harvesting of adult stem cells may be necessary in an *in vitro* meat production system and as such obtaining ADSCs from subcutaneous fat is far less invasive than collection of myosatellite cells from muscle tissue.

Matsumoto et al. reported that mature adipocytes can be dedifferentiated *in vitro* into a multipotent preadipocyte cell line known as dedifferentiated fat (DFAT) cells, reversion of a terminally differentiated cell into a multipotent cell type [68]. These DFAT cells are capable of being transdifferentiated into skeletal myocytes [69] and appear to be an attractive alternative to the use of stem cells. This process known as 'ceiling culture method' certainly seems achievable on an industrial scale, but [70] has put forth the argument that many of the claims of transdifferentiation, dedifferentiation and multipotency of once terminally differentiated cells may be due to abnormal processes resulting in cellular look-alikes.

Safe media for culturing of stem cells

In vitro meat would need an affordable medium system to enjoy its potential advantages over conventional meat production and that medium must contain the necessary nutritional

components available in free form to myoblasts and accompanying cells. Myoblast culturing usually takes place in animal sera, a costly media that does not lend itself well to consumer acceptance or large-scale use. Animal sera are from adult, newborn or fetal sources, with fetal bovine serum being the standard supplement for cell culture media [71]. Because of its *in vivo* source, it can have a large number of constituents in highly variable composition and potentially introduce pathogenic agents [72]. The harvest of fetal bovine serum also raises ethical concern and for the generation of an animal-free protein product, the addition of fetal calf serum to the cells would not be an option and it is therefore essential to develop a serum-free culture medium. Commercially available serum replacements and serum-free culture media offer some more realistic options for culturing mammalian cells *in vitro*. Serum-free media reduce operating costs and process variability while lessening the potential source of infectious agents [73]. Improvements in the composition of commercially available cell culture media have enhanced our ability to successfully culture many types of animal cells and serum-free media have been developed to support *in vitro* myosatellite cell cultures from the turkey [74], sheep [75] and pig [76]. Variations among different serum-free media outline the fact that satellite cells from different species have different requirements and respond differentially to certain additives [77]. Ultroser G is an example of a commercially available serum substitute containing growth factors, binding proteins, adhesin factors, vitamins, hormones, mineral trace elements and has been designed specially to replace fetal bovine serum for growth of anchorage-dependent cells *in vitro* [30,78]. succeeded in using a serum-free medium made from Maitake mushroom extract that achieved higher rates of growth than fetal bovine serum and recently it has been shown that lipids such as sphingosine-1-phosphate can replace serum in supporting the growth and differentiation of embryonic tissue explants. In most cases, serum-free media are supplemented with purified proteins of animal origin [79].

Indeed such media have already been generated and are available from various companies for biomedical purposes; however, their price is incompatible with the generation of an affordable edible product. Therefore, a cell culture medium has to be developed that does not contain products of animal origin and enables culturing of cells at an affordable price.

Safe differentiation media for producing muscle cells

For stem cell culturing, it is important that these cells remain undifferentiated and maintain their capacity to proliferate and for the production of cultured meat a specific and efficient differentiation process initiated with specific growth factors is needed. An appropriate array of growth factors is required to growing muscle cells in culture in addition to proper nutrition and these growth factors are synthesized and released by muscle cells themselves and, in tissues, are also provided by other cell types locally (paracrine effects) and non-locally (endocrine effects). The myosatellite cells of different species respond differentially to the same regulatory factors [54], and as such extrinsic regulatory factors must be specific to the chosen cell type and species. Furthermore, formulation may be required to change over the course of the culturing process from proliferation period to the differentiation and maturation period, requiring different set of factors. A multitude of regulatory factors have been identified as being capable of inducing myosatellite cell proliferation [80], and the regulation of meat animal-derived myosatellite cells by hormones, polypeptide growth factors and extracellular matrix proteins has also been investigated [76,77]. Purified growth factors or hormones may be supplemented into the media from an external source such as transgenic bacterial, plant or animal species which produce recombinant proteins [81]. Alternatively, a sort of synthetic paracrine signaling system can be arranged so that co-cultured cell types can secrete growth factors which can promote cell growth and proliferation in neighboring cells. Appropriate co-culture systems like hepatocytes may be developed to provide growth factors necessary for cultured muscle production that provide insulin-like growth factors which stimulate myoblast proliferation and differentiation [82] as well as myosatellite cell proliferation in several meat animal species *in vitro* [77]. Typically, investigators initiate differentiation and fusion of

myoblasts by lowering the levels of mitogenic growth factors and the proliferating cells then commence synthesis of insulin-like growth factor-II, which leads to differentiation and formation of multinucleated myotubes [83] and stimulate myocyte maturation [84]. So the successful system must be capable of changing the growth factor composition of the media. Currently the most efficient method to let (mouse) stem cells differentiate into skeletal muscle cells is to culture them in a medium that contains 2% horse serum instead of 10 or 20% fetal calf serum. However, for the generation of cultured meat, it is essential that the cells are cultured and differentiated without animal products, so a chemically defined culture medium has to be developed that enables the differentiation of stem cells to skeletal muscle cells.

Tissue engineering of muscle fibers

The possibility to form a three-dimensional structure of cells is restricted in the absence of blood flow that provides oxygen and nutrients to the cells and removes metabolic end products. Because of the limitations in nutrient diffusion, the *in vitro* culturing of cells is limited to only a few layers of cells. A solution to this problem may be provided by culture of cells on edible or biodegradable synthetic or biological scaffolds which would provide shape and structure to the engineered tissue. Another solution would be the processing of these thin layers of cells into a meat based product. Alternatively, deformable micro-carrier beads of edible (non-animal) material may be developed that enable production of secondary myotubes in suspension which may be used as an animal protein ingredient in a wide variety of products. Alternatively, products of animal origin with a meat-like appearance and texture can be made by addition of fibroblasts (for firmness) and fat cells (for taste) to the myotubes.

Scaffolds

As myoblasts are anchorage-dependent cells, a substratum or scaffold must be provided for proliferation and differentiation to occur [85]. Scaffolding mechanisms differ in shape, composition and characteristics to optimize muscle cell and tissue morphology. An ideal scaffold must have a large surface area for growth and attachment, be flexible enough to allow for contraction as myoblasts are capable of spontaneous contraction, maximize medium diffusion and be easily dissociated from the meat culture. A best scaffold is one that mimics the *in vivo* situation as myotubes differentiate optimally on scaffold with a tissue-like stiffness [86] and its by-products must be edible and natural and may be derived from non-animal sources, though inedible scaffold materials cannot be disregarded. New biomaterials may be developed that offer additional characteristics, such as fulfilling the requirement of contraction for proliferation and differentiation [87]. Thus challenge is to develop a scaffold that can mechanically stretch attached cells to stimulate differentiation and a flexible substratum to prevent detachment of developing myotubes that will normally undergo spontaneous contraction.

Edelman et al. proposed porous beads made of edible collagen as a substrate [36] while as Van Eelen et al. proposed a collagen meshwork described as a 'collagen sponge' of bovine origin [27]. The tribeculate structure of the sponge allows for increased surface area and diffusion, but may impede harvesting of the tissue culture. Other possible scaffold forms include large elastic sheets or an array of long, thin filaments. Cytodex-3 micro-carrier beads have been used as scaffolds in rotary bioreactors but these beads have no stretching potential. One elegant approach to mechanically stretch myoblasts would be to use edible, stimuli-sensitive porous microspheres made from cellulose, alginate, chitosan, or collagen [36] that undergo, at minimum, a 10 percent change in surface area following small changes in temperature or pH. Once myoblasts attach to the spheres, they could be stretched periodically provided such variation in the pH or temperature would not negatively affect cell proliferation, adhesion, and growth. Jun et al. have found that growing myoblasts on electrically conductive fibers induces their differentiation, forming more myotubes of greater length without the addition of electrical stimulation [88] but use of such inedible scaffolding systems necessitates simple and non-destructive techniques for removal of the culture from the scaffold.

Furthermore, there are greater technical challenges in developing a scaffold for large and highly structured meats due to the absence of vascular system. There is a need to build a branching network from an edible, elastic, and porous material, through which nutrients can be perfused and myoblasts and other cell types can then attach to this network. [36] acknowledge that a cast of an existing vascularization network, such as that in native muscle tissue, can be used to create a collagen network mimicking native vessel architecture. Taking this a step further Borenstein et al., has proposed an approach to create such a network by creating a cast onto which a collagen solution or a biocompatible polymer is spread and after solidification seeding the network with endothelial cells [89]. Following dissolution of the polymer mold, successful proliferation could theoretically leave behind a network of endothelial tissue, a branched network of micro-channels, which can be stacked onto each other to form a three-dimensional network onto which one could grow myocytes.
A synthetic vascular system would then require a circulation pumping system and a soluble oxygen carrier in the medium to be fully functional. But at this moment creation of these artificial vascular networks does not translate well into mass production due to the microfabrication processes required. Alternatively Benjaminson et al., proposed an attempt to create a highly structured meat without a scaffold by solving the vascularization problem through controlled angiogenesis of explants [30].

Another important factor is the texture and microstructure of scaffolds as texturized surfaces can attend to specific requirements of muscle cells, one of which is myofiber alignment. This myofiber organization is an important determinant for the functional characteristics of muscle and the textural characteristics of meat. Lam et al. cultured myoblasts on a substrate with a wavy micropatterned surface to mimic native muscle architecture and found that the wave pattern aligned differentiated muscle cells while promoting myoblast fusion to produce aligned myotubes [90]. While using scaffold-based techniques for meat culturing, micro-patterned surfaces could allow muscle tissue to assume a two-dimensional structure more similar to that of meat of native origin. Riboldi et al. utilized electrospinning, a process that uses electrical charge to extract very fine fibers from liquids, by using electrospun microfibrous meshwork membranes as a scaffold for skeletal myocytes [91]. These membranes offer high surface area to volume ratio in addition to some elastic properties. Electrospinning creates very smooth fibers, which may not translate well into a good adhesive surface and coating electrospun polymer fibers with extracellular matrix proteins, such as collagen or fibronectin, promotes attachment by ligand-receptor-binding interactions [91]. Electrospinning shows promise for scaffold formation because the process is simple, controllable, reproducible and capable of producing polymers with special properties by co-spinning [91].

Production of meat by the scaffold-based techniques also faces a technical challenge of removal of the scaffolding system. Confluent cultured cell sheets are conventionally removed enzymatically or mechanically, but these two methods damage the cells and the extracellular matrix they may be producing [92]. However, thermoresponsive coatings which change from hydrophobic to hydrophilic at lowered temperatures can release cultured cells and extracellular matrix as an intact sheet upon cooling [93]. This method known as 'thermal liftoff,' results in undamaged sheets that maintain the ability to adhere if transferred onto another substrate [93] and opens the possibility of stacking sheets to create a three-dimensional product. Lam et al. have presented a method for detaching culture as a confluent sheet from a non-adhesive micropatterned surface using the biodegradation of selective attachment protein laminin [94]. However, culturing on a scaffold may not result in a two-dimensional confluent 'sheet' of culture. The contractile forces exerted after scaffold removal by the cytoskeleton of the myocyte are no longer balanced by adhesion to a surface that causes the cell lawn to contract and aggregate, forming a detached multicellular spheroid [93]. To remove the culture as a sheet, a hydrophilic membrane or gel placed on the apical surface of the culture before detachment can provide physical support and use of a fibrin hydrogel is ideal for skeletal muscle tissue because cells can migrate, proliferate and produce their own

extracellular matrix within it while degrading excess fibrin [94]. These two-dimensional sheets could be stacked to create a three-dimensional product as suggested by Van Eelen et al. [27].

Industrial bioreactors

Production of *in vitro* meat for processed meat-based products will require large-scale culturing in large bioreactors as stem cells and skeletal muscle cells require a solid surface for culturing and a large surface area is needed for the generation of sufficient number of muscle cells. Cultured meat production is likely to require the development of new bioreactors that maintain low shear and uniform perfusion at large volumes. The bioreactor designing is intended to promote the growth of tissue cultures which accurately resemble native tissue architecture and provides an environment which allows for increased culture volumes. A laminar flow of the medium is created in rotating vessel bioreactors by rotating the cylindrical wall at a speed that balances centrifugal force, drag force and gravitational force, leaving the three-dimensional culture submerged in the medium in a perpetual free fall state [95]. This improves diffusion with high mass transfer rates at minimal levels of shear stress, producing three-dimensional tissues with structures very similar to those *in vivo* [96]. Direct perfusion bioreactors appear more appropriate for scaffold-based myocyte cultivation allowing flow of medium through a porous scaffold with gas exchange taking place in an external fluid loop [97]. Besides offering high mass transfer they also offer significant shear stress, so determining an appropriate flow rate is essential [96]. Direct perfusion bioreactors are also used for high-density, uniform myocyte cell seeding [98]. Another method of increasing medium perfusion is by vascularizing the tissue being grown. Levenberg et al. had induced endothelial vessel networks in skeletal muscle tissue constructs by using a co-culture of myoblasts, embryonic fibroblasts and endothelial cells co-seeded onto a highly porous biodegradable scaffold [99]. Research size rotating bioreactors have been scaled-up to three liters and, theoretically, scale-up to industrial sizes should not affect the physics of the system.

Adequate perfusion of the cultured tissue is required to produce large culture quantities and it is necessary to have adequate oxygen perfusion during cell seeding and cultivation on the scaffold as cell viability and density positively correlate with the oxygen gradient in statically grown tissue cultures [98]. Adequate oxygen perfusion is mediated by bioreactors which increase mass transport between culture medium and cells and by the use of oxygen carriers to mimic hemoglobin provided oxygen supply to maintain high oxygen concentrations in solution, similar to that of blood. Oxygen carriers are either modified versions of hemoglobin or artificially produced perfluorochemicals (PFCs) that are chemically inert [100]. Many chemically modified hemoglobins have been developed but their bovine or human source makes them an unfit candidate and alternatively, human hemoglobin has been produced by genetically modified plants [101] and microorganisms [102].

Fields

Proliferation and differentiation of myoblasts have been found to be affected by the mechanical, electromagnetic, gravitational, and fluid flow fields [39,87]. Repetitive stretch and relaxation equal to 10% of length, six times per hour increase differentiation into myotubes [103]. Myoblasts seeded with magnetic microparticles induced differentiation by placing them in a magnetic field without adding special growth factors or any conditioned medium [104]. Electrical stimulation also contributes to differentiation, as well as sarcomere formation within established myotubes [39].

Atrophy and exercise

One of the potential problems associated with cultured meat is that of atrophy or muscle wasting due to a reduction of cell size [105] caused by lack of use, denervation, or one of a variety of diseases [106,107]. Regular contraction is a necessity for skeletal muscle and

promotes differentiation and healthy myofiber morphology while preventing atrophy. Muscle *in vivo* is innervated, allowing for regular, controlled contraction whereas *in vitro* system would necessarily culture denervated muscle tissue, so contraction must be stimulated by alternate means. It might be possible that mechanical or electrical stimulation can promote growth and structure of the cultured meat as newly formed myotubes in culture start to contract spontaneously [108] or as a matter of fact, myooids also contract spontaneously at approximately 1 Hz. once formed [43]. So exercise by electrical stimulation might be a viable solution to overcome atrophy in an *in vitro* meat production system. Cha et al. have found that administration of cyclic mechanical strain to a highly porous scaffold sheet promotes differentiation and alignment of smooth muscle cells [109]. Edelman et al. and Van Eelen et al. proposed mechanical stretching of scaffolds and expandable scaffold beads to fulfill the requirement of providing contraction [27, 36]. De Deyne noted that external mechanical contraction is less effective than electrical stimulation in promoting muscle development [87]. Electrical stimulation, feasible on a large scale, induces contraction internally as opposed to passively and aids in differentiation and sarcomere formation. Even growth on electrically conductive fibers without application of electrical stimulation sufficed in reaping the benefits of induced contraction [88].

Neuronal activity can be mimicked by applying appropriate electrical stimuli *in vitro* cultures [110] and has proven to be pivotal in the development of mature muscle fibers [111]. It has been shown that induction of contractile activity promoted the differentiation of myotubes in culture by myosin heavy chain expression of different isoforms and sarcomere development [112,113]. Electrical stimulation can provide a non-invasive and accurate tool to assess the functionality of engineered muscle constructs [114]. Functional muscle constructs will exert a force due to active contractions of the muscle cells by generating a homogeneous electrical field inside the bioreactor but so far, these forces generated by engineered muscle constructs only reach 2−8% of those generated by skeletal muscles of adult rodents [47]. Thus, functional properties of tissue-engineered muscle constructs are still unsatisfactory at this moment.

Mechanotransduction is the process through which cells react to mechanical stimuli and is a complex mechanism [115,116] that is another important biophysical stimulus in myogenesis [117]. It is mainly by means of the family of integrin receptors that cells attach to the insoluble meshwork of extracellular matrix proteins [118] transmitting the applied force to the cytoskeleton. The resulting series of events shows parallels to growth factor receptor signaling pathways, which ultimately lead to changes in cell behavior, such as proliferation and differentiation [115]. Muscle growth and maturation is affected by different mechanical stimulation regimes and the application of static mechanical stretch to myoblasts *in vitro* results in a facilitated alignment and fusion of myotubes, and also results in hypertrophy of the myotubes [117]. Furthermore, cyclic strain activates quiescent satellite cells [119] and increases proliferation of myoblasts [120]. Thus all these results indicate that mechanical stimulation protocols affect both proliferation and differentiation of muscle cells and different parameters that presumably influence the outcome of the given stimulus are percentage of applied stretch, frequency of the stimulus and timing in the differentiation process.

Senescence

In vitro meat production system based on satellite cells still imposes a challenge of senescence that can be tackled either by starting fresh cell culture whenever needed or by immortalizing cell culture or by using embryonic stem cell cultures. Fresh satellite cells can be extracted without harming the animal donors [27] from time to time to start new cell cultures, although animal slaughter is a more common practice [54]. A second approach involves modification of the cells in culture so that senescence can be overcome by involving the ectopic expression of the gene for the telomerase enzyme [121]. An additional expression of an oncogene may be required to overcome senescence [122–125], but this method falls within the domain of genetic modification, which might severely hinder consumer acceptance. A third approach

involves embryonic stem cells which are pluripotent and apparently have an unlimited capability for division [126] and thus, embryonic stem cell culture derived from a single donor can be theoretically propagated unlimited but embryonic stem cells have to differentiate to muscle cells before they can be used.

Food processing technology

New food processing technologies need to be developed to make *in vitro* meat based products attractive and that will depend on the starting material whether suspensions of small myotubes, myofibers on scaffolds or microspheres, etc., are utilized. The first *in vitro* meat based product may be developed using small pieces of cultured muscle fiber as raw material.

Risks of contamination

Cultured meat will be safer and more sustainable than conventional meat but production may be less safe because of risks of contamination. Cultured meat may have a completely different risk profile to conventional meat, and much attention will have to be paid to the safety of added substrates and other compounds of the culture medium. So, there are fewer risks with respect to microbial contamination but more risk of contamination of substrates.

Future efforts in culturing meat will have to address the limitations of current techniques through advances that make cultured cells, scaffolds, culture media, and growth factors edible and affordable.

CONCLUSION

Continuing the production of meat by current methods is going to further aggravate the problems and *in vitro* meat production system seems to be an appealing alternative and is becoming increasingly justifiable in light of the sizable negative effects of current meat production systems. *In vitro* meat production is a sustainable, humane and safer system that offers a number of benefits including animal welfare, process monitoring, environmental considerations, efficiency of food production in terms of feedstock, decrease in intense land usage and greenhouse gas emissions. Furthermore, the composition, flavor and functional role of cultured meat could be better controlled. Thus *in vitro* meat holds great promises as an alternative to traditionally produced meat provided consumer resistance can be overcome. However, *in vitro* meat production on an industrial scale is feasible only when a relatively cost-effective process creating a product qualitatively competitive with existing meat products is established and provided with governmental subsidization like that provided to other agribusinesses.

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PART 21

Clinical Experience

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CHAPTER 80

Current State of Clinical Application

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INTRODUCTION

The overall impact of tissue engineering in biomedical science has been fairly diverse in nature. Directly and indirectly, it has enhanced our understanding of the structure-function relationships within normal and pathological tissues and of multi-dimensional physiologic processes. It has also broadened the possibilities for testing pharmacological therapies [1]. Still, the ultimate benchmark for the success of tissue engineering, as a multidisciplinary field, remains its ability to generate new and more effective therapies for patients afflicted with severe tissue loss and/or organ failure [2]. Based on this measure, only a handful of tissue-engineered products have reached the bedside. By and large, thus far these clinically tried tissue-engineering therapies have demonstrated mostly adequate safety, with only modest, if any, therapeutic benefits in small, defined patient populations, or even simply through anecdotal data [3]. Therefore, it is safe to say that tissue engineering has yet to fulfill its promise for the vast majority of patients in need [4].

As discussed in previous chapters, tissue-engineering technologies fall into a variety of ever expanding strategies, including (but not limited to): simple delivery of isolated cells (cell transfers); tissue-inducing substances and/or devices; extracorporeal and/or encapsulation techniques (closed systems); and transplantation of cells within matrices (open systems). This chapter outlines some of the present challenges in the clinical development of the field, while offering a concise, general outlook on the current state of clinical application of so-called open systems tissue engineering. More detailed reviews of the clinical experience involving the many other tissue-engineering approaches are discussed elsewhere in this book.

CURRENT CHALLENGES

Practical considerations

Timing is an inherent limitation of many tissue-engineering applications, not the least open systems. Although autologous approaches obviate the risk of immunologic rejection, they typically involve weeks, if not months, of processing in order to culture a sufficient number of cells for a given composite construct. Yet time is often an unaffordable luxury for many

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patients, particularly those with life-threatening conditions and/or for whom limited treatment alternatives currently exist.

Another practical barrier has been the relatively high cost of these technologies. Because elaborate and expensive infrastructures are often necessary for the development and manufacture of engineered tissues, products designed to address relatively rare disease processes are often difficult to remain sustainable in the long term. Indeed, many firms with considerable interest in tissue engineering and regenerative medicine therapies have exited the market despite early studies suggesting efficacy. Production within Good Manufacturing Practice (GMP) facilities are a prerequisite for Food and Drug Administration (FDA) approval of cell-based therapies, which, in turn, cannot be pursued without a critical mass of highly trained personnel [5–7]. Furthermore, certain tissues require preconditioning in complex bioreactors, which may not be readily amendable to scaled-up manufacturing and shipping [1]. All of these issues translate into a chronic difficulty in establishing multicentric clinical trials, which are in turn essential for the widespread application of new therapeutic strategies.

Regulatory matters

Despite the common goal of introducing safe tissue engineered products within the clinical arena, most have perceived the regulatory constraints imposed on the field to constitute a significant barrier towards clinical translation [8]. In the United States for example, the FDA has a long history of being notoriously slow at initiating and conducting product approval processes in tissue engineering. This problem has been attributed, at least in part, to the lack of clear regulatory frameworks and occasionally to uncertainties regarding whether tissue engineered products should be classified as mechanical implants, biological materials, or both. Furthermore, unlike many other biotechnology developments, cell-based tissue engineering technologies are rather unique in that they need to be thoroughly tested for infectious pathogens, tumorigenic potential, and immune reactions (the latter even in autologous applications, stemming from cell processing and/or scaffold composition). Engineered tissues also need to be rigorously studied in animals prior to clinical application. Depending on the particular product, such testing may require costly large animal models as a means to provide final proof of principle and safety to the FDA prior to implantation in humans [9].

For many companies devoted exclusively to tissue-engineering technologies, such regulatory burdens and delays, combined with strict reimbursement policies and intermittently poor business models, have often conspired to a commercially untenable enterprise. A few American biotechnology companies have been able to seek different tactics by accumulating clinical data overseas at lower costs, given that many other countries tend to have far less stringent regulatory procedures with regard to the marketing and clinical application of novel medical products than those enforced in the United States. Recently, this has been evidenced by several US regenerative medicine firms initiating their pilot tissue-engineering clinical trials in countries such as Mexico, Argentina, Korea, and Poland [10,11].

Scientific limitations

The rapidly changing technologies within the broad, multidisciplinary field of tissue engineering have sometimes made clinical evaluations fairly difficult. For example, the ideal cell type or even cell source for many clinical applications remain undetermined. In many cases, while differentiated autologous cells would be ideal, their use simply may not be a viable option in humans, either because of current isolation and expansion limitations (e.g., hepatocytes, neurons, cardiomyocytes, pancreatic islet cells) or because they tend to dedifferentiate over time (e.g., chondrocytes). Each cell type has its own unique advantages and disadvantages, which are discussed in depth in other chapters. Human embryonic stem (hES) cells and induced pluripotent stem cells (iPS) are relatively new and potent alternative sources for tissue

engineering, but also carry potential tumorigenic behavior, peculiar immunologic limitations, or have remained hampered by social and ethical constraints, particularly in the case of hES cells. Even autologous cells in culture may not be completely free of pathogens, since the culture media often requires xenogeneic growth factors, such as fetal bovine serum, for optimal growth, or because certain cells can only propagate consistently on murine feeder layers. At this time, infectious risks cannot be completely eliminated with these xenogeneic techniques.

Another scientific limitation is the lack of an optimal biomaterial for many clinical tissue-engineering applications. Many of the currently available synthetic scaffolds are still metabolized by the body leaving a significant foreign-body reaction behind. These conditions can lead to a reduction in the diffusion of nutrients and waste products, fibrosis, and other complications. Additionally, the cytotoxic effects of macrophage-generated nitric oxide can reach and destroy the transplanted cells. Thus, it is not surprising that most of the scaffolds that have been implanted in humans to date are derived from natural scores (e.g., bone, dermis, interstinal submucosa). Unfortunately, at the same time, natural scaffolds have been associated with unfavorable mechanical properties (e.g., rapid or inconsistent degradation, low or erratic tensile strength). Further, some chemicals used in the decellularization process are known to negatively affect the properties of the scaffold.

For these reasons, there remains a continued interest in the development of novel biocompatible synthetic biomaterials amongst materials scientists and others. For example, electrospinning is a somewhat novel, alternative approach for creating scaffolds that can be made with finely tuned biomechanical specifications. The advantages of this technique include the ability to make scaffolds with high porosity as well as high surface area to volume ratio, while mimicking the dimensions and structure of native collagen and elastin fibrils. Finally, scaffolds impregnated with growth factors or specific peptide sequences may also allow for better control of the surrounding microenvironment. Indeed, these newer synthetic materials, discussed in depth in other chapters, will be instrumental in helping to broaden the types of engineered tissues that can be rendered viable for human use.

Vascularization hurdles

Typically, any tissue greater than 1 cm in thickness cannot rely solely on vascular ingrowth from the host's vascular bed in order to remain viable *in vivo*. It is therefore not surprising that most successful clinical applications involving tissue engineering have dealt with the implantation of either surface lining (e.g., skin); simple, hollow organs (e.g., bladder); or relatively small, vessel-like conduits (e.g., blood vessels, trachea). A major challenge in the clinical implementation of more sizable and complex engineered structures and organs such as liver, heart, kidney, and lungs continues to revolve around how to best optimize the blood supply to the graft after implantation. One interesting, already preclinical approach to address this problem has been to create a preformed microcirculation within the engineered scaffold itself [12]. This strategy has been employed by Vacanti and his colleagues using microelectromechanical systems (MEMS) technology in a large animal model [13]. Preliminary work has enabled this group to develop a robust computational model of the vascular circulation, which includes the fractal nature of network topology, the rheology of blood flow through this computational system, and the mass transfer of oxygen and nutrients across the vascular bed. Vascular channels can be etched onto silicon wafers, which can then be transferred to biodegradable polymer systems. Multiple monolayers of this architecture can then be stacked to form three-dimensional structures. Another described approach to organ revascularization has involved seeding of the vasculature of a decellularized organ with endothelial cells in a bioreactor prior to anastomosis of the engineered graft, albeit so far only in rodent models [14]. Conceivably, some of these approaches might possibly become instrumental in facilitating the formation of other complex accessory networks (e.g., neural, lymphatic, biliary) between the host and engineered tissue itself. Two other approaches to enhance construct

vascularization, namely gene therapy and scaffold-based encapsulation of angiogenic growth factors (e.g., vascular endothelial growth factor), have also been pursued in preclinical models [15].

CLINICAL APPLICATIONS

As with many other novel medical technologies, translation of tissue-engineering applications from the bench to the bedside has not been easy, and many hurdles still need to be surmounted. Over the last decade, we have seen a number of tissue-engineered products that have either been abandoned following Phase I/II clinical trials, or have failed in Phase III clinical testing [16]. Nevertheless, there remains a slow but steady introduction of tissue-engineering products into the clinical arena. Below is a small yet illustrative sampling of some of the clinical experiences with open systems tissue engineering within the past decade. It is by no means purported to be a comprehensive, all-encompassing list.

Cardiovascular repair

Congenital heart disease is the leading cause of neonatal death due to congenital anomalies. In particular, single ventricle anomalies are especially threatening, often requiring several life-saving operations within the first few months of life in order to prevent irreversible congestive heart failure. A commonly performed procedure in these patients involves separation of the pulmonary circulation from the systemic circulation by creating a cardiac total cavopulmonary connection with a biocompatible synthetic conduit (e.g., Dacron, du Pont de Nemours, Wilmington, DE; or Gore-Tex, W.L. Gore & Associates, Newark, DE). Unfortunately, these synthetic conduits are prone to thromboembolic complications, are susceptible to infection, and do not grow with the patient through childhood. As a result, some of these patients are forced to undergo risky re-operations to revise the conduit. These procedures are associated with significantly higher rates of mortality and morbidity.

A tissue-engineered blood vessel conduit composed of autologous cells seeded on a biodegradable scaffold would be a significant advance towards avoiding the many disadvantages associated with synthetic conduits. In both large animal models (lambs) and humans, investigators have shown that tissue-engineered blood vessels can be made using a tubular scaffold composed of polyglycolic acid woven with either ε-caprolactone or L-lactide [17]. These scaffolds are seeded with autologous bone marrow mononuclear cells for two hours prior to implantation (Fig. 80.1). In 2001, Shin'oka and colleagues in Japan described their initial case report — a 4-year-old girl who developed total occlusion of her right pulmonary artery after a pulmonary artery angioplasty and a Fontan procedure, and subsequently underwent reconstruction with an autologous vessel graft engineered from cells obtained from a peripheral vein [18]. The construct remained patent for more than seven months after implantation. Between 2001 and 2004, a total of 25 children with congenital heart disease with a mean age of 5.5 years at the time of implantation were followed for over nine years postoperatively with tissue-engineered grafts as described above, with encouraging results [19].

This early experience supported the overall feasibility and safety of tissue-engineering technologies within pediatric cardiac surgery but also highlighted the problem of graft stenosis secondary to neotissue formation in approximately 15 to 20% of patients (Fig. 80.2) [20]. Fortunately, this complication has not been associated with any known clinical sequelae and has generally been amendable to percutaneous angioplasty. After further, more extensive studies in animal models by Shin'oka and Breuer, the first US patients were finally approved to receive tissue engineered vascular implants by the FDA in 2012. Whether the next generation of tissue-engineered blood vessel conduits for pediatric cardiac repair will be associated with lower long-term morbidity remains to be determined [21].

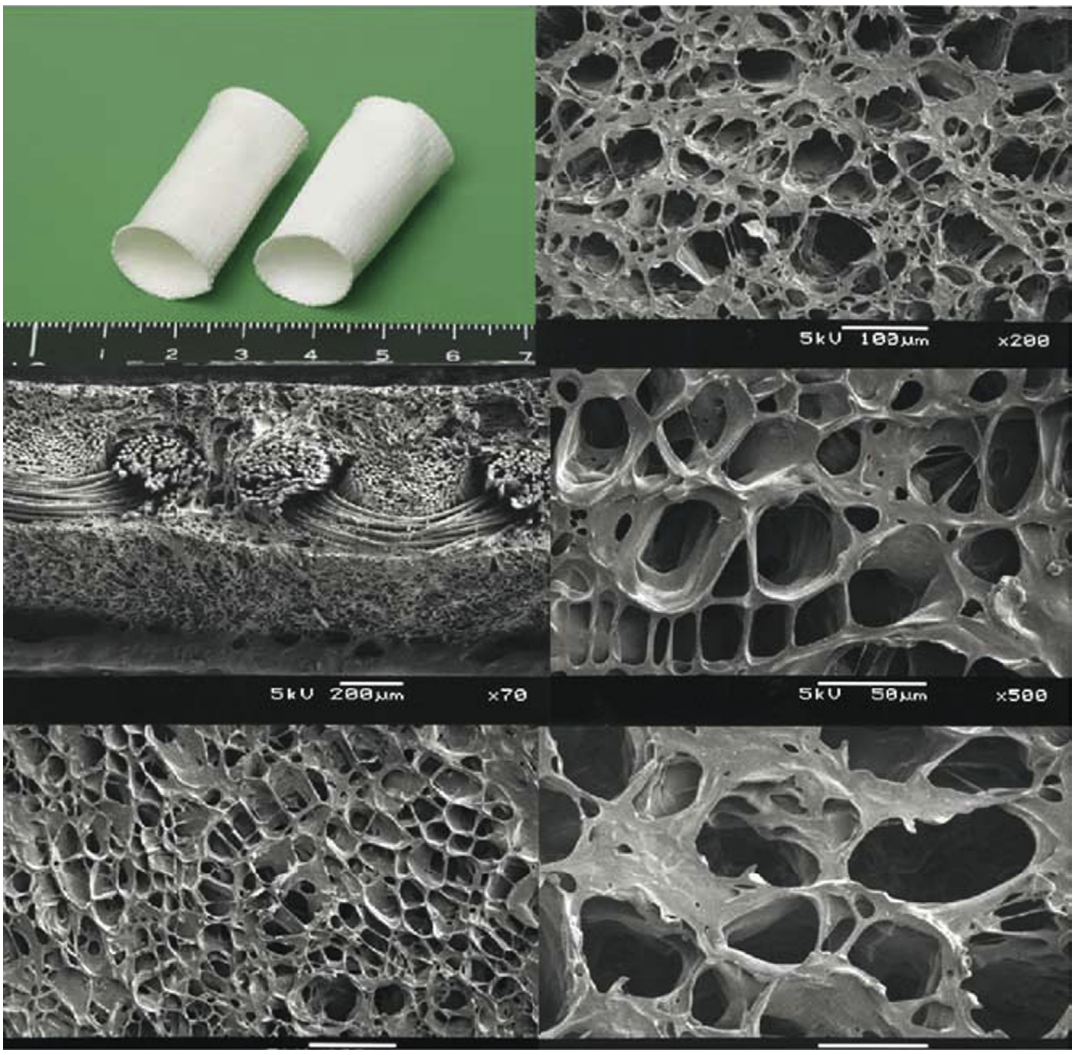

FIGURE 80.1

Macroscopic and scanning electromicroscopic appearance of biodegradable scaffolds used for cardiovascular tissue engineered conduits. The scaffolds are based on a copolymer of lactide acid and polycaprolactone. *(From Shin'oka et al. (2005).)*

Recently, clinical reports from Germany have demonstrated some success with tissue-engineered heart values when implanted into the lower pressure, right ventricular outflow tract. The rationale for tissue engineering of heart valves is similar to that for pediatric cardiovascular conduits – to provide an implant that has long-term durability and growth potential while avoiding thromboembolic complications. In a study of two pediatric patients, a tissue-engineered pulmonary valve was created following decellularization of a human donor valve with trypsin/EDTA with subsequent seeding using autologous endothelial progenitor cells isolated from peripheral blood [22]. The construct had been preconditioned for up to 21 days in a bioreactor prior to implantation. After a mean follow-up of 3.5 years, the tissue-engineered valves functioned well and seemed to have increased in dimensions over time based on echocardiography, in parallel with the overall somatic growth of the child. In a separate study of 11 patients, autologous endothelial progenitor cells were isolated from forearm vein and were used to seed valve allografts with good long-term results at 10 years follow-up [23]. Since most conventional unseeded allografts tend to fail between 15 and 20 years after implantation, longer follow-up will be required to ultimately define the utility of tissue-engineered heart valves in cardiac surgery.

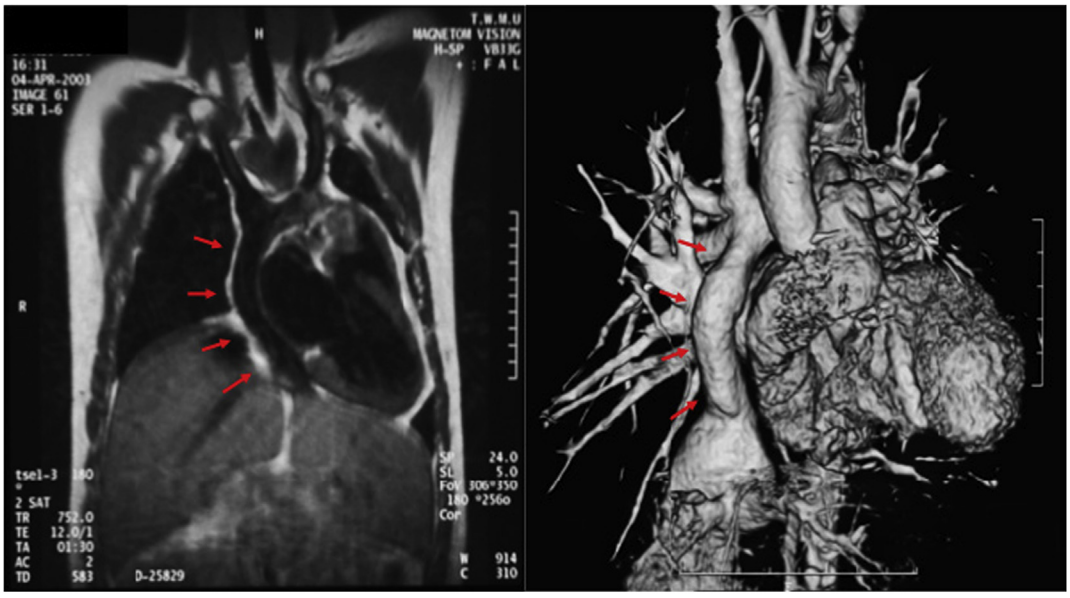

FIGURE 80.2

Growth potential of human tissue-engineered vascular grafts. (a) Magnetic resonance image (MRI) 9 months following implantation. (b) Three-dimensional computed tomography angiogram of an engineered graft one year after implantation. Red arrows indicate location of the implant. *(From Duncan and Breuer (2011).)*

VASCULAR REPLACEMENT

Tissue-engineered small-diameter conduits capable of withstanding arterial blood pressures are of great interest to vascular surgery, largely because of the epidemic of atherosclerotic peripheral vascular disease in the United States and other developed countries [24]. Unfortunately, the use of such conduits has yet to achieve widespread success in humans thus far. This has been due to a variety of factors, including the relatively easy availability of autologous grafts, usually taken from the saphenous vein or radial artery. As a result, the need for an alternative vascular conduit capable of long-term function has not been as compelling for most vascular surgical applications. Nevertheless, certain patients may be ideal candidates for a tissue-engineering approach, either because of the lack of adequate donor tissue, or because of a prior history of graft thrombosis. In a recently completed adult trial, vascular conduits were investigated as arteriovenous shunts for dialysis in patients with end-stage renal disease (Cytograft Tissue Engineering, Novato, CA) [11]. In that study, autologous fibroblasts and endothelial cells were isolated from skin and superficial vein biopsies, respectively. Fibroblast sheets were then wrapped around stainless steel mandrels (4–8 mm diameter) and matured for 10 weeks in culture. The endothelial cells were then seeded into the lumen prior to implantation. Despite early success following implantation in a high-risk population, graft patency of these tissue-engineered vessels at three months was only 60%, implying that immunogenicity and thrombogenicity have not been completely eliminated with the use of autologous endothelial cells. Another caveat of this approach has been the extended production time required to fabricate these grafts (over seven months).

Airway reconstruction

Despite the relative rarity of surgical diseases involving airways, tissue-engineered airway conduits have been of considerable interest for the treatment of fetal, neonatal, and adult tracheal and other airway anomalies for well over a decade [25–29]. Currently, tracheal reconstruction, except for primary resection, often leads to suboptimal functional results

and is associated with substantial morbidity and mortality. Although the trachea appears to resemble a simple hollow conduit at first glance, its structure and biomechanical characteristics are actually fairly complex and demanding. The ideal tissue-engineered substitute needs to have a unique combination of properties, including being airtight, non-immunogenic, non-collapsible, well-vascularized, well-epithelialized, and conducive to sustained chondrogenesis [30].

Based on previous work done primarily in large animal models, the early clinical experience with tissue-engineered tracheas has been arguably promising. To date, a number of patients, mostly in Europe have undergone tracheobronchial replacement with a cellularized tissue-engineered construct without the need for immunosuppression, with interesting results [28]. Both decellularized airway and synthetic scaffolds have been used, typically seeded with autologous bronchial epithelial cells and bone marrow-derived mesenchymal stem cells and/or differentiated chondrocytes.

Like many of the vascular conduits implanted into humans, a limitation of the current approach in tracheal engineering has been the extended length of time required to fabricate these constructs. To address this problem, more recent techniques have attempted to streamline this process by using nano-composite polymer and growth factor-induced endogenous stem cell mobilization to enhance engraftment and remodeling *in vivo* [31]. However, regardless of the approach, long-term follow-up, a better understanding of how the airway changes and remodels over time *in vivo*, along with comparisons in controlled Phase III trials will be essential before more widespread application of this technology can be justified.

Urinary tract repair

Traditionally, complex urologic reconstruction of the bladder, ureter, and urethra has relied on the use of heterotopic autologous grafts such as stomach, intestine, or colon. None of these structures constitute ideal urologic replacements as they lack urothelium and are associated with significant morbidity, including urolithiasis, metabolic disturbances, and malignant degeneration. As a result, investigators have long been evaluating tissue-engineering approaches for urologic reconstruction, leading up to some clinical experience.

The early clinical applications of tissue engineering in urology focused on bladder augmentation (cystoplasty). This procedure is commonly indicated for high-pressure, low compliant bladders secondary to spina bifida, spinal cord injury, or urge incontinence. Although anti-cholinergic medication and intermittent bladder catheterization can be effective, surgical reconstruction with intestine remains the current standard in severe cases. To date, tissue engineered bladders, referred to as Neo-Bladder Augment™ (Tengion, East Norriton, PA), have been implanted into several myelomeningocele (MMC) children with end-stage bladder disease, albeit only in a Phase I trial. In a report by Atala and colleagues, patients underwent a bladder biopsy ($1-2$ cm^2) through a small suprapubic incision; the muscle and urothelial layers were separated and expanded *ex vivo* in the laboratory [10]. After approximately two months, scaffolds composed of bladder submucosa with or without polyglycolic acid were seeded with both cell types and sutured to the dome of the bladder three to four days later. Post-operatively, bladder capacity seemed increased based on urodynamic studies at a mean follow-up of 46 months. Despite these early feasibility results, Tengion abandoned plans for Phase II trials and further work in 2011 based on a variety of factors, including limited to no clinical efficacy and the occurrence of serious adverse events. The trial was also hampered by the lack of a control group (i.e., standard enterocystoplasty), as well as the absence of data on innervation, in part because of the underlying voiding dysfunction within the MMC population.

In contrast to bladder augmentation, one area of urologic tissue engineering that has received considerable attention in recent years is urinary diversion for bladder cancer [32]. Bladder cancer is one of the most common cancers worldwide with over 20,000 urinary diversions

performed in the United States and Europe annually. The current standard for urinary diversion uses a piece of intestine to transport urine through a hole in the abdominal wall into a standard ostomy bag. As of early 2012, Phase I clinical trials at five US medical centers were ongoing for Tengion's tissue-engineered urinary conduit (Neo-Urinary Conduit™) in patients requiring urinary diversion following cystectomy for bladder cancer. Instead of using a segment of ileum, Neo-Urinary Conduit™ carries urine from the ureters to the skin surface using a polylacetate glycolate scaffold seeded with autologous smooth muscle cells isolated from autologous abdominal fat.

Similar techniques have been used to implant other tissue-engineered urological structures in humans, including tubularized conduits for posterior injuries of the membranous urethra secondary to trauma. In a Mexican study involving five boys, polyglycolic acid-based cellularized constructs were used to repair 5 cm long urethral injuries [33]. Further controlled analyses are needed to determine the role of precise tissue-engineered urethras as an alternative to buccal mucosa or skin in patients requiring urethroplasty. As it is the case in virtually all other reports involving clinical application of engineered grafts to date, the clinical experience with tissue engineering in urology lacks trials with suitable controls, and none of the products are approved by the FDA as of this writing.

CONCLUSIONS

Based on the classic open systems tissue-engineering paradigm of cells seeded onto biodegradable scaffolds, clinical application of engineered blood vessels, airways, heart valves, urinary tract, and other tissues is no longer considered fiction. A graduated approach, based on extensive background work by multiple disciplines testing these products in both small and large animal models, has enabled this technology to reach the bedside for selected patients with difficult problems requiring surgical reconstruction. However, much like the early clinical experience with human organ transplantation, the enthusiasm and excitement over the clinical possibilities uncovered by tissue engineering should be tempered until significant advancements, both in the laboratory and at the bedside, demonstrate better results in carefully controlled studies well beyond short descriptive case series or mere anecdotal data. The transition of many of the current approaches to tissue engineering towards sizable clinical scale remains to be seen, as the notion that off-the-shelf, fully cellularized 'products' can replace faulty tissues on a routine basis is still, for the most part, a dream. Perhaps this should not come as a surprise as this is still a relatively immature field.

Although the state-of-art in experimental tissue engineering continues to change rapidly, significant refinements in laboratory protocols and techniques are needed if further progress in the clinical arena is to be achieved. Persisting challenges including enhancements in scaffold materials and design, along with headway in xeno-free cell expansion, among other technical advances, will be essential to allow for optimal phenotypic control of the final tissue-engineered product. Furthermore, defining the ideal target population that can readily and safely benefit from tissue-engineering strategies in significant numbers remains challenging, to some extent due to the progress constantly taking place in other approaches to surgical reconstruction. Nevertheless, it is reasonable to hope that continued efforts in this vibrant, ever expanding multidisciplinary endeavor should contribute to broaden its impact, so as to eventually reach a large number of patients in need of tissue repair and/or replacement.

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CHAPTER 81

Tissue-Engineered Skin Products

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INTRODUCTION

Skin tissue engineering was perceived as the simplest tissue-engineering application and was the earliest to be explored. The culture of fibroblasts, derived from skin biopsies, was established early. The cells were comparatively easy to grow and were used to develop many of the early systems of tissue culture. The culture of keratinocytes was achieved by Rheinwald and Green in the late 1970s [1], and using a different system, by Karasek [2]. Skin biology was second only to immunology in the application of the techniques of molecular and cell biology to medical problems, which has led to great advances in the understanding of the physiology of skin. Examples of methods that have been applied include the use of classical techniques of molecular and cell biology and transgenic animals.

The skin acts as a barrier between internal structures and the external environment. In the 1980s it was realized that the skin, in addition to acting as a barrier, also has significant interaction with both the innate and the adaptive immune systems [3,4]. It is thus much more than a passive barrier and actively recruits defensive mechanisms to protect against infection, colonization by microorganisms and other external noxious entities.

With its long history of development, tissue-engineered skin substitutes have achieved market exposure and have some of the most extensive experience with the application of such products in a therapeutic setting. From its inception in 1979 [5], engineered skin was seen as potentially a clinical product and trials started in the 1980s. Preclinical testing was inaugurated, and demonstrated that fibroblasts were apparently not rejected and persisted for a considerable period in experimental animals [6–8]. This led to human clinical trials in the late 1980s and early 1990s. Tissue-engineered skin products first gained regulatory approval and appeared on the market in 1997. The first was TransCyte (Advanced Tissue Sciences, which was a non-viable burn product produced by tissue-engineering techniques). It was followed by Apligraf (Organogenesis) in 1998, the first tissue-engineered, viable, organotypic product, Dermagraft in 2000 and Orcell in 2001. At the same time, many applications of such products for *in vitro* testing applications and models of skin physiology were developed.

Much of the discussion in this article is based on experience with Dermagraft and TransCyte. Both products have a similar basis in growth of fibroblasts on three-dimensional scaffolds. The systems, however, differ substantially in detail.

Dermagraft is grown on 2 inch × 3 inch sheets of a knitted polylactide/glycolide scaffold under static conditions in a bag bioreactor. Following seeding, the cultures are refed with medium every few days until harvest after about two weeks. Initially, the fibroblasts proliferate rapidly,

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much as in monolayer, but as they become confluent, at about 8–10 days, they lay down increasing amounts of extracellular matrix. At harvest, medium is replaced with cryoprotectant, the bioreactor is welded closed without exposure of the product to the environment, boxed and frozen under controlled conditions. Dermagraft is stored below −65°C for up to six months. During the first month, sterility and analytical testing is performed and the paperwork completed to allow release of the product by the Quality Assurance Department. On thawing, which is performed by the end user, the cells of Dermagraft show 50–80% viability.

TransCyte was grown using a similar process on two 5 inch by 7 inch sheets of a knitted nylon (non-degradable) scaffold with a silastic backing in a continuous flow, hard bioreactor. This system has advantages in the ease of taking medium samples and in slightly superior cell growth but it adds considerably to the complexity of the system. At harvest, the cultures were rinsed, the bioreactors sealed from the system by welding, packaged and frozen without any care taken to maintain viability.

The three-dimensional structures formed by the cells under these conditions consist of cells embedded in extracellular matrix that they, themselves, have secreted. It comprises a complex series of molecules. The process of secretion may be thought of as formation of a foreign body capsule *in vitro*.

TYPES OF THERAPEUTIC TISSUE-ENGINEERED SKIN PRODUCTS

The development of tissue-engineered skin has taken two fundamentally different approaches using alternative methods for the three-dimensional culture of fibroblasts and a third which has intermediate characteristics. One is the use of fibroblasts suspended in collagen gel, in which the gel acted as a substrate for the growth of keratinocytes and forms a structure that has some resemblance to human skin. The second involves culture on a three-dimensional non-biologically derived, polymeric scaffold. Fibroblasts are responsive to signals from their environment and respond in quite different ways to the two types of culture. In a third system, fibroblasts are grown on a collagen sponge in serum-containing medium. Since collagen adsorbs fibronectin and vitronectin, this system initially shows cell adhesion to both collagen, fibronectin and vitronectin and the cultures show intermediate properties.

In collagen gel suspension, fibroblasts are surrounded by collagen, and respond through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins which are collagen receptors, by becoming quiescent and non-proliferative [9]. Characteristically, the collagen gel is contracted by the fibroblasts to less than 10% of its original volume in about 20 hrs, through the $\alpha_1\beta_1$ integrin. At the same time, many genes, notably those for collagen type I and proliferation, are repressed [10] and the cells show mechanosensitivity [11]. The collagen repression is mediated through the $\alpha_1\beta_1$ integrin [9]. If the gels are mechanically stressed, usually by preventing contraction with a ring, many genes, including collagen type I and proliferation related genes are somewhat induced relative to contracting gel fibroblasts, although not to the level of monolayer fibroblasts [11]. The cells also show a substantial upregulation of genes associated with inflammation such as the genes for IL-1, IL-6 and cyclooxygenase [12].

When grown on scaffolds, fibroblasts proliferate rapidly and then deposit large amounts of extracellular matrix. Initially, the conditions on the scaffold are not dissimilar from monolayer culture. The scaffold adsorbs proteins from the serum, which is a component of the medium still used for fibroblast culture. The proteins include fibronectin and vitronectin as in monolayer culture [13]. However, as the cells reach higher densities and the fibroblasts cease to proliferate, they proceed to lay down extracellular matrix at rates approaching their own weight per day. The matrix is loose connective tissue, much like the provisional matrix of granulation tissue. The cells are clearly very active and induce several genes, among the most notable of which are neutrophil chemoattractant chemokines, CXCL1, CXCL5, CXCL6 and CXCL8. They also induce the expression of IL-6 and IL-11. The conclusion from these

observations is that the fibroblasts act to recruit neutrophil granulocyte components of the innate immune system, which is important in responding to the most likely type of environmental insult to which the dermis is liable, physical injury followed by bacterial colonization.

COMPONENTS OF TISSUE-ENGINEERED SKIN GRAFTS AS RELATED TO FUNCTION

Scaffold

Several types of scaffold have been employed for tissue-engineered skin implants. They include collagen gels, knitted polylactate/glycolate fabrics (PLGA) and collagen sponges. As discussed above, the scaffold has profound effects on gene expression and the function of the fibroblasts.

The Dermagraft and TransCyte processes used scaffolds obtained as finished commercial products from other companies, which already had regulatory approval. While this simplified the development of the products, it ultimately proved an expensive decision. A solution to this is to obtain a second source of raw material that is not a finished product and establish its equivalence.

A further point here is that, while the use of already approved products as scaffolds may be an easy initial path, the available materials may not be ideal for the particular application envisaged. There remains a need to expand the range of scaffolds available for commercial tissue engineering. An example is enzymatically, rather than spontaneously, degrading scaffolds.

An interesting direction is the development of scaffolds incorporating a vascular capability. Revascularization of skin substitutes is a limitation in their application and the ability to be perfused by the host circulatory system would be a major advance. Scaffold with this type of capability may be made by decellularizing a perfused tissue and then using the extracellular matrix structure [14].

Keratinocytes

Keratinocytes generate the impervious surface of the skin (the stratum corneum) and also have been thought of as having a major role in defense against microbial colonization as activators of immune responses. As a consequence, keratinocytes have been included either alone or in combination with other components in many skin implants [15]. Their role in forming a physical barrier is important, but they also have the ability to produce antimicrobial peptides, such as β-defensin, psoriasin and cathelicidin [16,17] and a wide variety of cytokines capable of activating immune responses [18,19]. The epidermis contains antigen presenting cells that are capable of activating T-lymphocytes under suitable conditions to both cell mediated and humoral adaptive responses. This is largely a function of Langerhans cells, but it is possible that the keratinocytes also take part under special conditions. It has been argued that the application of a bag containing keratinocytes to a wound, that would be permeable to proteins, but not to cells, may be beneficial without incorporation of the cells into the patient (United States Patent 5972332).

It is notable that the keratinocytes show a higher expression of the fibroblast stimulating platelet-derived growth factor PDGF A chain gene than do fibroblasts. This may constitute part of the secretion of reciprocal paracrine growth factors by the epidermis and the dermis [20]. Such a pathway has been described by Fusenig, involving IL-1α secretion by keratinocytes, which stimulates secretion of FGF-7 (keratinocyte growth factor 1) and granulocyte-macrophage stimulating factor (GMCSF) that promote keratinocyte proliferation [21]. Such interactions may well be of importance during wound repair when proliferation of both types of cells is important [22].

In practice, allogeneic keratinocytes in skin implants appear to remain for some weeks and then disappear. This has been attributed to immunological rejection, but this is not clear

as keratinocytes, cultured to the numbers required for a skin implant, consist almost entirely of transiently amplifying cells that follow a differentiation pathway that leads to a modified form of apoptosis. They may, thus, be expected to be lost from the implant without the intervention of the immune system.

An approach that has been explored for increasing the coverage and survival of grafted epidermis is to mix allogeneic with autologous keratinocytes. This permits a comparatively small number of autologous keratinocytes, without extensive expansion, to cover a large area [23]. The allogeneic cells provide initial coverage, while the autologous population, which retains stem-like cells, provides the persistence. Most of the allogeneic cells are eventually lost, but the epidermis remains.

An alternative application of keratinocytes to wounds by spraying has been explored. The cells survive the experience and the technique provides a very simple and convenient way of applying them [24]. It is possible to envisage a system spraying largely allogeneic keratinocytes together with a fresh autologous keratinocyte suspension isolated intraoperatively from skin or scalp [25].

Fibroblasts

Fibroblasts are the major producers of extracellular matrix, discussed below. They are not antigen presenting cells and have been regarded as not having a major role in interactions with the immune system. However, their ability to secrete neutrophil attractant chemokines, CXCL-1, CXCL-4, CXCL-5 and CXCL-8, and the cytokines IL-6 and IL-11, discussed below, suggests that they may have a more important role in activating innate immune responses.

Fibroblasts do not appear to produce significant quantities of defensins, cathelicidin or other antimicrobial peptides. Their major antimicrobial activity appears to be through recruiting neutrophils.

Extracellular matrix

Fibroblasts express a large array of extracellular matrix genes. Proteins giving the 30 highest signals on Illumina expression arrays are listed in Table 81.1. Expressed genes represent collagens, types 1, 3, 5, 6, proteoglycans (decorin, lumican), thrombospondins, fibulins, fibronectin, tenascin C, metalloprotease inhibitors, lysyl oxidase, tissue factor inhibitor and Secreted protein, acidic, cysteine-rich (SPARC). It also includes matrix-bound growth factors, such as FGF-2 and TGF-β. The matrix includes multiple kinds of integrin ligand, including those for integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$ and proteins that are known to modify adhesion. It provides complex stimulation that, since it is secreted by fibroblasts, is presumably appropriate for cell migration. In systems where fibroblasts are cast in collagen gels, the initial signal is through the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin and that remains the dominant stimulus until the cells elaborate other signaling molecules.

Subcutaneous fat

Subcutaneous fat has received little attention thus far in the engineering of skin. However, it has been shown to undergo major structural changes during the hair cycle in the mouse and is a highly vascular organ. Fat-derived stem cells have been used successfully in the production of skin substitutes and may provide advantages over fibroblasts [26].

Components of the immune system

Inclusion of Langerhans cells has been accomplished in test systems but has not been applied to therapeutic products.

TABLE 81.1 Extracellular matrix proteins secreted by fibroblasts in three-dimensional culture, as a percentage of the protein giving the highest signal on an illumina expression array (SPARC)

Extracellular matrix protein	Signal as percent of SPARC
Collagen, type I, alpha 1	18.83%
Collagen, type I, alpha 2	36.51%
Collagen, type III, alpha 1	30.96%
Collagen, type IV, alpha 1	4.67%
Collagen, type V, alpha 1	12.54%
Collagen, type V, alpha 2	11.85%
Collagen, type VI, alpha 1	17.60%
Collagen, type VI, alpha 2	5.66%
Collagen, type VI, alpha 3	16.85%
Collagen, type VIII, alpha 1	6.61%
Collagen, type XVI, alpha 1	6.20%
Decorin	15.13%
Biglycan	5.92%
Lumican	42.19%
Secreted protein, acidic, cysteine-rich (SPARC, osteonectin)	100.00%
Testican	7.49%
Spondin 2	7.83%
Fibrillin 1	10.24%
Elastin microfibril interfacer 1	5.72%
Microfibrillar-associated protein 2	0.29%
Microfibrillar-associated protein 4	8.34%
Fibulin 1	0.27%
Fibulin 2	7.77%
Fibulin 2	7.77%
EGF-containing fibulin-like extracellular matrix protein 2	6.86%
Fibronectin 1	0.39%
Tenascin C (hexabrachion)	10.98%
Thrombospondin 1	30.53%
Thrombospondin 2	8.50%
Tissue factor pathway inhibitor 2	21.16%
Laminin, gamma 1	12.91%

Melanocytes

Other cells of the epidermis have been incorporated into test systems and have also been considered for transplant into epidermal tissue-engineered therapeutic products. An example is the inclusion of melanocytes as a treatment for vitiligo, which is a clinical problem in countries such as India. Systems including melanocytes have been used extensively in test systems for ultraviolet radiation (UV) protective preparations.

Adnexal structures

The self assembly of hair follicles and their use as a therapeutic product is discussed later in this chapter. Inclusion of sweat glands has been explored, but they have not yet been included in tissue-engineered constructs. The early fear that patients treated with keratinocyte allografts might have problems with temperature control has not eventuated.

COMMERCIAL PRODUCTION OF TISSUE-ENGINEERED SKIN PRODUCTS

Commercial production of tissue-engineered skin products takes place in a precisely controlled and highly regulated environment, which imposes many constraints if the process

is to be successful. These will be discussed under a series of headings, dealing with physical constraints, such as bioreactor design, growth system design, cell sources and with broader considerations of the nature of preclinical and clinical assessment, government regulation, reimbursement and the market place. It is central to a commercial enterprise that it ultimately make a profit if it is to be successful. Since tissue engineering is inherently an expensive undertaking, minimizing costs at all stages is essential.

Regulation

Regulation of tissue-engineered products by the FDA or other regulatory authorities is still developing. Since commercial products have only been available for less than 20 years and since they are very different from other medical products, establishing a regulatory pathway has been complex. Tissue-engineered skin substitutes do not fit readily into any of the standard FDA categories; drugs Center for Drug Evaluation and Research (CDER), biologics Center for Biologics Evaluation and Research (CBER) or devices Center for Devices and Radiological Health (CDRH). Classification is important as requirements, criteria and guidelines differ substantially between the centers. Skin implants were originally considered as dermal or skin replacements and classified as devices. As it became evident that growth factor activity played a part in their mode of action, considerations appropriate to biologics played an increasing role. There is now within FDA an Office of Combination Products responsible for assembling reviewers from different FDA centers to form a team to review such products. While tissue-engineered skin substitutes generally have more biochemical activity than would be expected of a pure device, they do not fit easily as a biologic. For instance, the concept of 'dose' is not straightforward. If a component of the activity of these materials is through secretion of growth factors, growth factor production would seem to be a reasonable release criterion. However, unlike a purified growth factor preparation, these products are living, and the secretion of growth factors may vary depending on conditions. The cells adjust their cytokine output to the environment in the wound bed. It may be possible to devise conditions under which secretion of a growth factor (for instance, vascular endothelial growth factor (VEGF)) is maximal (with optimal platelet-derived growth factor (PDGF) stimulation) and determine output under those conditions. To avoid such problems, a 'dose' of Dermagraft has been defined as a piece.

Also, at this stage, the composition of tissue-engineered skin can only be determined to a limited extent. Usually, it is desirable that a biologic contains well-defined constituents, including an active ingredient at a known dose and excipients. Thus far, studied components of skin implants have included those that are known and, to some degree understood, from other work. There is no comprehensive identification of constituents, and several that appear on expression arrays, such as lumican and collagen type 6, which are prominent, have received inadequate attention.

However, despite these difficulties, the FDA is placing increasing emphasis on the biological aspects of skin substitutes and overseeing them as biologics and as drugs.

Product development

Development of a commercial product should start with a Product Concept from which a Design Requirements document is developed. As an example, Table 81.2 shows the product concepts for Dermagraft and TransCyte. The product is then developed to meet the design requirements by a series of hierarchical, more and more detailed design processes that ultimately comprise the Design Master File. The requirements can be divided into groups: in each case, the first statement is concerned with the therapeutic purpose of the product, the next three describe intrinsic characteristics of the product and the last three are concerned with

TABLE 81.2 Design concepts for dermagraft and TransCyte

Dermagraft	TransCyte
Dermal replacement, later as a cytokine factory	Transitional covering for third degree burns to replace cadaveric skin
Allogeneic	Allogeneic
Viable product	Non-viable
No sterile fill: *i.e.*, bioreactor would be package	No sterile fill: i.e., bioreactor would be package
Long shelf life (~6 months)	Long shelf life
Suitable size	Suitable size (from Biobrane size)
User friendly presentation – modified bag	User friendly presentation – clamshell bioreactor

practical issues of importance to the final user. All these factors need to be considered from the earliest stages of development.

Overall concept

The initial application for Dermagraft was a dermal replacement for burn patients. Initial clinical application indicated that it provided little benefit and was slightly deleterious. As trials for chronic wound applications progressed, and it became evident that it was efficacious, the concept of Dermagraft as a cytokine source replaced the notion of a dermal replacement.

TransCyte, a replacement for cadaveric skin for covering third degree burns after debridement, was seen as a means to overcome problems with rejection and potentially with disease transmission.

Allogeneic cell source

The second item in both product concepts was that the products would be allogeneic. The decision whether a product will be allogeneic or autologous is fundamental to the entire development process. While not impossible, the development of a commercially successful (profitable), autologous, tissue-engineered product is very difficult. It requires separate, independent, tissue culture suites (hood, incubator, centrifuge, microscope and air conditioning), which will be occupied by a product for a single individual for the entire period of culture. It is a service industry, with complex logistics and high cost. As an alternative, it would be possible, in principle, to construct an automatic machine that would accept a patient's tissue and produce the tissue-engineered construct in the hospital with minimal intervention. The one advantage to an autologous process from a commercial point of view is the comparatively low requirement for safety testing and the lack of immunological rejection by the patient.

Allogeneic tissue engineering has advantages in using only one tissue culture facility per product type, allowing economies of scale and straightforward logistics. The major disadvantages are the very large amount of safety testing required for the master cell banks and the potential for immunological rejection. The first of these disadvantages is ameliorated by the infrequency of making master cell banks. In the case of Dermagraft and TransCyte, one master cell was used for more than 20 years, so the investment could be amortized over a long period. Required testing is determined by regulatory authorities and may be increased from time to time as new risks become known. It includes testing of the donor and, in the case of fibroblasts, the donor's mother for major human pathogens (Human Immunodeficiency Virus (HIV), hepatitis, cytomegalovirus (CMV), etc.). The cells isolated from the donor are tested for the major human pathogens, xenogeneic pathogens (which might be introduced from the reagents used in tissue culture), karyotype, identity (isoenzyme, variable number of tandem repeats (VNTR), single nucleotide polymorphisms (SNP), etc.), tumorigenicity, latent viruses,

etc. This is tested both on the initial Master Cell Bank and again on cells reisolated from final product (the End-of-Production Cell Bank).

Viability of product and no sterile fill

The viability of the product depends on its intended function and differs for Dermagraft and TransCyte. As discussed below, to package sterile material is a matter of some difficulty and it was decided, in this case, to avoid the issue. The bioreactor in which the tissue was grown was designed to form part of the final packaging.

Shelf life

Extended stasis preservation is important for commercial products including tissue-engineered products. It allows time for quality control testing, for off-the-shelf availability, and for distribution. In addition, the Dermagraft and TransCyte processes take 12 weeks from thawing a vial from the Manufacturer's Working Cell Bank (MWCB), cell expansion, three-dimensional growth and Quality Control (QC) testing. The difficulty of predicting the market so far ahead so as to minimize waste is greatly aided by extended shelf life. This was achieved through cryopreservation at −70°C. Other systems for long-term stabilization of tissue-engineered constructs have been explored with varying degrees of success, including trehalose, DMSO and 3-O-methyl glucose [27–29].

Size, user convenience

The remaining items in the product concept are related to convenience of use by the physician applying the product. This is an important item for a commercial product and needs much work in focus groups and thought. Thawing the product is already a major issue, requiring time and, in the case of TransCyte, careful estimation of the amount required to avoid wasting expensive material or having to wait for a piece to be thawed. The Dermagraft bioreactor was designed so that it would open easily and the tissue would be positioned (through the use of a Z-weld that attached opposite sides of the scaffold to opposite walls of the bioreactor) so as to be easily rinsed prior to use. The bioreactor is translucent to aid in marking out the piece to be cut out for implantation.

THE MANUFACTURE OF DERMAGRAFT AND TRANSCYTE

Overall schemata for the manufacture of Dermagraft and TransCyte are illustrated in Fig. 81.1. As can be seen, as much of the two processes as possible are identical. This economizes resources and reduces cost. In this case, the Master Cell Bank (MCB), MWCB, the cell expansion process and much of the release testing, are all in common. In addition, the clean room facility, all its ancillary equipment and monitoring are the same.

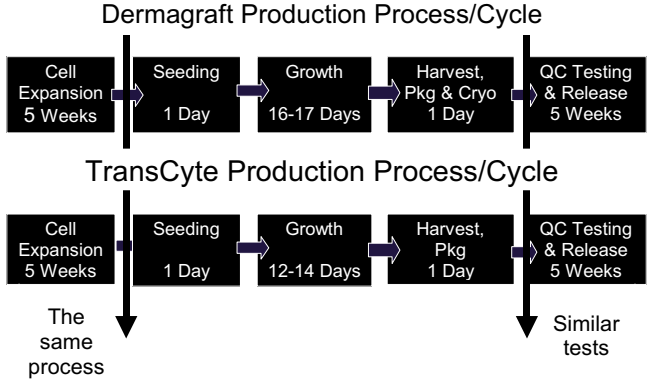

FIGURE 81.1
Overall comparison of the Dermagraft and TransCyte processes.

Cells

Several times per week, fibroblasts used in manufacture are recovered from liquid nitrogen storage (the MWCB) and expanded in roller bottles from fifth to eighth passage. This is a conventional procedure, but may not be ideal. Fibroblasts for Apligraf are expanded on beads in a mixed bioreactor. It has become evident that the proliferation of fibroblasts is improved at low oxygen (2–6%) [30] and, at this oxygen concentration, the development of a senescent phenotype may be reduced [31–33]. A tank bioreactor lends itself to active control of parameters such as oxygen tension and pH and is worth exploring before a system gets fixed by regulation. The cells should ideally be grown just to the beginning of stationary phase. Further incubation seems to be slightly deleterious.

Expansion of fibroblasts for Dermagraft and TransCyte manufacture was performed in roller bottles. These are well established for production of large numbers of cells under ambient conditions. Various other systems have been devised that may provide superior performance and greater control of the conditions of growth [34,35].

Medium

The Dermagraft and TransCyte systems are grown in Dulbecco's-modified Eagle medium, supplemented with 10% calf serum, non-essential amino acids and glutamine. All tissue culture beyond initial cell isolation from the foreskin is free of antimicrobial agents. Antimicrobial agents might hide otherwise significant contamination, which might only become evident in therapeutic application.

With the increased concern with the transmission of prion diseases, there has been great interest in developing serum-free systems. While serum-free media are available for keratinocytes, it has been found difficult to achieve such systems for fibroblasts. Serum-free fibroblasts media are commercially available, but while these have been successful in the research laboratory, transferring them to scaled-up systems and manufacturing has proved elusive. The major issue is that the cells do not grow to the extent required apparently because, early on, they acquire a senescent phenotype. It was early established that serial subculture of fibroblasts leads to senescence after about 60–80 population doublings. While this can be attained in serum-containing medium, experience with serum-free media have indicated a lifespan of about nine doublings. Since Dermagraft is made at about the 28th generation, which is about 10 doublings from the MWCB, 19 from the MCB, and implanted fibroblasts should not be too close to senescence, serum-free media do not provide sufficient proliferation potential.

The alternative solution to serum-free medium is to take extreme caution in the source and treatment of serum. By regulation, all materials used in manufacture must be traceable to their sources, but in addition, bovine serum is obtained only from countries that are free from bovine spongiform encephalopathy (BSE), which include Australia and New Zealand, from closed herds with a known pedigree history that includes no animals from outside the country. Serum is also irradiated sufficiently to eliminate possible viral contamination.

Bioreactor design

Many of the considerations of bioreactor design involve questions of mass transport, conditions under which cells are able to grow and so forth, much as in non-commercial tissue-engineering systems. A second series of considerations deals with the large-scale production process. These include ease of scale-up, ease of handling, minimal footprint and maximal automation. In addition, the use of the product by a community not experienced with products of this type is also important. This leads to features to make the product easy for the end user to work with and apply.

The bioreactor used for the growth of Dermagraft consists of a bag with eight cavities welded into it, each of which contains a sheet of the scaffold. Such bags are attached in groups of 12 to

manifolds, with a solid support, for cell, medium or cryopreservative addition, to form a system. Several systems may be connected and all fed at the same time to form a lot, which is the unit of manufacture. The bag reactors, systems and even lots may be assembled under clean, but not necessarily sterile conditions and sterilized by 25–43 Grays gamma radiation prior to seeding. From this point, the process only requires nine sterile connections, and is substantially automatic.

The TransCyte bioreactor systems are similarly assembled under clean conditions, complete with all associated tubing, and attached in groups of 12 to a manifold to form a system. This entire assembly is then sterilized by gamma radiation as described above. Several systems can then be connected to form a lot. In this case, the inlets to each bioreactor are individually passed through a peristaltic pump to provide even flow. (Attempts to use constrictions to control flow were found to be inferior.) Again, as with the Dermagraft system, once set up and seeded, the TransCyte system is substantially automatic.

Automation in tissue-engineering systems is an important feature. Apart from reducing labor costs, automation greatly reduces the number of errors that have to be investigated, requiring time, effort and cost. While operator errors are generally less serious than machine failures, they are much more numerous and still require individual investigation. Backup systems can be installed to minimize the impact of machine failure.

THE DERMAGRAFT AND TRANSCYTE PRODUCTION PROCESSES

The production of Dermagraft and TransCyte started with the cryopreserved Master Cell Bank that was stored at passage three in liquid nitrogen. About four times per year, cells were taken from this bank and expanded in roller bottles to fifth passage when they were stored again as the MWCB. This was tested to ensure identity to the Master Cell Bank. Several times per week, cells were taken from the MWCB and expanded to eighth passage in roller bottles when they were seeded to the three-dimensional bioreactors. This process is conventional, large-scale tissue culture. It uses batch feeding and exposes the cells frequently to atmospheric oxygen. As discussed elsewhere in this article, fibroblasts grow better and senesce less rapidly in a lower oxygen environment.

On seeding, the bioreactors were manipulated to ensure even distribution of the cells. In the case of Dermagraft, the bioreactors were rolled; in the case of TransCyte, each side was seeded successively under static conditions. The medium was replaced one day after seeding and then the cells fed every 2–4 days until harvest.

The time of harvest was determined from the glucose metabolism of the cultures. In the case of TransCyte, glucose consumption and lactate production were determined daily and interpreted as notional ATP turnover, assuming a P:O ratio for mitochondrial oxidative phosphorylation of 3. The resulting values were correlated with the performance of the system relative to release specifications and appropriate target values selected. In the case of Dermagraft, there was a concern that lactate release from the degradation of the scaffold might interfere with the ATP turnover estimation, so the time of harvest was determined on cumulative glucose utilization alone.

At harvest of Dermagraft, medium was replaced by cryopreservative solution that consisted of 10% dimethylsulphoxide in phosphate buffered saline, supplemented with calf serum and the individual pieces of Dermagraft sealed, removed from the surrounding bag, packaged, cooled slowly at a rate that fell within design parameters and frozen. It was then stored in a freezer set to −75°C. The time between cryopreservative addition was at least 4 hr and was validated to a 12 hr delay. This time interval did not appear to affect the viability of the cells and it was conjectured that it allowed induction and synthesis of stress proteins in response to the high osmotic pressure.

Release specifications

The release specifications include sterility, for bacterial, fungi, mycoplasma, and endotoxins as well as for analytical criteria that ensure consistency of the product. Dermagraft uses four analytical release criteria, (3-(4,5-Dimethylthiazol-2-yl)-2-5-diphenyltetrazolium Bromide) (MTT) reductase activity before freezing (in-process MTT) and after thawing, collagen content by Direct Red binding [36] and DNA content. MTT reductase was used to determine the metabolic activity of the product, collagen as a surrogate for the amount of extracellular matrix and DNA content was used to establish adequate cellular content. MTT reduction was an assay that was established early in the development of Dermagraft. Since then, several other substrates measuring essentially the same activity have been developed, several of which are less toxic to the cells than MTT and can be used repeatedly. It would be wise in the development of a new product to examine these early and select a method carefully. MTT reductase is frequently viewed as measuring mitochondrial activity. However, 70% of the activity is extramitochondrial [37] and it is better regarded as a general measure of cellular activity. The MTT reductase assay was originally developed to evaluate toxicity or cell proliferation, where values obtained after treatment were compared with control values. In determining the metabolic activity of tissue-engineered products, this is not possible and a great deal of fruitless effort has been expended in trying to obtain a standard for the MTT assay. MTT determination before and after cryopreservation might be interpreted in the same manner as in toxicity assays. However, changes occur in the viability and metabolic activity of cells for up to three days after cryopreservation, due to necrosis, apoptosis and repair, so the MTT values are better regarded as independent absolute determinations rather than relative values.

In the case of TransCyte the microbiological criteria and the collagen and DNA determinations are similar to those for Dermagraft. However, as the cells are non-viable in the final TransCyte product, there is no MTT determination. Determination of MTT reductase prior to final freezing (in-process MTT) was replaced by release specifications applied to ATP consumption as described above.

Definition of specifications was initially within three standard deviations of the process performance. This was refined by clinical experience. In the case of MTT, it was evident during the clinical trial that the specifications needed to be refined, requiring a mid-trial correction and ultimately an additional clinical trial.

Distribution and cryopreservation

The use of −70°C cryostorage for Dermagraft was a compromise between an ideal solution and practicality and turned out to have several benefits. If the goal was 100% cell viability and indefinite shelf life, the ideal solution is likely to have been storage at liquid nitrogen temperatures, probably using a vitrification procedure in which high concentrations of cryoprotectant and very rapid cooling is used to achieve the formation of a glass with minimal ice crystallization. While the technical difficulties of achieving sufficiently rapid cooling of a therapeutically useful structure and problems with the brittleness of plastics at low temperatures can probably be overcome, the method would still entail transport at liquid nitrogen temperatures and safety issues. Cryopreservation at dry ice temperatures provided adequate shelf life and allowed shipping in a container capable of maintaining −65°C for 4 days. This allowed distribution throughout the world and would generally permit 2−3 days leeway for patient and physician scheduling at the site of use. If a center had a −70°C freezer, storage at this temperature would allow much longer shelf life at the treatment site.

In addition to logistical advantages, the induction of stress proteins by the cryopreservation procedure [38], combined with the suboptimal viability recovery meant that necrotic cell debris, including heat shock proteins, were released from lysed cells. Such proteins activate

macrophages through CD91 and toll-like receptors [39]. While such necrotic products are likely not to be scarce in a chronic wound, this property may have contributed to the efficacy of Dermagraft.

While discussing cryopreservation, an important point that has received little attention is the thawing process. In the case of Apligraf, the thawing process involved solutions that had to be replaced, requiring a procedure of several hours, which was performed at the company. This provided shelf life, but not off-the-shelf convenience, and required additional resources from the company. Therefore, the procedure was not continued. In the case of Dermagraft, thawing is performed in the physician's office, out of the control of the company.

During the controlled freezing process, after the majority of the osmotic water removal has occurred, the cell contents reach a eutectic and freeze. While most of the intracellular material forms a glassy state, small ice nuclei may be formed. The slow growth of these nuclei during −70°C storage is probably what limits shelf life. During thawing, conditions arise that allow the growth of ice crystals, the nuclei enlarge and, if they become big enough, will kill the cells. It is important to take the cells through this stage as rapidly as possible to minimize ice crystal growth. So rapid thawing, straight from the −70°C storage is critical. The procedure that was adopted involved a thawing tub with a thermometer, which could be filled with warm water from the tap, or a water bath at 37°C. A few seconds unprotected in ambient air was sufficient to reduce viability drastically.

Problems with commercial culture for tissue engineering

In large-scale tissue culture, procedures are followed that differ from those found in a research laboratory. As a result, problems have arisen from time to time that have no counterpart in culture on a smaller scale. Frequently, it is difficult to establish the cause unequivocally because of the scale and cost of the experiments that would be required. When such a problem occurs, it is usual to assemble all those with potentially valuable ideas, analyze the incident with respect to changes in procedure (new batches of medium or other raw materials, newly implemented changes, etc.) and prioritize possible solutions. Usually the most likely and cheapest suggestions are explored first. These tend to be implemented more or less at the same time and immediately. This means that, when the problem ceases, as it usually does, it is very difficult to determine which suggestion solved it. At best, the solution relies on a plausible rationale.

Examples of the causes of such occurrences include light damage to medium and deterioration of cell banks. It has been known for many years that medium is light sensitive and will degrade if left exposed to daylight or fluorescent light. However, such an event is never seen in the research laboratory, as medium is normally stored in a refrigerator in the dark. In commercial operation, it is sometimes necessary to make up medium a few weeks in advance and store it in a cold room. If the lights in the cold room are fluorescent and are left on, deterioration of medium will be observed. Indeed, the Dermagraft plant was illuminated with yellow light to avoid such problems.

In a research laboratory, cell stocks frozen in liquid nitrogen are manipulated rarely, a few times per year. In commercial scale tissue engineering, vials from the MWCB are required several times per week. While one or two vials are identified and removed, the remainder of the cell bank stock taken out of cryostorage is warming up. We noticed that, after some years of this, the fibroblasts could be shown to demonstrate a decreased lifespan. This was attributed to ice recrystallization during periodic warming to perhaps −120°C to −90°C during recovery of other vials. It is, thus, necessary to store the MCB independently of the MWCB and to devise a method of recovering selected vials with minimal disturbance to other vials.

CLINICAL TRIALS

The design and implementation of clinical trials is specialized and beyond the scope of this discussion. The process requires a great deal of time and expense. In the case of the CDRH division of the Food and Drug Administration (FDA), the initial trial is a pilot or feasibility trail, equivalent to a Phase I trial in the CBER or CDER. This consists of a small number of patients (6–20) per treatment regime, and is primarily concerned with safety. Any information obtained on efficacy in a trail of this magnitude is unlikely to be statistically significant but may provide useful information on the size and variability of the therapeutic effect that is valuable in design of the subsequent, pivotal trial.

The pivotal trials (equivalent to Phase II trials in biologics and devices) involve much larger numbers of patients (50–500 in the case of devices) which is determined by a sample size calculation, based on the results from the pilot trial, to give about 80% probability of a significant result ($p < 0.05$) if one exists.

Trials with chronic wounds take about two years or more to complete. The design phase, deciding on protocols, obtaining institutional approval for multiple centers and preliminaries to the recruitment of patients is likely to take at least six months. The actual trial involves screening many more patients than will actually take part in the trial, as many fail to conform to exclusion criteria, which reduce irrelevant and interfering factors, established in the design phase. With a trial involving a 12 week follow-up period, most should be completed within 18 months to two years. There is then a period of six months while the data are checked, discrepancies resolved and the results of the trial evaluated. This period could be shortened substantially by logging the patient outcomes to an internet site and checking them on line as they are generated.

The design of the clinical trial should be conducted in close consultation with the relevant regulatory authorities. It is critically important that all aspects should be thoroughly discussed and agreed beforehand. In the case of Dermagraft, failure to follow advice from the FDA precisely led to considerable delay and additional expense.

Considerations in the selection of clinical trials include the size and value of the potential market, the expected efficacy of the product and the difficulty of completing the trials. Five major areas of chronic wounds include venous stasis ulcers, diabetic foot ulcers, arterial ulcers, pressure (decubitus) ulcers and all other chronic wounds. Of these, venous stasis ulcers represent the largest market. Diabetic ulcers are a valuable market, as, while smaller in number, the consequence of failure to heal such an ulcer is limb amputation, an expensive and seriously debilitating procedure. It is very difficult to perform a trial on decubitus ulcers, as the patients are frequently elderly, very infirm and may need approval from a guardian. Such trials are very arduous, may be difficult to complete and are best undertaken by a specialized group with well-developed access to such patients. In no cases does the treatment of chronic wounds with skin implants address the underlying cause of the ulcer, which lies in venous insufficiency, metabolic abnormalities, reduced arterial supply or repeated ischemia. This is particularly evident in ulcers caused by arterial insufficiency. While the angiogenic activity implant may be capable of improving vascularity in the region of the wound, little may be gained if blood supply to the limb is inadequate.

The pivotal clinical trial for Dermagraft for diabetic ulcers was initiated in 1994. Dermagraft gave a statistically significant increase in healing of 19.1% that was similar to results obtained in similar trials with other products. These data were used in combination with mechanism of action and clinical instruction to support the clinical use of Dermagraft commercially. Since then, it has grown to a several $100M/year business.

The indications for which the product may be marketed (its labeling) are strictly limited by the clinical trials and new trials are required to extend them. However, in certain categories,

there may be insufficient patients to support both a clinical trial and a commercial market. Examples are monogenetic congenital diseases, where, although the conditions may be very distressing, the entire population may number in the hundreds or a few thousand. An example in the chronic wound field is epidermolysis bullosa (EB). For such diseases, the FDA has a category, Humanitarian Device Exemption (HDE), which allows much reduced clinical testing. It does, however, require Institutional Review Board (IRB) approval from each institution where it will be used. In the case of dermal implants, such a route has been used for Apligraf and Orcell for all genetic forms of EB, and for Dermagraft for the dystrophic forms of EB. The category, 'all other chronic wounds' includes many miscellaneous conditions, such as pyoderma gangrenosum, necrobiosis lipoidica diabeticorum, for which there is too small a patient population to perform a trial, but would be expected to benefit from treatment with Dermagraft or TransCyte. The HDE pathway may not be appropriate or available. However, since the FDA does not regulate the practice of medicine, some approach to these patients may be obtained through off-label use, although this cannot be formally promoted by the manufacturer.

IMMUNOLOGICAL PROPERTIES OF TISSUE-ENGINEERED SKIN

Immunological rejection of tissue-engineered products is, at the time of writing, a controversial question. As was clearly established by Medawar in the 1940s, allogeneic transplants of whole live organs invariably cause immunological reactions that lead to rejection [40]. However, experience with some cultured cells and tissue-engineered constructs indicates that, in some cases, no clinically significant rejection occurs. At this point, some hundreds of thousands of patients have been implanted with Dermagraft, in many cases several times to the same patient, without a single example of immunological rejection and experience with Apligraf has been similar. The fibroblasts from Dermagraft have been found to persist in the wound site for six months and survival for about a month been obtained with Apligraf [41].

There are two major pathways of transplant rejection, direct and indirect. The direct pathway involves recognition of donor histocompatability antigens (HLA) by the host immune system leading to acute rejection over about two weeks. This involves both HLA, of which Class II molecules, such as HLA-DR, are the most important and also co-stimulatory molecules on the transplant, CD40 and the CD80 group. These molecules are not normally expressed by fibroblasts. However, in monolayer tissue culture, the presence of γ-interferon, which may be present in chronic wounds, will cause induction of HLA-DR, CD-40 and genes involved with the physiological function of Class II HLA (antigen presentation). In three-dimensional culture, many of the cells show a selective response to γ-interferon, that does excludes induction of these molecules [42]. The indirect pathway of rejection involves display of transplant antigenic peptides by host antigen presenting cells (macrophages, tissue dendritic cells and endothelial cells). This gives rise to chronic rejection which may cause destruction of the transplant over many months. While it cannot be excluded that this may occur, Dermagraft fibroblasts have been detected at 6 months from the time of implantation and no clinical evidence for chronic rejection has been observed.

Acute rejection is primarily an attack on the endothelial cells of the vascular system, which are antigen presenting cells and do express the component of the antigen presentation system (HLA Class II, CD40, CD80). It is possible that the lack of rejection of tissue-engineered skin products is related to the absence of such cells. Indeed, adding antigen presenting cells (in this case, B lymphocytes) to a tissue-engineered construct has restored susceptibility to immunological rejection [43].

COMMERCIAL SUCCESS

There are many contributors to the commercial success of a product. They include a satisfactory clinical trial, a good rationale for the performance of the product, convenience

in use, a reasonable price, adequate reimbursement from third party payers, sensitivity to complaints, efficient distribution. Some of these require long-term effort and must be initiated early in the development process.

New products frequently find themselves selling into a conservative and skeptical market that arises from the hype that is involved in developing and funding the product. The first requirement in overcoming this view is a satisfactory clinical trial. This process is determined by regulatory bodies. The major criteria for the American authorities (FDA) are safety and efficacy. Unless the product is safe and efficacy is extraordinary, clinical results need to be backed up by a mechanism of action that, while it may not be fully established, must provide a logical reason for using the product. In the case of Dermagraft, this has led to a great deal of fundamental wound healing research.

Equally important is the convenience of use, cost and process for reimbursement of the physician applying it. Convenience of use and cost are functions of bioreactor and process design, and company structure and are discussed elsewhere. Third party reimbursement and health economics are major and specialized subjects that are outside the scope of this article. However, a major component of establishing satisfactory reimbursement in the United States involves the Center for Medicare and Medicaid Services (CMS). Government authorities with similar responsibilities are involved in other countries. Their criteria, in contrast to the FDA, are reasonableness and effectiveness/cost ratio. Both the FDA-type and CMS-type criteria have to be met for a product to be successful.

An activity that would prove very valuable in establishing the performance of a product is a system for tracking the clinical performance subsequent to regulatory approval to market the product. While complaints and adverse events are carefully tracked and reported, performance has not been. It may, however, become a regulatory requirement to confirm safety, efficacy and cost effectiveness with a larger patient population than possible in a clinical trial. It is also valuable to the manufacturer because, while anecdotes of positive clinical experience may be heartening, they do not provide objective quantitative data. This was not performed in the cases of Dermagraft or TransCyte, making it difficult to assess the actual performance of the product in clinical use.

Mechanism of action

Understanding the mechanism of action of a product required some understanding of the underlying pathology of the disease the product is intended to treat. Wound healing, and particularly the etiology and maintenance of chronic wounds was poorly understood during the development of Dermagraft and TransCyte. Many phenomena have been observed, particularly in chronic wounds. The problem is sorting out how they relate to one another, which are etiological, and which are symptomatic; the difference between cause and response. The problem was less acute in the case of TransCyte as the product was only designed as a temporary covering for third degree burns.

Changes from normal skin observed in chronic wounds include lack of keratinocyte migration, capillary cuffing, protease activity , abnormalities in macrophage activation, diminished vascular supply and senescence in fibroblasts [44]. In developing a rationale for the action of Dermagraft, the first aspects considered were vascular supply and keratinocyte migration. Much study was undertaken on the angiogenic activities of Dermagraft and it was shown that the tissue produced VEGF and hepatocyte growth factor. It was also shown to be angiogenic in the chick chorioallantoic membrane assay and to increase blood supply to diabetic ulcers in patients *in vivo* using laser-Doppler techniques. Thus, a case could be made that one mode of action was through its angiogenic effect.

The high protease activity of chronic wounds was combined with the lack of keratinocyte migration to hypothesize that the extracellular matrix in a chronic wound was degraded to the extent that the migration substrate was lacking. Indeed it was demonstrated that fibronectin

was degraded by neutrophil elastase [45]. This led to work on the properties of Dermagraft and TransCyte as substrates for keratinocyte migration.

During comprehensive surveys of genes induced by three-dimensional culture, it was found that the most highly upregulated gene was IL-8. Other ELR CXC chemokines were also highly induced. This led to studies of the secretion of these proteins and to changes in their production that might relate to the etiology of chronic wounds and the possible role of Dermagraft. It was found that Dermagraft secretes IL-8, sometimes in large quantities. The secretion is extremely variable. Monolayer fibroblasts also secrete CXCL-1, CXCL-5 and CXCL-6 (gro-α, ENA-78 and GCP-2 respectively) that are all chemoattractive for neutrophils and activate them to a bactericidal phenotype. At the same time, it was observed that bacterial products (lipopolysaccharide) inhibit keratinocyte migration. This led to the hypothesis that a major function of fibroblasts in a wound context is the recruitment of neutrophil leukocytes to destroy colonizing bacteria and that failure of this system in chronic wounds results in the establishment of bacterial contamination that leads to failure of re-epithelialization. It is known that fibroblasts in chronic wounds display a senescence-like phenotype [46] that grows slowly and is unresponsive to growth factors. The condition is probably related to stress-induced premature senescence caused by ischemia reperfusion injury, metabolic abnormality, extravasated red cells and the inflammatory conditions of the chronic wound. It has been found that the production of CXCL-1, CXCL-5, CXCL-6 and CXCL-8 decline in senescent fibroblasts. Thus, in chronic wounds, a decline in the ability of the fibroblasts to recruit and activate neutrophils allows wound colonization by bacteria and failure of keratinocyte migration to close the wound. Chronic wounds seem to involve an arrest of normal healing at about the stage of neutrophil immigration. On this hypothesis, a major role of Dermagraft is to provide non-senescent fibroblasts that are able to respond to the presence of bacteria with appropriate secretion of neutrophil chemoattractant chemokines.

In the case of TransCyte, the formation of a foreign body capsule-like material *in vitro* leads to lack of ingrowth of the scaffold into the wound through a host foreign body reaction, and, thus, comparative ease of removal. The ability of TransCyte to reduce the pain of second degree burns is common to many occlusive dressings.

A remarkable feature of dermal implants is that they do not appear to have led to an increase in infection. Indeed the general experience has been a slight reduction in infections. Preparations with keratinocytes included secretion of antimicrobial peptides, but this is not known to be the case for fibroblasts. The most likely explanation is a combination of fibroblasts secretion of neutrophil chemoattractant CXC chemokines and the provision of an extracellular matrix substrate for leukocyte migration.

FUTURE DEVELOPMENTS

The major direction of development in skin products is towards simpler, possibly non-viable systems. Inclusion of live cells in a product entails many issues such as the use of allogeneic cells, cryopreservation, distribution, inconvenience to the customer (for instance, the thawing procedure) and many manufacturing problems. Hence, studies were initiated to explore the possibility that a possibly less effective but substantially less expensive product could be developed. This is a direction that has also been explored by other companies in the field.

It is a general principle that value is likely to be obtained by exploiting a technology base as far as possible. A major product of fibroblasts is collagen, in this case human. As a non-viable product, human collagen was extracted from the extracellular matrix laid down by fibroblasts and used, by injection, for the treatment of wrinkles.

An alternative to cryopreservation that has been explored is the possibility of storage by desiccation. Many natural systems, such as plant seeds, tardigrades, *Artemia*, yeast have developed the

ability to survive desiccation and, in that state may be able to survive extreme conditions of temperature, vacuum, radiation and time [47]. Many factors appear to be involved in this ability [48], but one that has attracted attention is trehalose, which is a disaccharide (1,1,α,α diglucose) that is produced in large quantities by many desiccation-resistant organisms. It was found that TransCyte could be dried at room temperature in the presence of trehalose, irradiated to 25–40 Grays to provide terminal sterilization and would recover its structure and wound adhesion on rehydration. Similar results could be obtained by careful lyophilization. While some success has been obtained in the retention of viability of human cells in the desiccated state, it is not yet possible to obtain the shelf life (at least one month and preferably at least six months) required for a tissue-engineering application. It is also questionable whether the radiation treatment possible with a non-viable product would be appropriate with living cells, and an aseptic drying and packaging system would have to be devised.

Thus far, the therapeutic applications of skin tissue engineering have encompassed acute and chronic wounds and comprise dermal components alone or dermal components with epidermal structures. In the future, it is likely that adnexal structures will be added. Remarkable success in this direction has been achieved with hair follicles [49], where it has been found that hair follicle-inducing cells can be cultured so that they retain their properties and, mixed with hair follicle-derived keratinocytes, will, on injection form a hair. This has also been achieved with human cells. The remarkable feature of this system is that the entire organ, hair follicle, sebaceous gland and erector pili muscle is formed. This constitutes one of the few examples of true organogenesis observed using adult cells.

CONCLUSION

Experience with the commercial production of tissue-engineered skin has developed many principles that are important beyond the simple development of a tissue-engineered product. Most of these are based on well-known concepts used in other manufacturing processes, as applied to tissue engineering. They include:

1) Optimized cryostorage of Master Cell Banks with minimal access
2) Intermediate storage of expanded cell banks (MWCB)
3) Use of allogeneic cells
4) Bioreactors that permit scale-up, minimize footprint
5) Minimal aseptic connections
6) Maximal automation, to minimize errors
7) Avoidance of aseptic fill, so that the bioreactor forms part of the final package
8) Bioreactor designed for end user convenience
9) Cryostorage

While it may not be possible to incorporate all of these principles, as many as possible should be used. The major problem with tissue-engineered products in the market place is cost and all these principles are directed towards reducing cost. Some, such as the use of allogeneic cells, may be difficult to implement. While experience with fibroblasts indicates that immunological rejection is not a major issue, this is not known for other cells. The possible use of allogeneic cells should, however, be checked, preferably in animals other than mice, because the advantages for a commercial product are great. Optimal cryostorage is a compromise between ideal tissue survival and other factors, such as ease of distribution.

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CHAPTER 82

Tissue-Engineered Cartilage Products: Clinical Experience

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INTRODUCTION

Building upon the first clinical implantation of autologous cultured chondrocytes by Peterson and Brittberg just over 25 years ago [1], the field of articular cartilage repair continues to advance. While the repair of damaged articular cartilage remains a long-standing challenge, significant clinical progress has been made since the third edition of this textbook. This progress includes an improved understanding of the spectrum of cartilage injury, comorbid conditions, and the importance of the subchondral plate and joint homeostasis, as well as further long-term experience with first-through third-generation autologous cultured chondrocyte cell-based repair. Awareness has increased for the need to address concomitant pathology such as joint instability and malalignment to optimize treatment success and the importance of rehabilitation for development of high-quality repair tissue.

Progress has also been made in improving the quality of study design and conduct in the cartilage repair field. Additional guidance for the cartilage repair field has become available in recent years. This includes the European Medicines Agency (EMA) reflection paper on *in vitro* cultured chondrocyte containing products for cartilage repair of the knee [2], the Food and Drug Agency (FDA) guidance for industry for products intended to repair or replace knee cartilage [3,4], and the International Cartilage Repair Society (ICRS) guidelines for the design and conduct of clinical studies in knee articular cartilage repair [5]. Notable in these guidances is the emphasis on the need for long-term follow-up, prospective study design with rigorous controls, and clinical outcomes for pain and function as the most important and clinically valid endpoints.

The challenges of articular cartilage repair relate to both the biologic characteristics of the tissue and the epidemiology of cartilage injury. Articular cartilage lacks intrinsic capacity for repair. The clinical strategy for autologous cultured chondrocyte cell therapy derived from the observations that articular cartilage is avascular and has a limited supply of cells adjacent to injuries to mediate a repair process [6]. The articular chondrocytes that maintain normal cartilage homeostasis are embedded in an extracellular matrix that limits a cell's ability to migrate to a zone of injury and carry out tissue repair.

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Articular cartilage injuries are common, especially in the knee, and are often associated with damage to other joint structures. There is a spectrum of clinical presentations, ranging from some lesions that may be asymptomatic on initial diagnosis, often in the setting of an incidental finding during an arthroscopy initially indicated for another more evident injury, to lesions that may cause disabling symptoms. The public health impact of these latter types of lesions is magnified because they often occur in relatively young adults and may progress to end-stage arthritis. There clearly is a subset of patients with articular cartilage injury that are very disabled. For example, in a study of patients with focal cartilage defects of the knee, the extent of pain and impairment in function experienced was rated worse in comparison with ratings from patients with anterior cruciate ligament (ACL)-deficient knees, and quality of life was rated at the same level to that reported by patients scheduled for knee-replacement surgery [7].

Although the incidence and prevalence of symptomatic full-thickness chondral injuries in the general population have not been established, it is known that cartilage lesions are a common finding at knee arthroscopy. Curl et al. [8] reviewed records from 31,516 knee arthroscopies and reported Outerbridge Grade III cartilage lesions in 41% and Outerbridge Grade IV lesions in 19%. They reported an incidence of 5% Grade IV lesions when considering only lesions on the femoral condyle in patients less than 40 years old. Other authors have reported a similar incidence of full-thickness cartilage lesions in other arthroscopy series [9–11].

Cartilage injury in the knee is often associated with other injuries, especially ACL rupture, meniscus tears, and patella dislocation [12–15]. For example, Maffulli et al. reported that 42% of patients in a series of 378 patients with ACL tears had associated articular cartilage injuries [12]. In a multicenter registry of 1,095 knee cartilage injuries, the etiology of the injury was associated with falls (25.9%), sports (27.4%), activities of daily living (21.2%), and motor vehicle accidents (5.5%), and the etiology could not be identified in 20% of cases (Cartilage Repair Registry Volume 7, 2001). In this same study, the onset of symptoms was noted to be acute in 64.6% of cases and gradual in 35.4% of cases. Another distinct etiology for articular cartilage lesions is osteochondritis dissecans (OCD) [16,17].

Since the mid-1990s, treatment algorithms have evolved that reflect the range of cartilage lesion presentations, the different indications for different treatment modalities, and the outcome data [18–21]. While these algorithms may differ in specific detail, they are directionally similar and base treatment guidelines on such factors as lesion size and location,

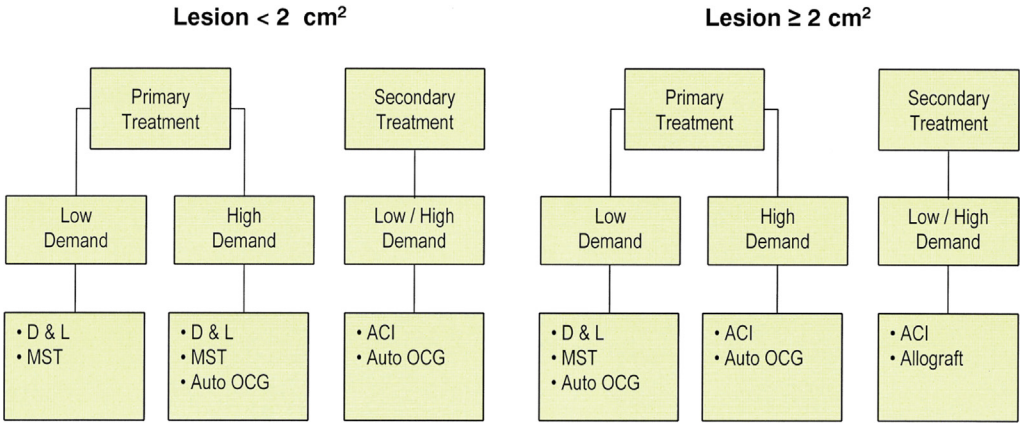

FIGURE 82.1
Illustrative example of current treatment algorithms for repair of symptomatic cartilage lesions. The algorithm gives an overview of treatment options based on lesion size, patient functional demands, and prior surgical history. Given other factors and the complexity of decision making for individual patients, the authors of this algorithm, caution against a mechanistic "menu-driven decision making process." D&L = Debridement & Lavage; MST = Marrow Stimulation Technique; Auto OCG = Autologous osteochondral graft (Cole and Farr 2001).

Patient Factors	Co-Morbidity Factors	Lesion Factors	Procedure Factors
• Symptoms • Age • Expectations • Demand • Prior treatments • General health, systemic disorders	• Ligaments • Meniscus • Alignment • Bone	• Size • Location • Containment • Onset & duration • Etiology	• Arthroscopy • Mini-arthrotomy • Arthrotomy

FIGURE 82.2
Choosing the most appropriate and optimal cartilage repair treatment for an individual patient depends upon multiple factors.

prior surgical history, and patient expectations and functional demands. Fig. 82.1 is provided as an illustrative example of one of these treatment algorithms [19]. Clinical decision-making for a given individual patient can be complex, and the multiple factors to evaluate and consider are outlined in Fig. 82.2. In decision-making for treatment of articular cartilage lesions, it is critical to consider the whole joint and the whole patient.

While it is beyond the scope of this chapter to review the outcome literature for all articular cartilage treatment modalities, it is helpful to understand where traditional first-line arthroscopic treatments and the cell-based tissue-engineering experience fit in current treatment algorithms. In general, first-line arthroscopic treatments are used in less severe lesions, and autologous chondrocyte implantation (ACI) is used in more severe lesions and failed first-line treatments (Fig. 82.3). The impetus for developing ACI came from the recognition that while traditional treatments such as debridement and marrow stimulation techniques (Pridie drilling, abrasion arthroplasty, microfracture) may provide some benefit in smaller and/or less symptomatic lesions, they tend to be less effective and less durable in larger and/or more symptomatic lesions due to production of fibrocartilage which has weaker mechanical properties than native cartilage [6,22,23]. A recent, evidence-based, systematic analysis of microfractures showed decreasing knee function and increasing failure rate were associated clinical limitations in the long-term [24].

CLINICAL EXPERIENCE WITH FIRST-GENERATION ACI

Initial experience

Lars Peterson and colleagues, in Gothenburg, Sweden, pioneered the clinical application of autologous cultured chondrocytes to repair symptomatic articular cartilage lesions in the knee

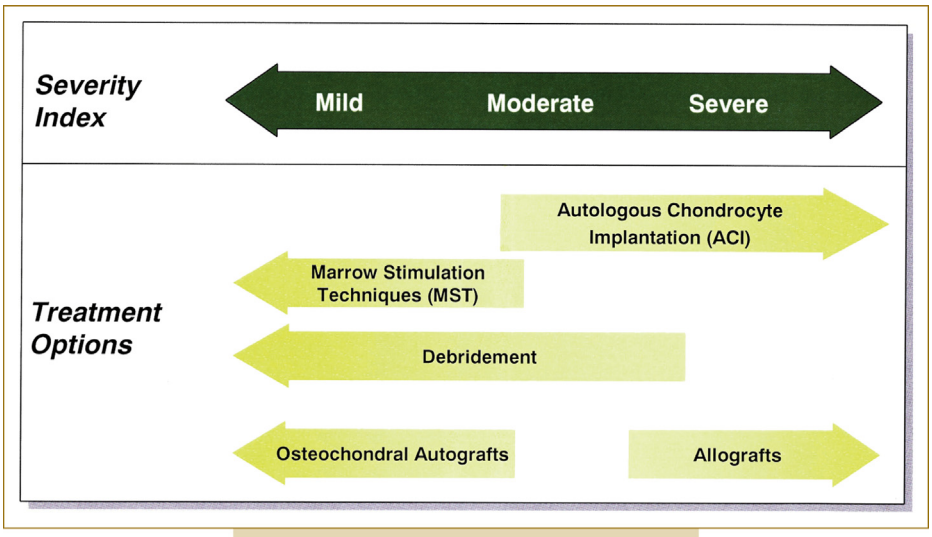

FIGURE 82.3
There is a wide range of clinical presentations of articular cartilage injury in the knee. Treatment options reflect severity based on a combination of patient, lesion, and co-morbity factors. The clinical experience with ACI has been at the more severe end of the spectrum of full thickness articular cartilage injury.

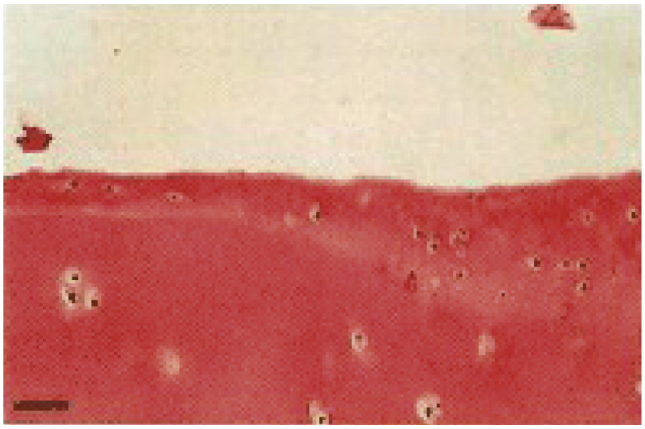

FIGURE 82.4
Histologic section from a biopsy of cartilage repair tissue 36 months after treatment with autologous chondrocyte implantation of an articular cartilage defect on the femoral condyle. From Brittberg et al (1994). *Copyright 1994 Massachusetts Medical Society. All rights reserved.*

joint. The initial results in 23 patients with a mean follow-up of 39 months were published in a landmark paper in 1994 [1]. Results were reported for clinical outcome scores, macroscopic appearance during second-look investigational arthroscopies, and histology evaluation of repair tissue biopsies (Fig. 82.4). Clinical results were good to excellent in 14 of 16 lesions (87.5%) on the femoral condyles. Eleven of 15 biopsies (73%) on the femoral condyles had the appearance of hyaline cartilage. Initial results in the sevem patella lesions were less promising. Later experience with patella implants, in which patella alignment was addressed concurrently with cell implantation, yielded better results [25,26].

Cell processing and surgical technique

The basic techniques and promising results reported in this initial study became the basis for a larger long-term experience at Peterson's center as well as the commercialization and multicenter experience with ACI. In 1995, Genzyme Corporation established (and subsequently obtained an FDA license for) a commercial GMP (cGMP) facility in the United States (US) to perform the isolation and expansion of chondrocytes from cartilage biopsies. Also in 1995, a multicenter registry was established to track outcomes of ACI and other cartilage procedures. In 1997, the FDA approved Carticel® (autlogous cultured chondrocytes) as the first cell therapy in orthopedics. More than 20,000 patients have now been treated with Carticel®. Other commercial cell-culturing facilities have been established outside the US.

ACI, the only FDA approved cell-based cartilage repair product, is a two-stage procedure. In the first stage, a 200 to 300 mg specimen of cartilage is harvested arthroscopically from a lesser weight-bearing area of the trochlear ridge or the intercondylar notch. In the US, the procedure is to send this sample to the Genzyme facility (Cambridge, MA), where it is enzymatically digested and the chondrocytes isolated, expanded through primary and secondary culture, and then cryopreserved. Once the facility has been notified of an implantation date by the surgeon and the hospital, the cryopreserved cells are thawed and cultured to expand the cell population *in vitro*. The final product, a suspension of approximately 12 million cells, is shipped for implantation in a second-stage surgery (Fig. 82.5). All implants are tested for appropriate chondrocytic morphology, sterility, endotoxin, and cell viability as part of the release specifications. The extensive process validation and sterility-testing procedures for this unique, patient lot-specific manufacturing process have been described in detail elsewhere [6,27–29].

The cells are implanted via an arthrotomy, in which an autologous periosteal flap is harvested from the proximal tibia or distal femur, and sutured over the defect and the cells are injected into the defect under the patch after testing for a watertight seal. Fibrin glue is used to ensure a watertight seal (Fig. 82.6).

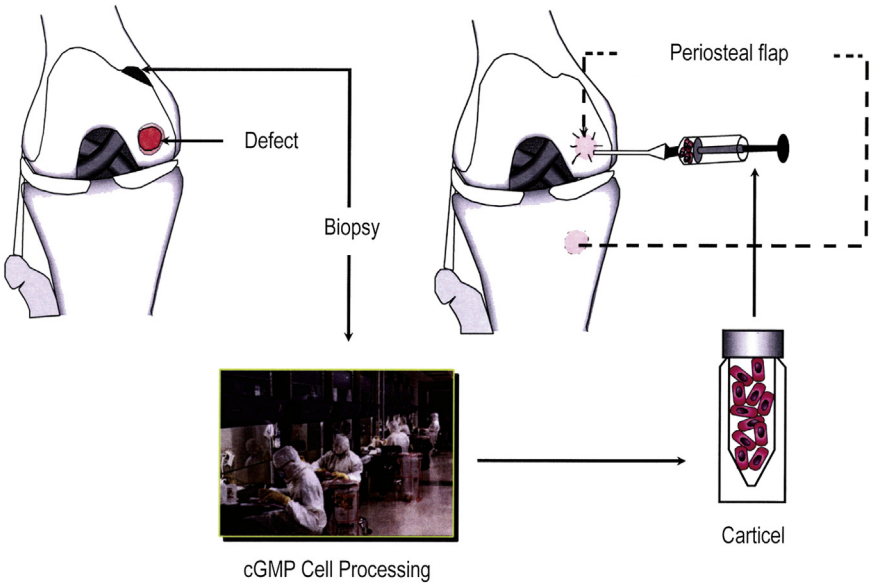

FIGURE 82.5
Flow diagram for Carticel® (autologous cultured chondrocytes) patient lot specific cartilage biopsy harvest, cGMP cell processing, and surgical implantation. *Copyright 2002 Genzyme Corporation. All rights reserved.*

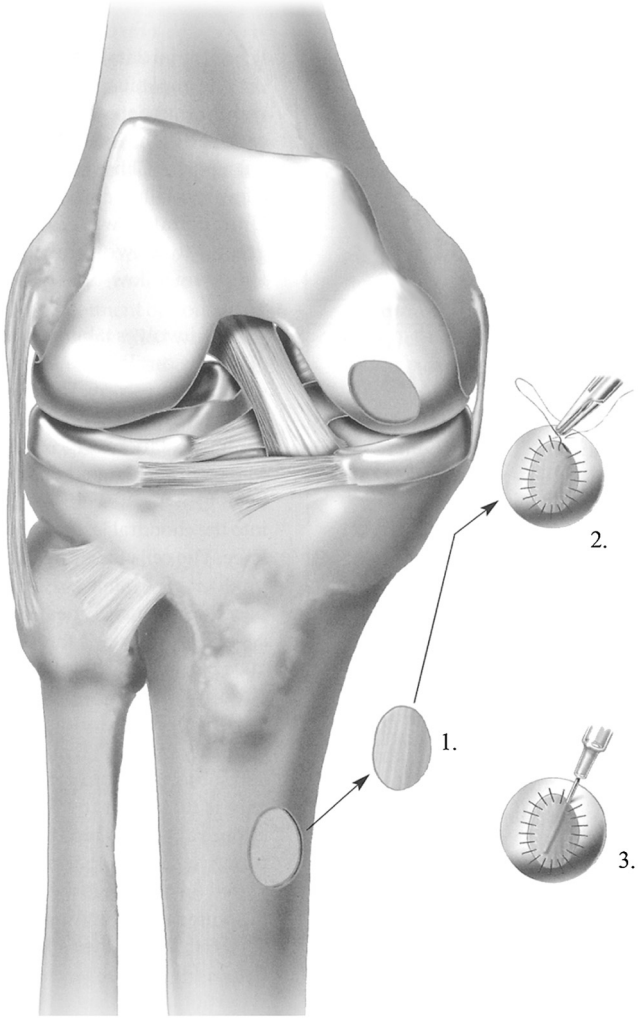

FIGURE 82.6
Surgical implantation of 1st generation ACI: figure illustrates (1) harvest of periosteal patch, (2) suturing of periosteal patch over cartilage defect on femoral condyle and (3) catheter in place for injection of autologous cultured chondroctyes under sutured patch. After chondrocyte injection, catheter is removed and the catheter entry site is sealed with additional suture. *Copyright 2006 Genzyme Corporation. All rights reserved.*

Long-term outcomes in the Swedish series

Peterson et al. [25] reported outcomes for 94 patients implanted between 1987 and 1995, with only one patient lost to follow-up. The patients were divided into five treatment groups based on defect classification and concurrent ACL procedures. Baseline characteristics and mean follow-up varied by defect category. In general these patients had large, chronic lesions and multiple prior surgeries. The mean defect size ranged from a 4.2 cm^2 in isolated femoral lesions to 4.7 cm^2 in OCD lesions. Mean follow-up ranged from 2.7 years in the multiple-lesion group to 4.2 years (range: 2 to 9) in the isolated femoral condyle group. Outcome results based on patient assessment varied by defect category, with isolated femoral condyles 92% improved, femoral condyles with ACL reconstruction 75% improved, OCD lesions 89% improved, multiple lesions 67% improved, and patella defects 68% improved.

Subsequent studies have provided evidence on durability of ACI treatment from 2 to 20 years post-treatment [30–32]. Long-term treatment durability from 10 to 20 years post-ACI (mean follow-up of 12.8 years) was assessed in a study of 341 patients who received patient-reported outcome measures by mail [32]. Of the 224 patients who responded (mean defect size of 5.3 cm^2), 74% reported their status as better or the same as previous years and 92% reported being satisfied with the ACI treatment. Improved average assessment scores from preoperative to follow-up timepoints included the Lysholm (from 60.3 to 69.5), the Tegner-Wallgren (from 7.2 to 8.2), and the Brittberg-Peterson (from 59.4 to 40.9). The authors reported that final treatment outcome was not associated with age at time of ACI, lesion size, or history of marrow stimulation treatment or meniscal injury.

Other studies of ACI

Zaslav et al. evaluated ACI treatment outcome in 154 patients who had failed at least one prior non-ACI procedure for the repair of a full-thickness articular cartilage defect of the knee [33]. For the majority of patients, the defect was located on the medial femoral condyle (67%); the mean defect size was 4.6 cm^2. The authors described the patient population as very challenging with a mean pretreatment modified Cincinnati Knee Rating System score of 3.0 for pain and 3.3 for overall knee condition (0 = severe, 10 = normal). At four years post-treatment, 76% of the 126 patients who completed the study had a successful treatment outcome while 24% were considered treatment failures. Subsequent surgical procedures (SSPs) were reported for 49% of patients; need for SSP was not associated with treatment failure. Mean improvements from pretreatment were significant for all outcome measures assessed in the study. The authors concluded that ACI can be expected to provide a durable improvement in pain and function for patients with moderate to large cartilage defects who have failed a prior cartilage repair treatment.

Cole et al. reported on a subset of patients with OCD from the Zaslav et al. study [34]. Of the 40 patients with OCD treated with ACI, 80% completed the 4-year study follow-up and 35% underwent a SSP. From pretreatment to four-year follow-up, significant mean improvements in pain and function were observed in this challenging patient population. For example, the mean modified Cincinnati Knee Rating System score for overall knee condition rose from 3.1 ($\pm$ 1.1) at pretreatment to 6.8 ($\pm$ 2.0) at follow-up. The authors conclude that the patients' clinically relevant and statistically significant improvements have shown ACI to be a viable treatment for OCD. Thirty-five percent of patients experienced an SAE which most commonly involved new areas of cartilage involvement, graft hypertrophy, graft complication, chondromalacia, graft delamination, and arthrofibrosis. Further discussion of safety aspects of ACI treatment is provided below in the section on complications of ACI.

Fu et al. [35] directly compared two Cartilage Registry Repair cohorts at three-year follow-up: a cohort of 58 debridement patients and a cohort of 58 ACI patients. The two cohorts had similar demographics and defect pathologies at baseline. However, more ACI patients failed a previous

debridement or marrow stimulation procedure than the debridement patients. The mean total defect area for the debridement cohort was 4.5 cm^2, and for the ACI cohort it was 5 cm^2.

Fifty-six ACI patients and 42 debridement patients completed follow-up outcome assessments; 81% of the ACI patients and 60% of the debridement patients reported median improvements of 5 and 2 points, respectively, in the overall condition score. ACI patients also reported greater improvements in the median pain and swelling scores than debridement patients. The treatment failure rate was the same for both ACI and debridement patients. Based on the *a priori* definition of treatment failure, there were three (5.2%) failures in the debridement group and four (6.9%) in the ACI group. The authors concluded that:

> 'Although patients treated with debridement for symptomatic, large, focal, chondral defects of the distal femur had some functional improvement at follow-up, ACI patients obtained higher levels of knee function and had greater relief from pain and swelling at 3 years.'

Micheli et al. [17] reported on ACI outcomes in children and adolescents. Thirty-seven registry patients with a mean lesion size of 5.2 cm^2 and a mean age of 16 at implant were in the cohort. Despite the young age of these patients, they were similar to adult ACI cohorts, in that the majority (70%) had undergone at least one prior knee procedure before the index arthroscopy for cartilage specimen harvest. Thirty-two patients completed follow-up assessments at a mean follow-up of 4.3 years. Twenty-eight patients (88%) reported a favorable outcome at a minimum of two-year follow-up. These results are encouraging, however, no cell therapy product for cartilage repair is indicated in the US for use with pediatric patients. The first Advanced Therapy Medicinal Product (ATMP) approved in Europe by the EMA for cartilage repair (ChondroCelect, TiGenixNV), is also indicated for adults only.

Knutsen et al. [36] in Norway conducted a four-center, 80-patient randomized controlled trial comparing ACI to microfracture at a two-year follow-up. Endpoints were clinical outcome scores (Lysholm, VAS pain score, and SF-36) and a histology score. Both groups were improved at two years, with no statistically significant difference between the ACI and microfracture groups in Lysholm, VAS pain score, or SF-36 mental health subscale (results reported in aggregate, not stratified by lesion size). The authors do report a statistically significant difference in the SF-36 physical component score in favor of the microfracture arm; however, the authors also report different SF-36 physical component mean scores at baseline between the arms.

The authors report that microfracture outcomes varied by lesion size with lesions smaller than 4 cm^2 associated with better clinical results. With regard to the histology comparison, the authors conclude:

> 'There was a tendency in our study for the ACI procedure to result in more hyaline repair cartilage than the microfracture procedure, but this was not a significant finding with the numbers available.'

The authors' overall conclusion was that:

> 'because microfracture is a relatively simple 1-stage procedure, it may be more suitable for a primary first-line cartilage repair of a local contained defect. In patients in whom microfracture has failed and in those with bigger, non-contained defects, ACI may be a better option.'

At the five-year follow-up, both treatment groups continued to show significant clinical improvement [37]. There were no significant differences between the ACI and microfracture groups in clinical scores or failure rate at five years post-treatment. The authors report that there was no correlation between histological score and clinical outcome. Further, there was no significant difference in histological score when comparing patients with and without treatment failure. The authors report that none of the patients with treatment failure had the

best histological scores. The authors conclude that in their chronic patient group, both treatments resulted in satisfactory outcome in the majority of patients at five years.

In the United Kingdom, Bentley et al. [38] conducted a single-center, 100-patient randomized controlled trial comparing two ACI arms (ACI with periosteal flap coverage and ACI with type I/III collagen membrane coverage) to mosaicplasty, with a mean follow-up of 1.7 years. Outcome measures included clinical scores, second-look arthroscopy with ICRS scoring, and histology.

The overall results favor ACI over mosaicplasty in all measured parameters, including clinical outcome, histology, and ICRS scores on follow-up arthroscopy. Overall, 88% of the ACI patients vs. 69% of the mosaicplasty patients were graded as 'excellent or good result.'

Study strengths include that it is a prospective, randomized design with follow-up arthroscopy evaluation as well as clinical outcome scores. Mean lesion size was 4.66 cm^2. Mean duration of symptoms was 7.2 years, and all but six patients had undergone previous surgical interventions. Limitations of the study include that while the study showed better scores for ACI vs. mosaicplasty in all parameters, the differences in clinical scores were statistically significant for only one anatomic location, the medial femoral condyle.

The authors conclude:

> 'There is definite evidence, however, that ACI is valuable for selected patients in that it gives healing of hyaline cartilage in chondral and osteochondral defects. It can also dramatically reduce the symptoms of pain and disability. The continued use of mosaicplasty, however, appears to be dubious.'

The results of follow-up of these 100 patients at a minimum of 10 years (94% follow-up rate) continue to show significantly better outcome for patients treated with ACI compared to mosaicplasty [39]. Functional outcome assessed with the modified Cincinnati score was significantly better for the ACI group; no significant difference between the treatment groups was seen for the Stanmore-Bentley score. There was a significant difference in rate of treatment failure favoring ACI treatment (17%) over mosaicplasty (55%). The authors state, 'The timing of more rapid deterioration after two years in the mosaicplasty patients might reflect the known pattern of deterioration of fibrocartilage.'

A Phase 3 pivotal study comparing an ACI treatment (ChondroCelect, TiGenix NV) to microfracture was designed to show superiority of structural outcome and non-inferiority of clinical outcome at one year post-treatment [40]. The study showed that the chondrocyte implantation technique resulted in superior structural repair at the one-year follow-up, with no clinically significant difference observed between the two treatments. At the three-year follow-up, mean clinical improvement from baseline was significantly greater for ACI versus microfracture, while assessment by MRI did not reveal significant differences between treatment groups in structural repair [41]. The authors observed that patients with shorter duration between symptom onset and ACI treatment had significantly better clinical improvement compared to microfracture treatment.

There are several other published studies on ACI from other centers. They are too numerous to review in detail here. In general, the results are similar to the studies just discussed. A few specific additional points are worth noting with regard to special populations or indications treated with ACI and health economics of this technology. Mithofer et al. [42] reported on ACI to treat adolescent athletes. Overall, they report a 96% rate of return to high-impact sports and 60% achieving an athletic level equal to or higher than the preinjury level. Return rate to preinjury sports correlated with shorter preoperative symptom duration and a lower number of prior operations. This suggests that earlier intervention may optimize cell therapy outcomes, an especially important consideration in younger patients.

Minas has reported successful outcomes in young adults with complex joint pathology for which ACI was performed as an alternative to an artificial joint arthroplasty [26,43]. Further, while the vast majority of ACI cases are in the knee, there are reports of successful use in other joints, especially the ankle. While the patient lot-specific manufacture of a live cellular product with stringent quality controls is inherently expensive, favorable health economics were demonstrated in three studies that assessed economic parameters [44–46].

Complications of ACI

Patients who undergo ACI may require SSPs on the treated knee. The majority of these procedures are arthroscopic interventions. Such procedures frequently have no findings causally attributable to ACI. Frequently encountered findings following the original ACI method include graft or periosteal hypertrophy (18%), delamination (5%), and arthrofibrosis (3%) [47]. In a study comparing the original ACI method with collagen-covered autologous chondrocyte implantation (CACI, a second generation treatment method similar to ACI but with the periosteal flap replaced by a collagen membrane), re-operation due to symptomatic graft hypertrophy was reported for approximately 36% of patients treated with the original ACI method and for none of the patients treated with CACI at one year post-treatment [48]. A systematic review aimed at comparing rates of failure, re-operation, and complications between different methods of ACI treatment indicated that although the overall failure rate is low for all methods of ACI, ranging from 1.5 to 7.5%, the original ACI method using a periosteal cover is associated with higher rates of failure, unplanned operation, hypertrophy, and delamination [47].

Multiple prior surgeries and a history of ligament reconstruction or meniscal procedures concomitant with ACI appear to be risk factors for a post-ACI subsequent procedure. The frequency of arthroscopic intervention after ACI is likely due to a combination of factors, including the associated arthrotomy necessary for implantation, complex knee pathology, including comorbid conditions, concurrent procedures and prior surgical history, and a hypertrophy of the graft or periosteal patch in some patients.

Summary of first-generation ACI clinical experience

Multiple factors make direct comparisons or data synthesis of these varied studies challenging. These factors include differences in study design, data collection instruments, length of follow-up, evaluation criteria, treatment failure definitions, and patient populations. However, bearing these factors in mind, these different studies are generally consistent and complementary in supporting the following overall conclusions regarding the efficacy of first-generation ACI:

- ACI has demonstrated successful clinical outcomes in challenging patients where other treatment modalities, such as debridement and microfracture have been less successful.
- ACI has demonstrated successful clinical outcomes in patients who have failed prior to cartilage repair procedures.

While these are important advances, first-generation ACI has limitations. These include the harvest and suturing of a periosteal flap to secure the implanted chondrocytes in the defect. Not only does this prolong the operative time and require a more extensive arthrotomy, some authors have postulated that the periosteal flap may contribute to the formation of hypertrophic tissue, the most common complication of first-generation ACI [38,49]. These limitations have led to efforts to develop improved technology for chondrocyte implantation. Goals include maintaining the benefits of achieving biological cartilage resurfacing and functional repair tissue while improving surgical delivery and reducing complications.

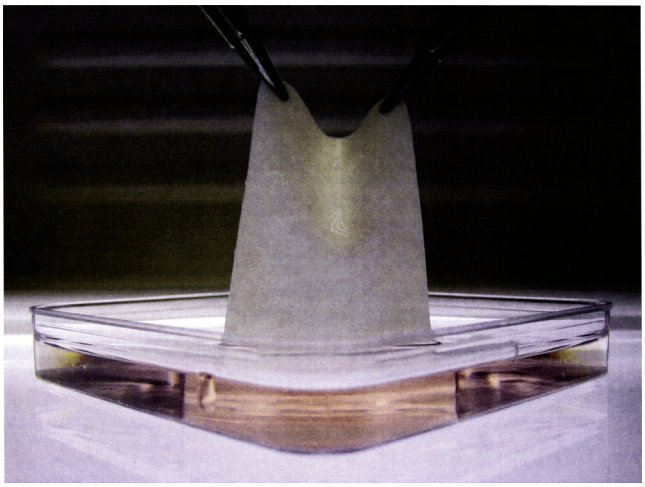

FIGURE 82.7
Third generation autologous cultured chondrocyte graft technology. In Matrix-induced Autologous Chondrocyte Implantation (MACI®), the cultured chondrocytes are seeded on 4×5 cm Type I/III collagen membrane. This eliminates the need for the periosteal patch and extensive suturing use in first generation ACI and facilitates less invasive surgical implantation. *Copyright 2005 Genzyme Corporation. All rights reserved.*

CLINICAL EVOLUTION OF ADVANCED-GENERATION ACI

Significant clinical experience with advanced-generation ACI has been obtained in Europe and Australia. The first clinical step was to replace the autologous periosteal flap with a porcine type I/III collagen patch. This has been termed CACI, or collagen-covered ACI. Early results suggested clinical and histological results similar to periosteum-covered ACI, but with a lower incidence of post-implant hypertrophy [38,48,50].

With CACI, the chondrocytes are still delivered to the defect in a liquid suspension that requires suturing a collagen patch in place with a watertight seal. Development of matrix-induced autologous chondrocyte implantation (MACI®, Genzyme Biosurgery), a third-generation ACI product, began as a further improvement on the CACI technique in markets outside the US (Fig. 82.7) following the acquisition of Verigen Corporation by Genzyme. MACI® optimizes the delivery of cultured chondrocytes by seeding them onto a purified, resorbable, porcine-derived collagen type I/III membrane (ACI-Maix™), thereby removing the need for the harvest, placement, and suture of a periosteal flap to retain the suspension of chondrocytes in the cartilage defect (as required for ACI). At implant, the MACI® cell-seeded membrane is trimmed to the size and shape of the cartilage defect, implanted cell-side down into the defect, and secured in place using fibrin sealant. Controlled seeding of the cultured cells directly on the type I/III collagen membrane eliminates the need for extensive suturing of a periosteal or collagen patch and facilitates a simpler, more rapid, and less morbid operation to implant the autologous cultured chondrocytes. With another membrane-assisted product, Hyalograft C® (Anika Therapeutics), a patient's chondrocytes are seeded on a hyaluronic acid scaffold. Hyalograft C® is positioned in the defect using a delivery device where it is fixed to the subchondral bone without the use of fibrin glue or sutures [51]. The membrane-assisted product CaReS® (Arthrokinetics) uses a type I collagen gel, into which a patient's chondrocytes are mixed. The product is cut to slightly larger than the cartilage defect and positioned into the defect using fibrin glue [52].

CLINICAL EXPERIENCE WITH THIRD-GENERATION ACI

Several different types of membrane and construct are used in third-generation ACI products and marketed outside the US. With MACI® the cultured, characterized chondrocytes are seeded on a 14.5 cm^2 porcine type I/III collagen membrane. In Hyalograft C®, chondrocytes are seeded on a 2×2 cm Hyaff 11 membrane, an esterified derivative of hyaluronan [51]. Other biomaterial approaches to delivering chondrocytes for cartilage repair are being pursued by several entities, but have limited clinical experience to date. Although products such as MACI®, Hyalograft C®, and CaReS® are currently marketed in Europe, the transitional phase of the

ATMP Regulation will expire on 30 December 2012. Both MACI® and Hyalograft C® have pending applications with the EMA for Marketing Authorization as ATMP [53].

A recently reported study compared Hyalograft C® delivered by arthroscopy (n = 22) with an earlier developmental form of MACI that used a Chondro-Gide membrane delivered by arthrotomy (n = 39) in patients over 40 years of age [51]. At one year post-treatment, a significant ($p = 0.049$) improvement on one measure of clinical outcome suggested a faster improvement for those treated with arthroscopic Hyalograft C®. At 2 and 5 years post-treatment, both groups improved clinically and no significant difference in outcome was seen. There was no significant difference between groups in treatment failure and no severe product-related Adverse Events (AEs) occurred during the study or follow-up period. The authors concluded that:

> 'a clinical improvement was found in patients more than 40 years old, … with good results lasting at the medium-term follow-up. However, the results were inferior with respect to those previously found for younger populations, and the failure rate at medium-term was also higher.'

The first comparison of CaReS® (n = 10) versus microfracture (n = 10) treatment for patello-femoral cartilage defects was recently reported [52]. At three years post-treatment, clinical improvement from baseline was statistically significantly better for those treated with CaReS®, however, clinical outcome at three years post-treatment was not significantly different between the treatment groups. The authors reported that the comparable results for CaReS® with microfracture call for a larger prospective randomized study comparing the two treatments.

Of the third-generation ACI products, MACI® has the largest accumulated clinical experience, including a just completed Good Clinical Practise (GCP), randomized, comparative Phase III pivotal clinical trial. More than 8,000 patients have been implanted with MACI® to date. A number of studies reporting on the efficacy and safety of MACI® with follow-up to two and five years post-treatment have been completed.

In a study by Ebert et al, 41 patients treated with MACI® were followed for five years post-treatment [54]. The treated lesions had a mean surface of 3.0 cm^2 (range: 1 to 9). Of the 41 patients, 85% had undergone one or more previous surgical procedures due to knee pain and/or other symptoms. The results reported are for the 35 patients with complete five-year follow-up data. The mean Knee Injury and Osteoarthritis Outcome Score (KOOS) score for each of the five subscales improved significantly over the five years following treatment. Most improvement in knee pain, symptoms, and activities of daily living took place in the first year following surgery; most improvement in knee-related quality of life and sport and recreational activities took place in the first two years. Improvement was maintained through five years post-treatment. Significant improvement over time was also observed for the SF-36, 6 minute walk test, and knee extension range-of-motion. The authors report that MACI® treatment results in functional tissue infill to 24 months and improved clinical outcome that were maintained to five years following MACI® treatment.

In a recent publication, results of five year follow-up of 21 patients treated with MACI® were reported [55]. For each of the five KOOS subscales, significant improvement was observed ($p < 0.05$), and maintained up to five years post-treatment in 90.5% of the patients. Significant improvement up to five years post-treatment was also reported for other patient-reported outcome measures including the International Knee Documentation Committee (IKDC) and the modified Cincinnati. Positive evidence of structural repair was demonstrated by MRI at five years post-treatment. The MRI composite score significantly improved up to five years post-treatment ($p < 0.001$); complete defect fill (83%) and graft integration (82%) were reported for the majority of patients. The authors conclude:

> 'The MACI® procedure was shown to be a safe and effective treatment for symptomatic, traumatic chondral knee defects in this study.'

A large GCP, randomized, comparative, multicenter study has recently been completed in Europe comparing patients treated with MACI® vs. microfracture (SUMMIT study, Genzyme Biosurgery; ClinTrials.gov Identifier: NCT00719576). It is expected that the results will be published in 2013. The study design closely follows the guidelines for the study of cartilage repair currently available from the EMA, the FDA, and the ICRS, including clinical outcome of pain and function as the co-primary efficacy endpoint. Outcome up to two years post-treatment has been assessed and five-year follow-up is ongoing.

The clinical development of third-generation products has built on the earlier ACI experience by addressing some of the limitations of the original ACI and continuing to advance the field of cellularly-based therapies for cartilage repair. These products were designed to remove the need for the harvest, placement, and suture of a periosteal flap and thereby provide implantation of autologous chondrocytes for cartilage repair with less invasive techniques. In general, studies of third-generation ACI have shown significant improvement in pain and function, structural evidence of repair tissue maintained in the longer-term, and few adverse safety findings. The next steps for clinical development may include arthroscopic application and expansion of treatment to joints other than the knee.

CONCLUSIONS

Further progress in treating articular cartilage lesions is likely to come from a number of approaches, including continued improvement of autologous cultured chondrocyte implantation. Given the range of clinical presentations outlined in the chapter introduction, it is unlikely that a single technology will be applicable to the entire spectrum of articular cartilage pathology. More likely is an array of diagnostic and therapeutic tools to enable orthopedic surgeons to effectively treat each stage of cartilage injury and comorbid injury. There is a continuum from early chondral injury, identified when it may still be relatively asymptomatic, to symptomatic focal cartilage injury, to end-stage arthritis. At the early end of this continuum is a need to better identify and mitigate the risk of progression of disease.

Third-generation ACI focuses on improved delivery of autologous cultured chondrocytes for treatment of symptomatic cartilage lesions. This is well along in clinical development. Additional work is needed to assess the applicability of cell therapy approaches to arthritis and to joints other than the knee. This is likely to focus on indications where traditional arthroplasty has the most limitations (e.g., younger, athletic patients, need for revision, and bone stock loss).

The clinical applications of cell therapy tissue engineering to date have focused on autologous cultured chondrocytes. As discussed elsewhere in this book, there is extensive research on a wide range of other cell sources, such as adipose cells, embryonic stem cells, mesenchymal and induced pluripotent stem cells, and allogeneic chondrocytes. These have the potential to expand the clinical applications to other cell types. Cell optimization and modification strategies using gene therapy or growth factors to mediate cartilage repair with different cell types are also under investigation and have the potential to broaden the scope of cell therapy clinical applications.

Developing and producing surgical implants with live cells poses scientific, logistical, commercial, and clinical challenges that are very different from the challenges with the development and production of more traditional orthopedic implants. To the extent that future cell therapy applications in cartilage repair require *in vitro* cell processing on a manufacturing scale, the experience with Carticel®, MACI®, Hyalograft C®, and CaReS® demonstrate the feasibility of manufacturing a high-quality cell product for surgical implantation. The clinical success with first-through third-generation ACI in patient populations with very symptomatic and challenging cartilage lesions has laid the foundation for further progress in cell-based orthopedic tissue engineering in general and cartilage repair in particular.

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CHAPTER 83

Bone Tissue Engineering: Clinical Challenges and Emergent Advances in Orthopedic and Craniofacial Surgery

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INTRODUCTION

Unlike softer skeletal tissues, bone has inherent regenerative capabilities that enable self-repair of fractures and limited tissue loss. However, when the bone loss due to trauma or tumor removal is too extensive, bone defects do not completely heal and require surgical intervention. In orthopedics, the clinical management of a critical diaphyseal defect (a large discontinuity in bone that cannot spontaneously regenerate) continues to be a major challenge. The two available treatment options are limb salvage or amputation, and both have high rates of self-reported disability [1,2]. These clinical scenarios can be complicated further by infections and/or non-union fibrosis. Moreover, these problems and the high morbidity and associated costs of care are not limited to the civilian population, but represent a major concern in the ever-increasing non-lethal combat casualties [3]. The treatment of diaphyseal bone defects has advanced over the years from amputation to reconstruction with bone grafts (vascularized, cancellous, autologous, allograft) to distraction osteogenesis with Ilizarov devices to the emergent translational possibilities of tissue engineering and regenerative medicine [4].

Similarly, oral cancer is a major reason for mandibulectomy and maxillectomy, and major neurosurgical procedures typically involve craniotomy. *In vivo* bone formation is a major challenge in various craniofacial reconstructions and dental procedures that involve jawbone [5,6]. Currently, major mandibular defects can be repaired with an autologous vascularized bone from a variety of donor sites including fibula, scapula, iliac crest, or rib. A major disadvantage of this technique is that harvesting these grafts always creates a skeletal defect at

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the donor site, and induces significant secondary morbidity. Other therapeutic options including allografts, xenogenic tissue grafts, synthetic materials, or prosthesis unfortunately do not yield consistent and efficient regenerative repair and are fraught with complications [7]. Significant progress in tissue engineering could yield more favorable outcomes than current clinical approaches used to repair craniofacial defects.

CELLS, SCAFFOLDS, AND BIOFACTORS: FROM *FUNCTIONAL* TO *TRANSLATIONAL* TISSUE ENGINEERING

Regenerative medicine approaches based on engineering cells and biomaterial scaffolds into 'spare-part' tissues promise to shape the future of reconstructive surgery and organ transplantation. To date, use of growing *functional* engineered tissues *in vitro* for subsequent implantation into tissue defects *in vivo* remains experimental, despite some early clinical successes [8]. In this approach, combinations of cells and bioactive molecules are seeded onto three-dimensional biomaterial scaffolds [9–14]

Cells can be retrieved from a variety of sources, including embryonic stem cells, postnatal and adult stem/progenitor cells, or the most recently discovered induced pluripotent stem cells (iPS). The common approach in engineered tissue regeneration has been to isolate cells from tissue biopsies or aspirates, manipulate them and reintroduce them into the host [12]. For bone regeneration, multiple cell sources have been investigated, including fresh bone marrow aspirates [7]; purified, culture-expanded bone marrow mesenchymal stem/progenitor cells [15], osteoblasts and cells that have been modified genetically to express osteogenic factors such as rhBMP [16], umbilical cord blood cells [17], adipose derived stem/progenitor cells [18], or embryonic stem cells [19]. Perceived advantages and disadvantages of these cell sources in bone tissue engineering have recently been reviewed [6].

One of the pivotal challenges of cell transplantation is the cost and complexity associated with the development of experimental strategies into regulatory approved products. Intra-operative cell processing, while immune from regulatory approval, can only serve as a *point-of-care* service for one patient at a time. Once the cells are manipulated off site, regulatory approval is automatically required. Cell transplantation has encountered a number of barriers towards clinical translation, including potential immune rejection for non-autologous cells, pathogen transmission, potential tumorigenesis, costs associated with packaging, storage and shipping, shelf life and reluctance of physicians and insurance in clinical adoption [6]. Cell survival in the host is also an unsettled issue, regardless of the cell source, and there is debate on whether the transplanted cells are regenerative *per se* or simply act as a pleiotropic source of factors and signals, especially in their ability to regulate inflammation [19a]. These barriers will continue to be challenges for the implementation of engineered bone as a clinical treatment in foreseeable future. An alternative paradigm is to activate endogenous stem cells to participate in bone regeneration. A case in point is periosteal progenitors cells, which are activated by injury and play an indispensible role in fracture repair [20,21]. Whether simple mobilization and homing of endogenous stem cells to the defect site will suffice for regeneration and have advantages over exogenous cell transplantation remains to be proven.

The assembly of the cells into the required three-dimensional (3D) form of the bone defect requires a scaffolding biomaterial that delivers and retains the cells, and potentially stimulates and guides their induction of tissue regeneration. The minimum requirements of biomaterial scaffolds in addition to sustenance of *Form* (3D shape and size) include *Fixation* (securing the attachment to the host bone and minimization micro-motion), *Function* (establishment of temporary or permanent mechanical load bearing), and *Formation* (provision of appropriate porosity for mass transport, revascularization, osteoinduction and osteoconduction) [22]. Additional biocompatibility characteristics must also be met in biomaterial scaffolds including

the lack of immunogenicity and toxicity. Furthermore, scaffolds can be enhanced by surface functionalizing to elicit affinity to cell binding and interactive modulation of the cells' response, and can be designed for localized, controlled delivery of various bioactive molecules.

Scaffolds can derive from native tissues and biological polymers and/or synthetic polymers, and can be fabricated using a variety of conventional techniques (reviewed in [23]). Among these techniques, solid freeform fabrication (SFF) offers distinct advantages in enabling exquisite control of the scaffold form and internal architecture based on medical image-guided 3D modeling of the bone defect [24]. Recently, 3D-bioprinting has been enabled by the commercial availability of low temperature, high resolution, multi-injector 3D printing systems, which were originally developed for rapid prototyping applications. This technology has been successfully adapted for bone tissue engineering with biocompatible and osteoinductive calcium phosphate powder and biocompatible binder system for CT-guided 3D printing of patient-specific scaffolds [25]. The multi-injector capabilities of a *colored* 3D printer potentially allow the embedding of combinations of biofactors and molecules within the scaffold with spatial control, which can be attractive in scenarios that might require spatiotemporal control over release kinetics. However, a recent review of scaffolds for bone tissue engineering has painted a bleak picture for translational progress of the field [22], which remains riddled with technical challenges of designing, manufacturing, and functionalizing scaffolds, regulatory approval barriers, business challenges related to meeting identifying niche markets and generating large initial investments necessary to sustain the business through the long-drawn-out regulatory process, and intellectual property (IP) lifecycle issues that must protect the product long enough beyond the regulatory process to recoup the investment and make these products commercially viable.

Delivery of biofactors and molecules can alter cell signaling in the defect milieu and has been shown to influence the outcome of regeneration. A popular paradigm in tissue engineering suggests that reactivating developmental factors and signaling might be necessary for true regeneration of the lost adult tissue [26]. However, it is unclear whether the complex developmental signaling gradients and cascades need to or can be replicated faithfully in postnatal tissue repair. Regardless, our understanding of the developmental biology of the musculoskeletal system, and more specifically endochondral and intramembranous bone formation in the embryo provides us with a plethora of information about factors, which when applied individually can enhance bone regeneration. This latter, simpler approach is preferable for therapeutic translation.

A case in point has been the discovery of bone morphogenetic proteins (BMP), which were discovered by Marshall Urist and touted for their osteoinductive properties [27–29]. Basic science studies using transgenic mice in which individual BMPs have been selectively knocked out from the limb skeleton have identified BMP-2 as a critical factor in the innate regenerative capacity of bone [30]. The combination of recombinant human BMP-2 on an absorbable collagen sponge (ACS) carrier has been one of the most studied systems in preclinical and clinical investigations, and represents one of the most significant therapeutic orthopedic discoveries [31]. With supporting level 1 clinical trial data, rhBMP-2/ACS (INFUSE® Bone Graft) is commercially available, at the time of this writing, for three Food and Drug Administration (FDA)-approved clinical indications including spinal fusion, open tibial fractures with an intermedullary (IM) nail fixation, oral and maxillofacial augmentation (sinus augmentations, and alveolar ridge augmentations for defects associated with extraction sockets) [31]. However, the efficacy of INFUSE Bone Grafts requires supraphysiological concentrations of BMP-2, and numerous adverse events have been filed at the FDA and reported in the literature in approved indications and off-label uses [32,33]. Therefore, the identification of effective doses of BMP-2 (and perhaps other osteogenic and vasculogenic factors) for the regeneration of critical bone defects, preferably with tolerable and subclinical side effects, remains a common challenge for tissue regeneration community.

An exciting prospect has been the discovery of the therapeutic value of systemic hormones such as parathyroid hormone (PTH) in fracture repair [34–37] and tissue engineering of critical diaphyseal defects in preclinical models [38–40]. This systemic delivery approach might overcome the challenges associated with local delivery, but remains to be clinically validated.

To sum, each of the individual components of the conventional tissue-engineering triad (cells, scaffolds, and biofactors) brings unique sets of challenges. Optimization of these *composite* constructs into *functional* tissue substitutes, is typically performed empirically in the laboratory *ex vivo* using cell and tissue culture models and *in vivo* using preclinical animal models. However, this approach has faced difficult barriers to translation from the bench to the bedside. A three-component medical product would have at least 3^{n_i} possible combinations of independent variables (where n_i is the number of possible variables associated with the i^{th} component of the three-component product), which makes the feasibility of testing the experimental matrix in a comprehensive investigation impossible. This has limited advances in the field to only incremental discoveries, despite exciting developments and breakthrough technologies that have been reported in small animal and preclinical models. The regulatory requirements of the multicomponent bone regeneration products have hindered and continue to slow down clinical translation. Nevertheless, innovative '*point-of-care*' regenerative approaches guided by the tissue engineering paradigm have been reported in the clinical literature with remarkable early successes.

CLINICAL SUCCESSES AND OPPORTUNITIES IN REGENERATIVE REPAIR OF DIAPHYSEAL DEFECTS

Segmental diaphyseal defects are common in long bone in scenarios involving tumor resection, trauma, and debridement following infections or atrophic non-unions. Previously, substantial bone defects have required amputation. In the 1960s an innovative technique for *in vivo* bone regeneration was developed in Russia by G.A. Ilizarov [41,42,62]. In the late 1980's this technique, referred to as distraction osteogenesis was extended into Western Europe and the United States. Distraction osteogenesis is a process in which a bone is gradually separated at an osteotomy site. The bone on either end of the osteotomy is stabilized with an external fixation device (Fig. 83.1a). The regeneration of bone involves three stages [43].

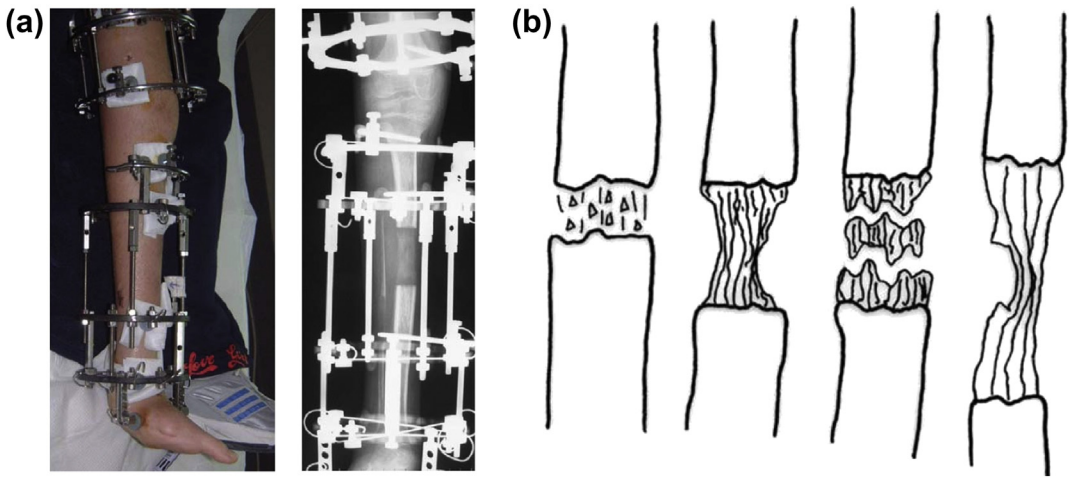

FIGURE 83.1

Bone regeneration using distraction osteogenesis. (a) Typical distraction osteogenesis hardware (frame and rings) is shown. (b) Distraction osteogenesis proceeds in stages, the first of which is the '*latency*' stage during which the injury and repair process initiates and fracture callus forms, followed by the '*distraction*' stage in which the bone is gradually distracted through the osteotomy site at a rate of approximately 1 mm per day resulting in a fibrous interzone composed of mesenchymal progenitors. The interzone eventually re-vascularizes, and the cells differentiate and form regenerate bone through intramembranous and endochondral ossification. Finally, in the '*consolidation*' stage, the immature bone that formed in the defect is remodeled into lamellar bone. *(Reproduced with permission from Catagni et al., 2011.)*

The initial stage is termed latency and consists of a 5–10 day period of during which the injury and repair process initiates and fracture callus forms. During the second, or distraction phase, the bone is gradually distracted through the osteotomy site at a rate of approximately 1 mm per day. Separation of the bone results in a repetitive tensile force in the distraction gap between the bone segments. In the center of the distraction a fibrous interzone composed of mesenchymal progenitors forms, while at each of the osteotomy surfaces bone the tissue is re-vascularized, and the cells differentiate and form regenerate bone through intramembranous and endochondral ossification. The final phase of the process is termed 'consolidation' in which the immature bone that formed in the defect is remodeled into lamellar bone (Fig. 83.1b).

Distraction osteogenesis has been used successfully to reconstruct limb defects resulting from tumor, trauma, infection, and developmental diseases [44–46]. The approach is also frequently used for the treatment of craniofacial defects [47]. The process of bone formation in distraction osteogenesis is dependent upon the mechanical stimulation of the healing bone callus that is generated by the tensile forces resulting from the daily separation of the bone fragments [43,48]. The tensile force results in the release of cytokines and growth factors that stimulate the proliferation and differentiation of mesenchymal stem cell progenitors and lead to angiogenesis and re-vascularization of the tissue. Growth factors associated with distraction osteogenesis include BMPs, VEGF, FGF, and Wnt signaling factors [49–51]. Thus distraction osteogenesis includes the various features associated the successful engineering of regenerate bone: stabilization of the skeleton, mechanical stimulation, recruitment and stimulation of stem cell populations, angiogenesis, and bone incorporation and remodeling.

Despite these advantages, distraction osteogenesis is not an ideal approach to regenerate bone. While effective, distraction osteogenesis has a high complication rate. The stabilization of the bone with an external frame connected to pins or wires inserted into the bone are associated with pin tract infections in nearly all cases. Bone lengthening is also associated with a risk of joint contractures or stiffness, nerve palsy, and limb deformity [52]. Moreover, the entire process, and particularly the consolidation phase, requires an extensive period of time. In patients with war-related bone loss, fixation was maintained at approximately 1.5 months per centimeter of bone regeneration [44].

One area of need for bone tissue regeneration involves device-associated infections. These are typically treated with a two-stage reconstruction approach. This approach involves complete removal of the hardware and tissue debridement, and later reconstruction after eradication of the infection with antibiotics [53,54]. The use of poly(methyl methacrylate) or PMMA cement spacers impregnated with antibiotic agents in two-stage reconstruction has been common practice in Europe and has experienced widespread clinical use in US hospitals over the past few years [55,56]. These temporary spacers, which are typically installed after tissue debridement, are intended to provide sustained local elution of effective tissue concentrations of antibiotics to treat the infection while maintaining limb length, preserving tissue stock, minimizing soft tissue fibrosis and contraction, and in the case of articulating spacers, maintaining joint mobility until the lost tissue is reconstructed [57].

An innovative regenerative approach to treat this complicated scenario follows the two-stage guidelines of the Masquelet technique [58], which described the *in situ* formation of a periosteum-like pseudosynovial membrane around temporary antibiotic-eluting bone cement implants, which if preserved can provide nutrition and vascularization during the regenerative phase of the reconstruction. Fig. 83.2a shows an X-ray of a classic fractured tibia with atrophic non-union and clinical evidence of osteomyelitis [4]. Stage 1 treatment of this case involved thorough debridement of the infected, necrotic bone and the placement of vancomycin-eluting polymethylmethacrylate cement beads in the resulting defect alongside the intramedullary nail (Fig. 83.2b). This resulted in eradication of the infection and the

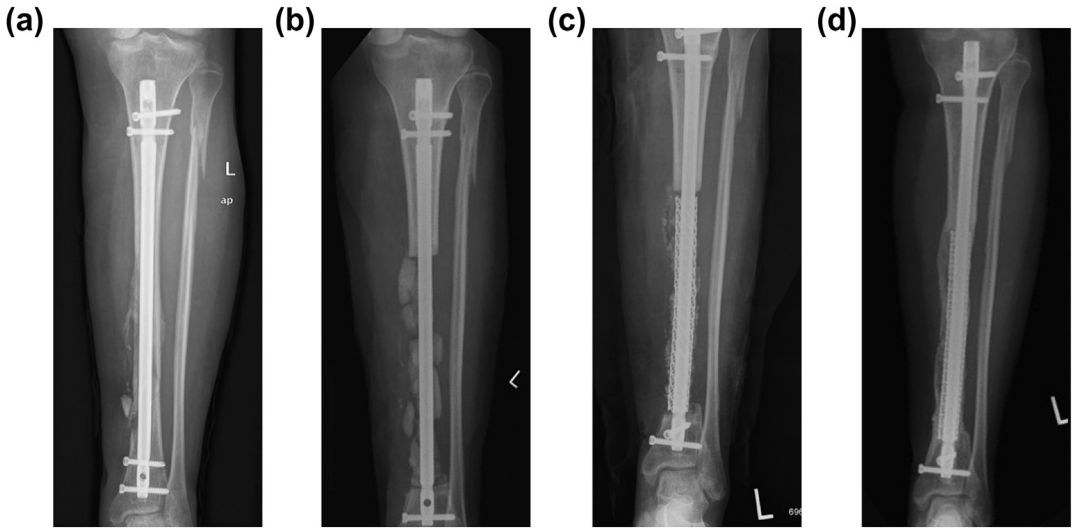

FIGURE 83.2

Tissue engineered reconstruction of a Methicillin-resistant Staphylococcus aureus (MRSA) infected tibial non-union. (a) Radiograph of the MRSA infected tibia five months after fracture, with no clinical evidence of healing. (b) Radiograph of the infected tibia after the 1st stage of the Masquelet technique, which involves resection of 17 cm of necrotic bone and insertion of vancomycin-eluting cement. (c) Radiograph following the second stage of the Masquelet technique, which involves exchanging the IM nail, with spinal cage support for INFUSE® Bone Graft and autologous cancellous bone reamings. (d) Radiograph of the tibia 1 year following the two-stage Masquelet reconstruction, showing bone regeneration in the massive tibial defect. *(Reproduced with permission from O'Malley and Kates, 2012.)*

endogenous formation of a pseudosynovial membrane over almost one year. The second stage then involved removal of antibiotic-eluting cement beads with careful preservation of the nutritive, induced biologic membrane, and subsequent installation of two titanium 15 mm diameter spinal cages to provide structural scaffolding for osteoinductive rhBMP-2 (INFUSE® Bone Graft) that was placed 'off-label' in the defect (Fig. 83.2c). To introduce BMP-2 responsive cells, autogenous cancellous bone and bone marrow were mixed with β-tricalcium sulfate granules (chronOS Bone Void Filler) and used to augment the defect, and the biological membrane was closed around the implanted biologics, morselized cells and tissue, and metallic scaffold. Four months following reconstruction, there was radiological evidence of new bone formation, and one year later there was resumption of independent ambulation with evidence of radiological and clinical healing [4] (Fig. 83.2d).

A similar approach to treat defects created by tumor resection exploits the endogenous osteoinductive properties of the periosteum and the osteoprogenitors it harbors. The concept is to circumferentially elevate a periosteal membrane off of healthy diaphyseal bone adjacent to the bone defect, and to then osteotomize the healthy bone (sans the periosteum) and transfer it to the defect out of the periosteal membrane. The retained periosteum with its soft tissue attachment maintains vascularity and osteogenesis can be augmented with cancellous bone grafts [59] (Fig. 83.3a). The feasibility of this approach was demonstrated in an osteosarcoma case by the reconstruction of an iatrogenically created defect in the fibula following lifting and *in situ* retention of its periosteal membrane (Fig. 83.3b). The fibula graft was transferred to augment regeneration of a surface defect in the tibia that resulted from resection of a low-grade surface osteosarcoma. The periosteum membrane that was left behind was activated *in situ* to serve as an osteoinductive and osteoconductive sleeve, and led to complete regeneration of the fibula defect within just six months (Fig. 83.3c).

The two approaches described above utilize the principles of tissue stabilization, recruitment/delivery of stem cells, bone tissue differentiation, angiogenesis and remodeling. The approaches also represent a presonalized *point-of-care* method of tissue engineering of critical

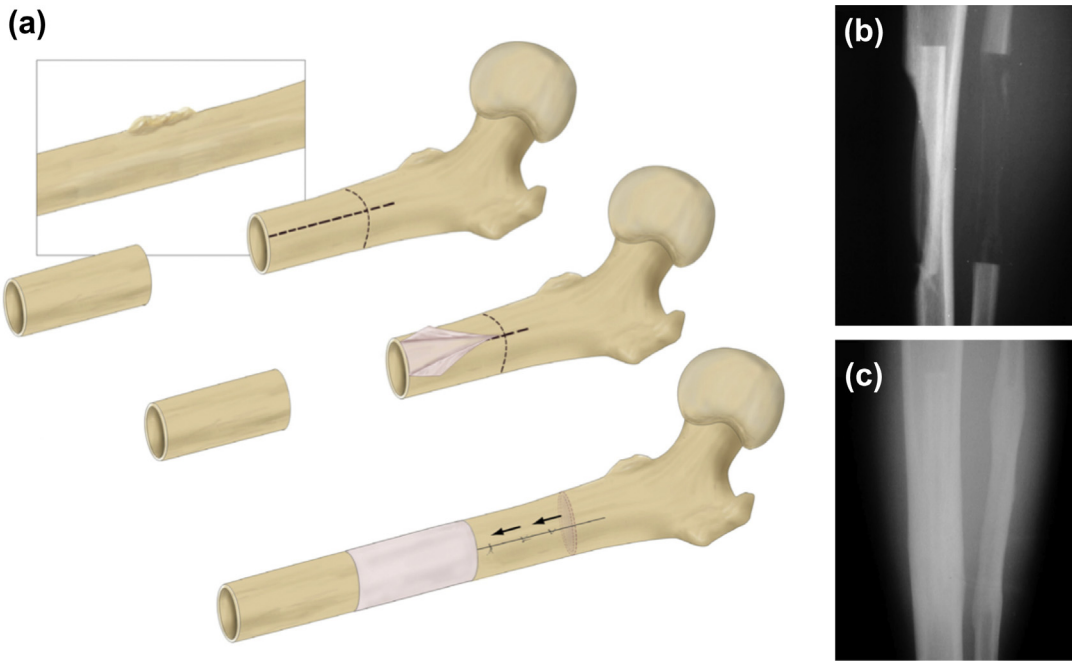

FIGURE 83.3

Exploiting the inherent regenerative capabilities of the periosteum to reconstruct the massive diaphyseal defects. (a) Schematic representation of the concept, which involves circumferential elevation a periosteal membrane off of healthy diaphyseal bone adjacent to the bone defect, and to then osteotomize the healthy bone (sans the periosteum) and transfer it to the defect out of the periosteal membrane. In theory, the retained periosteum with its soft tissue attachment maintains vascularity and osteogenic progenitors. (b) Radiograph showing the reconstruction of a tibia with malignant cortical tumor with an autologous, pedicled fibula transfer, following elevation and *in situ* retention of the fibular periosteum. (c) Complete periosteum-enabled regeneration of the fibula is achieved by 3 months post reconstruction. *(Reproduced from Knothe and Springfield, 2005, under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).)*

diaphyseal defects. They clearly borrow from the first principles of conventional tissue engineering, but circumvent the regulatory process via '*off-label*' use of several approved products (e.g., INFUSE, chronOS, bone marrow, etc). These cases represent individual success stories and demonstrate the safety and efficacy of biologics, scaffolds, and cells without *in vitro* manipulation; an alternative paradigm in tissue engineering that has been recognized for some time but one that has received less attention in the basic science literature. Advances in image-guided fabrication of biocompatible, osteoinductive scaffolds such as 3D bioprinting, which in theory enables simultaneous printing of biofactors and antibiotics within the patient-specific scaffold for sustained release can potentially lead to single stage reconstruction procedures, accelerated recovery time, and improved the clinical outcome.

CLINICAL SUCCESSES AND OPPORTUNITIES IN REGENERATIVE REPAIR OF CRANIOFACIAL DEFECTS

The face distinguishes one human being from another. When the face is disfigured because of trauma, tumor removal, congenital anomalies, or chronic diseases, there is a critical need for functional and esthetic reconstruction [6]. These scenarios lend themselves to image-guided scaffold fabrication to restore the original shape of the face or jawbone. Success in this approach was first demonstrated by a report in the Lancet [7]. In this case, the patient, who lost a significant part of their mandible due to cancer, underwent 3D computed tomography (CT) of the head and computer-aided design (CAD) to generate an idealized virtual replacement of the missing parts of the mandible (Fig. 83.4a,b). A titanium mesh scaffold was then

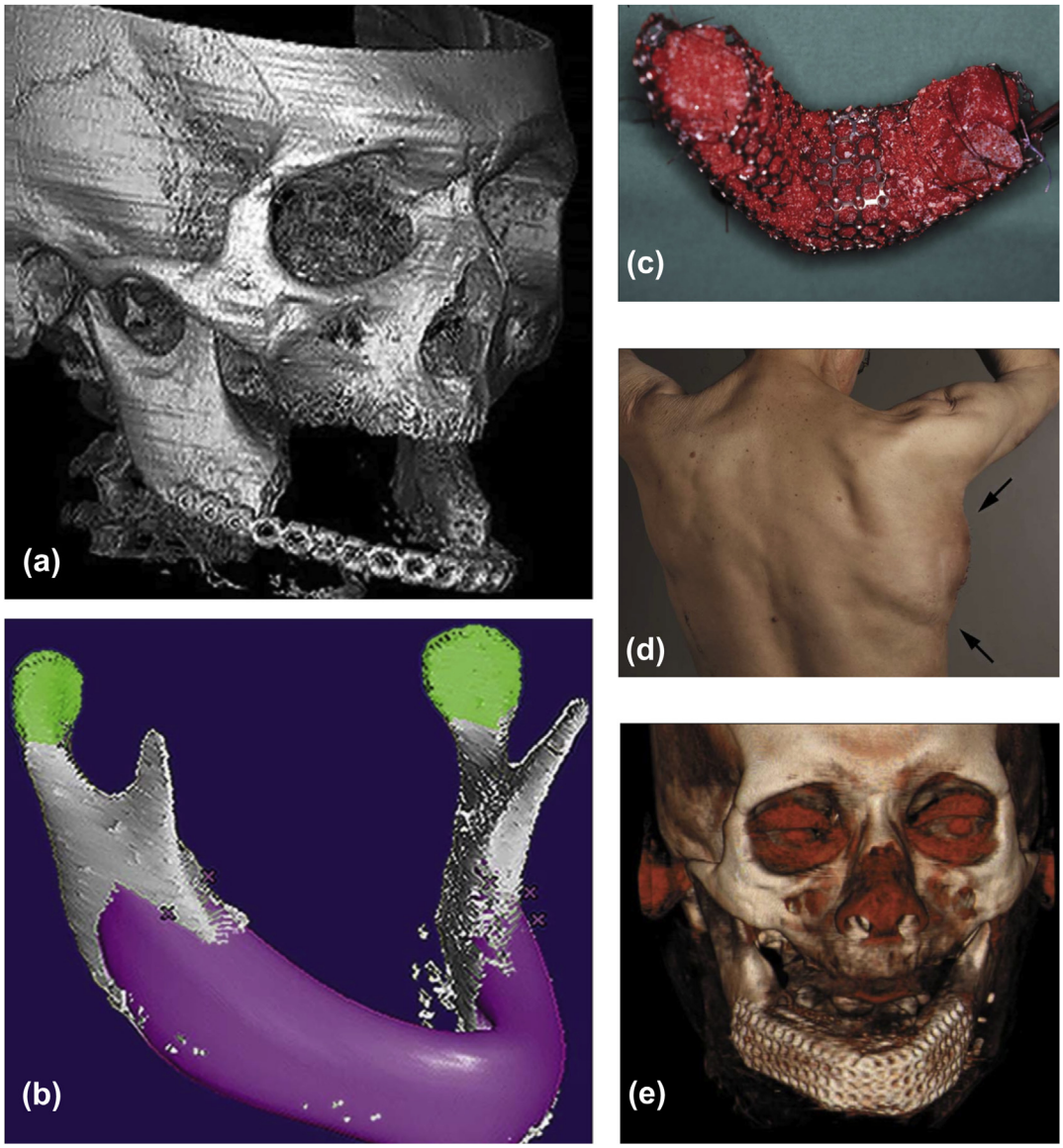

FIGURE 83.4
Image-guided tissue-engineered reconstruction of a massive mandibular defect. (a) The region of interest (jaw) is imaged using 3D computed tomography (CT). (b) The CT data is then fed to computer-aided design (CAD) software to generate an idealized virtual replacement of the missing parts of the mandible. (c) A titanium mesh is then formed in the shape of the missing bone model and augmented with BioOss hydroxyapatite blocks, OP-1 collagen implant, rhBMP-7, and autologous bone marrow aspirate. (d) The engineered mandibular graft is implanted in a heterotopic muscular pouch in the patient to establish vascularization and initial osteogenesis. (e) The graft was finally implanted orthotopically to reconstruct the mandibular defect. The patient had functional mastication and satisfactory aesthetic outcome. *(Reproduced with permission from Warnke et al., 2004.)*

formed in the shape of the model, and was filled with bone mineral blocks (BioOss-Blocks) and augmented with bovine collagen type 1 (OP-1 implant) soaked with recombinant human BMP-7, and an autologous bone marrow aspirate to provide BMP-responsive osteo-regenerative cells (Fig. 83.4c). The titanium mesh cage was then implanted heterotopically into a pouch of the patient's right latissimus dorsi muscle (Fig. 83.4d) to establish vascularization and initial osteogenesis prior to orthotopic implantation to reconstruct the mandible (Fig. 83.4e). Skeletal scintigraphy bone scans showed evidence of uptake indicative of viable bone metabolism within the engineered mandible at both the heterotopic and orthotopic implantation sites, and post-operative CT imaging demonstrated radiographic evidence of healing and incorporation, with patient reported functional improvements in

chewing and a satisfactory aesthetic outcome [7]. Other reports have since followed suit and demonstrated similar patient-specific, *point-of-care* reconstruction of the mandible with successful functional and aesthetic outcomes [60,61].

CONCLUSIONS

Despite regulatory bottlenecks, surgeons and scientists will likely, and should, continue to push the frontiers of what is surgically possible, armed with innovations in cell-free tissue engineering products, their skill and ingenuity, and most importantly their professional ethics and the vow they live by to do no harm. Cell-free refers to the recruitment of endogenous cells, including stem/progenitor cells, towards bone regeneration. Cell-free approaches arguably do not represent orthodox or conventional medical or tissue-engineering products from a regulatory or business standpoints, and the debates might not be settled by these n = 1 clinical reports, a far cry from randomized clinical trials required for level 1 data of safety and efficacy for regulatory approval. Nevertheless, these cases and similar ones not described herein successfully restored form, function, and quality of life to the afflicted patients. These *point-of-care* innovations might just be the bridge to wide clinical translatability and adoption of bone tissue-engineering approaches and products.

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CHAPTER 84

Tissue-Engineered Cardiovascular Products

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INTRODUCTION

Cardiovascular disease is by far the most common reason for morbidity and mortality in the Western world. It comprises malfunction of heart valves, blood vessels, and myocardium, all of which are subject to tissue-engineering programs. The development of tissue-engineered heart valves is fueled by the idea to create a 'growing valve' for children with congenital cardiac malformations and a 'totally autologous biological valve' for adults with acquired valve defects. Here the tissue-engineered product has to compare favorably to the existing spectrum of quite sophisticated and long-term approved biological and mechanical prostheses. Recent clinical trials with decellularized homografts, i.e., variations of standard procedures rather than truly tissue-engineered products, gave promising results [1–2]. Blood vessel engineering predominantly aims at generating small-diameter vessels (<6 mm; e.g., coronary arteries). Driving forces in the field are the low long-term patency rates of classical saphenous vein grafts and the paucity of suitable arterial vessels in coronary bypass surgery. The first fully engineered medium diameter blood vessels have been successfully applied in 10 patients with end-stage renal disease as arterio-venous fistulas for chronic hemodialysis, representing exciting progress in the field [3]. Myocardial tissue engineering aims at creating force-developing cardiac tissue patches to correct cardiac congenital malformations in children ('the growing patch') and replacing or supporting heart muscle function after myocardial infarction or in patients with cardiomyopathies. The total bioartificial heart remains a distant goal, despite some encouraging successes. Engineered human heart tissues are on the cusp of entering preclinical drug development [4] and the first patient with heart failure has been treated with a stacked cell sheet construct, not with cardiac but skeletal muscle cells [5]. The relatively slow progress of cardiovascular tissue engineering compared to e.g., skin engineering has two major causes. On the one hand, the risk is higher than in other fields. Failure of a heart valve, a coronary artery conduit, or a load-carrying heart muscle patch would have disastrous consequences. On the other hand, cardiovascular cells (myocytes in particular) cannot or not easily be

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propagated and therefore require the use of pluripotent stem cells with their inherent limitations.

This chapter gives an overview of current development of the three major tissue-engineered cardiovascular products (valves, vessels, and myocardium), but focuses on myocardial tissue patch engineering, being the field the authors are actively involved in. For more thorough reviews on valves and vessels see [6–9].

CLINICAL NEED FOR TISSUE-ENGINEERED CARDIOVASCULAR PRODUCTS

Cardiovascular medicine has been among the most successful medical fields since the mid-1980s. Its contribution to the increase in life expectancy in the United States is impressive (3.9 out of a total of 6 years) and outnumbers that of all other fields [10]. Yet mortality and morbidity from cardiovascular causes remain high, and it is unquestionable that new therapeutic paradigms must be introduced.

Heart valves

Malfunctioning heart valves are generally replaced surgically by either mechanical or biological prostheses, a procedure performed in more than 100,000 patients per year in the United States and 280,000 worldwide [11]. While valve replacement is often life-saving and has a tremendous impact on quality of life, drawbacks do exist. Mechanical prostheses require lifelong anticoagulation, with its potential hazards, and are still associated with a markedly increased rate of thromboembolism (4% per year [12]). Biological prostheses do not share these problems, but they have a limited half-life. None of the existing models grow, with the consequence that children with congenital valvular malformation have to be re-operated on several times during childhood and adolescence. The ideal tissue-engineered living valves would overcome all of these problems. Yet the strength of these arguments has to be critically weighed. In particular, the best biological prostheses, such as the aortic homografts and the Edwards pericardial valve, have a durability close to 20 years [13]. In fact, this will often be 'lifelong', given that the majority of patients undergoing valve replacement are over 50 years old. Moreover, surgical techniques have improved, and the high mortality rate associated with repeated valve replacements (formerly approximately 20%) has gone down considerably to approximately 4% [6], allowing the option of repeated use of bioprosthetic valves rather than implanting a mechanical valve. Thus, progress through tissue engineering appears most likely to be relevant in children with congenital malformations, where the availability of growing valves would constitute a major advancement. Most effort is directed towards this aim.

Blood vessels

Considering that the market for heart valves is large, the need for blood vessels is even larger. Every year, 500,000 procedures are being performed that involve small-caliber blood vessels just in the United States [7]. For many years, poly(ethylene terephthalate) (PET; Dacron) or poly(tetrafluorethylene) (PTFE; Teflon) grafts have been in clinical use for the replacement or repair of large-diameter blood vessels, such as the aorta and the carotid arteries, with good results [14,15]. In contrast, replacement of small-diameter vessels, such as coronary arteries, still requires venous or arterial autografts. The standard saphenous vein graft as used in coronary artery bypass grafting (CABG) has a high risk of acute or chronic vessel occlusion and requires a second surgical procedure. This does not apply to arterial autografts, such as the internal mammery artery, but this graft is often not available in sufficient number (e.g., for a three-vessel disease) or quality. Another important clinical application is arterio-venous (AV) fistulas needed for patients undergoing chronic hemodialysis, currently the most common vascular surgery in the US. Thus, there is a clear need for tissue-engineered small arteries.

Myocardial patches

Compared to current treatment options (drugs, devices, transplantation, prostheses, cell therapy), cardiac repair with tissue-engineered myocardial patches appears ambitious. Yet there are at least two areas where tissue engineering could prove to be valuable. The first, and maybe the most obvious, is reconstructing malformed hearts in children [16]. The other, quantitatively more important, is repairing infarcted or failing hearts in adults [17]. Heart malformations that might benefit from restoration using biological, autologous muscle patches include several forms of ventricular dysplasia, such as the hypoplastic left heart syndrome or tricuspid atresia with right ventricular dysplasia. Right now most of these infants undergo palliative surgery (a univentricular correction) ('Fontan-surgery'). Biologic active muscle patches might allow biventricular correction and true ventricular restoration. In addition, indications for a right ventricular outflow tract patch plasty are quite frequent in patients with pulmonary atresia or 'Fallot'-type malformations, which might also benefit from a biological solution. In contrast to cardiac malformations, myocardial infarction and its consequences belong to the most common diseases with huge socioeconomic impact. Present cell-based clinical studies employed bone marrow-derived cells and heart-derived progenitors in patients with relatively well-preserved left ventricular (LV) function after acute myocardial infarction and yielded mixed results [18]. The patient population included in these trials is large and easy to study, but not best-suited for tissue-engineering strategies, because it is 'not sick enough' and existing therapies are effective. Implantation of tissue patches could be rather considered in patients with chronic heart failure resulting from large myocardial infarctions. These patients might in fact benefit from a combination of tissue-engineered patch grafting, ventricular restoration surgery ('Dor-plasty'), surgical revascularization, and valve repair, although the data of the recently published 'Stitch'-trial are somewhat controversial with respect to the benefit of ventricular restoration [19]. A combination of the Dor-plasty and subsequent implantation of a contractile patch seems especially promising and would ideally enable functional regeneration of the ventricle [20]. Whereas it is hardly disputable that numerous patients would profit from a direct support of contractile function, the question is whether tissue-engineered patches could play a significant role in this scenario when compared to current alternatives.

1) Mechanical left ventricular-assist devices (LVADs) acutely support the pumping activity of the left ventricle and unload the heart, a process leading to 'reverse remodeling' and improved microvascularization [21], rarely even to recovery [22]. The established place of LVADs has been bridging to transplantation. Since the new third generation LVADs are much smaller in size, easier to implant and show less device-related complications, they are increasingly used as long-term, so-called 'destination-therapy'
2) Implantable defibrillators/cardioverters protect against lethal arrhythmias and thereby prolong survival [23], but in themselves have no effect on contractile function and heart failure symptoms. In contrast, the negative impact on patient's well-being is increasingly recognized [24].
3) Cardiac resynchronization therapy by means of biventricular pacing, in contrast, leads to improved heart function and has a documented effect on survival [25]. Yet only a limited number of patients (those with conduction abnormalities) profit from this new therapy.
4) The ACORN CorCap device, a bag-like polymer-mesh that prevents the heart from further dilatation [26] may be valuable in a limited subset of patients with large ventricles, but it is also hampered by severe complications, e.g., constrictive cardiomyopathy–like symptoms.

Thus, heart transplantation remains the only curative therapy for terminal heart failure. However, the number of candidates largely exceeds the number of suitable donor organs, and this ratio has not improved over the years [27].

When considering a tissue-engineering strategy for patients with terminal heart failure, three scenarios can be envisioned. The simpler one is to add patches of tissue-engineered heart

muscle on the diseased heart. A more elaborated and invasive one is to excise non-functioning myocardium and replace it with an engineered heart muscle patch. Obviously, the latter approach requires high mechanical stability of the patch to withstand systolic pressure, and this may be quite difficult to achieve. Finally, replacement of the entire heart by a fully engineered new heart is a thinkable, as yet unrealistic option. At this point, the addition of tissue-engineered patches to the heart seems to be the most realistic perspective.

CONCEPTS AND ACHIEVEMENTS IN ENGINEERING CARDIOVASCULAR PRODUCTS

The principal strategies in tissue engineering of valves, vessels, and myocardium are quite similar and have been developed in parallel, partly independently of each other (Table 84.1). They can be broadly categorized into five approaches:

1) Decellularization of native tissues (valves, vessels),
2) Re-cellularization of decellularized native tissues,
3) Cell seeding onto biodegradable polymer scaffolds,
4) Cell entrapment in naturally occurring biogels such as collagen I, Matrigel, and fibrin, and
5) The cell sheet technique.

Consistently, tissue formation can be significantly improved by mechanical loading and electrical stimulation.

Decellularized tissue

Decellularization of native tissues is a simple technique and, in fact, does not require any specific tissue engineering or cell biology procedure. Driven by companies such as CryoLife, decellularized valves and vessels entered the clinics decades ago. Conceptionally, the natural tissue provides the ideal 3D form, and decellularization is expected to reduce immune reactions directed against the cellular component of the valve. Indeed, immune responses have been detected in patients receiving cryopreserved allografts [28] and may limit the long-term function of the graft. This problem seems particularly relevant in children with their high immunological competence [29]. On the other hand, the process of decellularization, usually performed with hyper- or hypotonic solutions, mild detergents such as Triton X-100, sodium dodecylsulfate (SDS) or sodiumdesoxycholate, and proteolytic enzymes, exerts detrimental effects on the matrix that can give rise to thromboembolic complications and mechanical instability of the graft. Moreover, porcine extracellular matrix itself also seems to be immunogenic [30]. This may be one reason of the catastrophic failure of the first series of implantations of decellularized porcine valves in children in Vienna, Austria, in which at least three children died [31]. The exact number of patients that received these grafts has never been reported. Despite these disastrous results, a series of 50 patients in Berlin received such a graft during a Ross operation (replacement of the defective aortic valve by the endogenous pulmonary artery valve and implantation of the porcine valve in the pulmonary, low pressure position), with apparently good results [32]. However, there is evidence that a number of patients have indeed died from valve-related complications [6], casting serious doubts on the xenogenic approach. In contrast, decellularized, cryopreserved valved allografts (Synergraft, CryoLife) are a variant of the current gold standard of cryopreserved homografts and have been clinically evaluated in larger series of patients. As expected, these grafts did not induce immune responses [33] and compared favorably with standard cryopreserved allografts [1,34,35]. Lower peak transvalve gradients suggest better preservation and long-term outcomes, but the rates of insufficiency were similar [1]. More data are needed to draw definite conclusions. Another variation is freshly decellularized (unpreserved) pulmonary valves. In a recent report, they compared favorably with conventional cryopreserved homografts and valves made from glutaraldehyde-fixed bovine jugular vein [2]. Evidence was provided that the fresh

TABLE 84.1 Milestones in cardiovascular tissue engineering. The format was adapted from [8].

Approaches		First introduction	*In vitro*: animal cells	*In vivo*: animal model	*In vitro*: human cells	*In vivo*: human in animal	Clinical use
Blood vessels							
Cell-seeded synthetic grafts		1987	Dog autologous venous EC [104]	Dog [104]	Saphenous vein cells [105]	–	Infrainguinal [105–106]
Cell-seeded bioresorbable scaffolds		1998	Sheep artery/ venous cells on PG/PGA [107]	Sheep PA [107]	–	–	PA in 25 pts. [108–109]
		1999	Bovine SMC/EC on PGA+bioreactor [60]	Bovine in pig [60]	hTERT-modified SMC [110]	Human in baboon as AVF, dog in dog+/– autologous EC CABG [48]	–
Cell-seeded hydrogels	Collagen	1986	Bovine SMC and EC (collagen tube + Dacron mesh) [57]	Rabbit vena cava [111]	Human SMC, EC, FB [59]	–	–
	Fibrin	2003	Rat neonatal/adult [112]; Sheep SMC + EC [67], sheep SMC/FB in fibrin+PLA mesh [113]	Sheep jugular vein [67], sheep carotid artery [114]	Human dermal FB [66]	–	–
In vivo bioreactor		2007	–	Rat, rabbit [92]	–	–	–
Cell sheet based biological grafts (TESA)		1998	–	–	Human umbilical cord SMC, HUVEC, dermal FB [68]	Nude rats [58]	AVF in 10 pts. [3]
Heart valves							
Decellularized xenografts		1999	NA	Porcine PV in sheep [115]	NA	NA	Children, PV (stopped [31]; adults, Ross proc. [32]
Decellularized allografts		2001	NA	Sheep PV in sheep [116]	NA	NA	Children Ross/ RVOT, n=47 [1], n=342 [34], n=38 [2]
Cell-seeded decellularized allografts (bioreactor)		2006		Sheep PV repopulated with autologous EC [42]	Peripheral MNC-seeding of decellularized PV allograft, 21 d in bioreactor [42]		Children dec. allograft repopulated with peripheral MNC, n=2 [43]

Continnued

TABLE 84.1 Milestones in cardiovascular tissue engineering. The format was adapted from [8].—*continued*

Approaches	First introduction	*In vitro*: animal cells	*In vivo*: animal model	*In vitro*: human cells	*In vivo*: human in animal	Clinical use
Cell-seeded bioresorbable scaffolds	− Decellularization	2000	Sheep myoFB/EC on PGA/P4HB scaffolds, 14 d bioreactor [117]	Sheep TE-PV, 20 wks [117]	–	–
	+Decellularization	2012	Sheep myoFB on PGA/P4HB scaffolds, 28 d bioreactor, decellularization [49]	–	–	–
Heart muscle						
Cell-seeded bioresorbable scaffolds	Collagen sponges	1999	Fetal rat CM on collagen sponges [52]	Rat, infarcted heart [52]	–	–
	Alginate	2000	Fetal rat CM on alginate discs [53]	Rat, infarcted heart [53]	–	–
	PGA	1999	Neonatal rat CM on woven PGA discs [118]	–		
	Silk	2012	Neonatal rat CM on silk fibroin discs [56]	–	–	–
Cell-seeded hydrogels	Collagen	1997	Embryonic chick CM in collagen [61]	Rat, infarcted heart [65]	hESC/ hiPS-derived CM [119]	hESC-derived patch on athymic rat heart [120]
	Collagen/ Matrigel	2000	Neonatal rat CM in collagen/Matrigel rings [73]			
	Fibrin	2009	Neonatal rat CM in self-organizing fibrin layers [63]	–	–	–
In vivo bioreactor		2005	NA	Neonatal rat CM in fibrin [121] or Matrigel [92] in chamber on femoral A/V; transplant on heart [93]	NA	–
Cell sheet based completely biological grafts		2002	Neonatal rat CM on thermo-sensitive surfaces [72]	Multilayered sheet on infarcted rat heart [94]	–	hESC+adipocyte cell sheet in baboon [99]

AVF = AV fistula, CABG = coronary artery bypass graft, CM = cadiac myocytes, EC = endothelial cells, FB = fibroblasts, hESC = human embryonic stem cells, hips= human induced pluripotent stem cells, hTERT = human telomerase reverse transcriptase subunit, MNC = mononuclear cells, myoFB = myofibroblast, PA = pulmonary artery, PG = polyglactin, PGA = polyglycolic acid, PV = pulmonary vein, Ross proc. = replacement of defective aortic valve by endogenous PV and insertion of new PV, RVOT = right ventricular outflow tract, SMC = smooth muscle cells.

decellularized grafts exhibited improved freedom from explantation and low peak gradients, but again, in the absence of prospective randomization it is difficult to make definite conclusions.

Decellularized blood vessels are being used for decades already, mainly as AV fistulas for hemodialysis patients [36]. The decellularized bovine carotid artery was introduced in 1966, the glutaraldehyde-fixed, non-decellularized bovine mesenteric artery in 2003 (ProCol Vascular Bioprosthesis). It has been tested in several smaller and one multicenter trial in 183 hemodialysis patients with encouraging results [37]. The primary and secondary patency rates were higher than in patients receiving a prosthetic graft (36% vs. 28% and 60% vs. 43%). This compares favorably with cryopreserved saphenous or femoral vein grafts, marketed by Cryo-Life since the early 1990s, that exhibited inferior patency rates than prosthetic grafts when used as infrainguinal bypass material [38]. The decellularized bovine ureter (Synergraft SG100) has been successfully applied as an alternative to PTFE grafts as AV fistula [39]. However, recent papers reported high failure rates [40] and a case of massive aneurysm formation in two weeks [41], both possibly related to residual bovine cell material in the graft. These data recall the catastrophic failure of decellularized porcine valves [31] and the Federal Drug Administration (FDA) ordering Cryolife to recall distributed human tissue in 2002.

Recellularization of decellularized tissues

A more elaborated approach is to repopulate the decellularized valve with autologous, patient-derived cells *in vitro*. This procedure may restore the biology of the graft, reduce thromboembolic complications, immune responses, and mechanical deterioration over time, and ultimately allow the graft to grow with the recipient. Positive results have been reported from heart valve experiments in sheep [42] as well as from two children who received reseeded decellularized allografts in the pulmonary position [43]. However, evidence exists that normal allografts show similar age-related enlargement in the pulmonary artery position [44]. Moreover, the logistical challenges related to cell seeding and mechanic conditioning of the valves in bioreactors are considerable, potentially explaining why no further reports with this technique were published.

An ultimate form of tissue engineering would be to decellularize whole hearts and repopulate the matrix with heart cells. Indeed, such experiments have been successfully performed with rat and porcine hearts [45]. Simple Langendorff-perfusion (through the canulated aorta and the coronary arteries) of whole hearts with SDS and Triton X-100 resulted in structurally preserved hearts devoid of cellular DNA. One conceptional problem was that the beneficial integrity of the extracellular matrix of the coronary vasculature also prevented egress of cells from the vascular lumen into the empty matrix of the ventricular walls. Therefore, reseeding had to be done by injecting (neonatal rat) heart cells at multiple sites into the matrix. This resulted in measurable contractions and pressure development ($\sim$2–5 mmHg), but the tissue quality was limited. Despite the apparent appeal of the idea, such a totally bioartificial heart appears a rather unlikely option for therapy, because it would have to be implanted as a substitute for the diseased heart. This means that it would have to work perfectly at the time of implantation on the scale of a human heart. A simpler alternative would be to devise a repopulated heart muscle patch derived from decellularized heart pieces, potentially with a perfusing vessel. Such experiments are ongoing in different laboratories but have not been published.

Biodegradable polymer scaffolds

The second principal tissue-engineering approach is to seed cardiovascular cells onto porous, biodegradable polymer scaffolds. Conceptionally the polymer serves as a three-dimensional template for the desired organ, and the cells are expected to degrade the polymer and produce their own extracellular matrix. This 'conventional' tissue-engineering approach has the

advantage that the scaffold can theoretically be manufactured at any desired size, form, or microstructure and with various chemical and biological properties. A principal problem is the difficulty to find the right balance between premature polymer degradation, with the risk of rupture (of a blood vessel or valve), and too little degradation, preventing tissue formation. Two variations of this technique in the blood vessel field have recently made important progress towards clinical application (Table 84.1). A Japanese group have reported a series of implantations of tissue-engineered vascular grafts in the pulmonary artery position in children, performed since 2001 [46]. They used tubular scaffolds made from a porous polycaprolactone-polylactic (PLA] acid co-polymer reinforced by a woven polyglycolic acid (PGA) sheet and seeded it with endothelial cells from a peripheral vein or, more recently, directly at the time of surgery with bone marrow cells. After a mean observation time of 17 months grafts were all patent and showed evidence for limited growth. Parallel experiments in mice suggested that the bone marrow cells were very quickly replaced by macrophages and then by ingrowing endothelial and smooth muscle cells from the adjacent ends of the blood vessel [47]. The second approach is more elaborated in that tubular PGA scaffolds are seeded with allogenic (cadaveric) smooth muscle cells *in vitro* and cultured for extended periods [48]. During this phase, the smooth muscle cells degrade the PGA scaffold and replace it by their own extracellular matrix. After chemical decellularization, an extracellular matrix tube remains and can be stored until time of implantation ('off-the-shelf'). Alternatively, the tubes can be reseeded with autologous endothelial cells before implantation, a procedure likely more effective in small-diameter vessel replacement. The method was successfully tested in a baboon model for AV fistulas for hemodialysis, and also in dogs as coronary artery bypass [48].

A similar hybrid approach using polymer scaffolds and decellularization has recently been developed for heart valves [49]. The rationale for this approach is twofold. On the one hand, heart valves generated by cell seeding of polymer scaffolds show retraction of the leaflets over time, leading to valvular insufficiency [50,51]. This response is thought to be cell-mediated. On the other hand, generation and implantation of cell-seeded polymer valves require complicated logistics, not well compatible with clinical requirements. Decellularized cell-seeded valves, based on a quickly biodegradable PGA scaffold, can be stored long-term, appear to retain their mechanical properties and showed better functional properties when tested *in vitro* [49]. Testing in an animal model has not yet been performed.

Prefabricated, biodegradable polymer scaffolds have also been used for myocardial tissue engineering. Cardiac myocytes have been seeded on scaffold blocks made from porous collagen [52], alginate [53], PGA [54], PGA/PLGA-collagen co-polymers [55] or silk fibroin [56]. A common observation is that cells preferentially populate the surface of the matrix. Moreover, cells remain randomly oriented if they lack mechanical guidance as is the case in solid scaffolds. Both factors limit cardiac tissue formation and maturation. Much effort is directed toward newer matrix materials that may overcome the current limitations. Material sciences and nanotechnology are progressing rapidly, and it may be that in a collaboration between engineers, physicists, biotechnologists, biologists, and morphologists the ideal scaffold material for creating truly 'artificial' heart tissues will be designed.

Biopolymers

The third approach is based on the entrapment of the desired cardiovascular cell type in biopolymers such as collagen I, matrigel or fibrin and the provision of a suitable casting mold. These natural polymers form a gel in which the cells are trapped in a given 3D form. The main advantage of this approach is that collagen I and fibrin are approved materials in clinical use and clearly promote tissue formation. Weinberg and Bell generated blood vessels using this technique more than 30 years ago [57], and in the meantime many groups have adopted a similar approach using fibroblasts or smooth muscle cells to create blood vessels [58–60] or heart valves [6] and cardiac myocytes for myocardial patches [61–65]. A disadvantage of the

biopolymer approach is that the tensile strength of the resulting tissues is normally low. This can be advantageous in the case of cardiac patches which should not increase passive stiffness of the heart and thereby impede diastolic relaxation. But it is a clear shortcoming in case of heart valves and blood vessels. By culturing dermal fibroblasts in tubular fibrin gels for extended periods (7–9 weeks in a bioreactor), their burst strength could be increased to 1400–1600 mmHg, and suture retention rates were sufficient to successfully implant the grafts into rats or sheep [66]. Others have used this approach to generate low pressure constructs for the replacement of venous vessels [67]. No clinical application has been reported.

Cell sheets

The fourth tissue-engineering strategy differs from all others in that it goes without any scaffold material. In this approach, first described by Auger's group [68] vascular smooth muscle cells were grown on plastic culture dishes as normal monolayers until confluency, were then removed from the culture dish as a cellular sheet and wrapped around a porous mandrel to form the media, nourished from the inside through the mandrel. In the same manner, a second layer of fibroblast sheets was wrapped around the media to form the adventitia. Finally, the mandrel was removed and the tube populated with endothelial cells by installation and rotating overnight. These sheet-based blood vessels exhibited, for the first time, a very good burst pressure, much better than what had been reported from the cell-seeded fibrin or PGA grafts. Moreover, the engineered vessels showed vasoactive responses to several known receptor ligands [69]. Sheet-based blood vessels made exclusively from human fibroblasts and human endothelial cells have been tested successfully in non-human primates [58] and as AV fistulas in 10 patients with end-stage renal disease receiving hemodialysis [3]. A 75% patency rate in this high-risk population can be considered a very good value and represents one of the first successful applications of basic tissue-engineering principles in the cardiovascular field [70]. Recent efforts are being directed to simplifying the method, making it faster, cheaper and readily available to improve clinical applicability (for review see [8], Fig. 84.1). Proposed steps are to omit the endothelium (e.g., in AV fistula with high flow rates), to air-dry the grafts (thereby get rid of living cells), store them at −80°C, rehydrate before implantation and reseed with autologous endothelial cells [71] and finally to use allogenic instead of autologous fibroblasts, considering that many patients already received allogenic tissue-engineered skin (Appligraf® or Dermagraft®) without apparent immune responses. The most recent twist of the technique is to produce threads from fibroblast-made sheets and use textile technologies to assemble threads into three-dimensional structures [8]. Apparently, this technique allows the production of vascular grafts in relatively short periods with burst pressures and suture retention rates exceeding those of the original technique.

Cardiac myocyte sheets can also be stacked to form contracting three-dimensional myocardial tissues [72], but here the absolute need for living, force-generating myocytes that are metabolically very active impose other problems than in the vascular field. Details will be discussed below.

STATE OF MYOCARDIAL TISSUE ENGINEERING

Heart-like tissue structure

Early experiments with biodegradable polymer scaffolds such as polyglycolic acid, alginate, and preformed collagen sponges showed that cardiac cells survive on these preformed 3D structures, but do not form synchronously beating, force-developing heart-like structures [52–54], likely because the scaffolds prevented the intense cell-to-cell contacts necessary for tissue formation. In contrast, tissue-engineering approaches based on biopolymers such as liquid collagen [61], collagen/Matrigel [73] or fibrin/Matrigel [74] hydrogels or prefabricated collagen sponges + Matrigel [75] as well as the cell sheet technique [72] yielded spontaneously contracting myocardial tissues that exhibit a characteristic cardiac muscle-like structure,

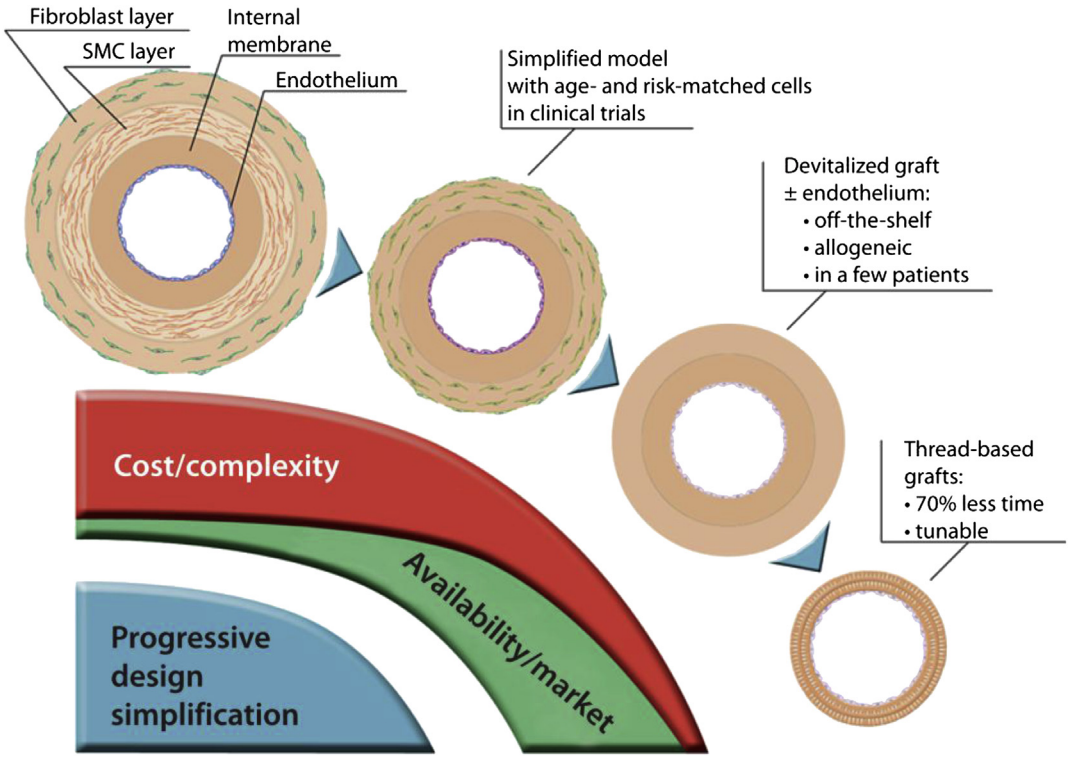

FIGURE 84.1
Evolution of a vascular tissue-engineering strategy (*tissue engineering by self-assembly;* TESA) [68] that led to the first application of a fully tissue engineered biological product in the cardiovascular field. The development went through a first phase in which a positive clinical outcome with a complex product was the major goal. Second and third phases are directed to make the process simpler, faster and cheaper. *(Adapted from [8].)*

both on the cellular and sub-cellular levels (Fig. 84.2). All of these approaches make use of the intrinsic capacity of immature cardiac cells to assemble into heart-like muscle structures spontaneously [76]. This process can be enhanced not only in a 3D growth environment, but also by mechanical loading [61,65,73,77], electrical stimulation [75], oxygenation and perfusion [65,78], growth factors [62], and non-myocytes [79] Other concepts for generating heart muscle *in vitro* are based on the formation of self-assembling cardiomyocyte aggregates/microtissues [80–82], cell seeding on foils [83] or fusion of small encapsulated tissue modules [84]. Applications of these alternative technologies are envisaged in basic cardiovascular science and drug screening rather than heart muscle repair *in vivo*.

Contractile function

Tissue-engineered heart muscle must have the capacity to contract and develop force. Reported values for muscle diameter-corrected force of contraction in tissue-engineered myocardium range between 0.08 and 66 mN/mm [17,24]. Native myocardium and single cardiomyocytes have been reported to develop up to 89 mN/mm^2 [85]. The variability and overall lower contractile force in tissue-engineered myocardium is mainly due to tissue heterogeneity, i.e., highly variable cross-sectional content of muscle and extracellular matrix. In engineered heart tissues (EHTs) developing ~5 mN/mm^2, ~10–30% of the cross-sectional area is composed of cardiomyocytes. Thus, specific muscle cross-sectional force would be 17–50 mN/mm^2. The notion that cardiomyocytes in EHTs develop similar absolute force as native cardiomyocytes is further substantiated by the observation of clearly anisotropic cardiomyocyte morphology with 'normal' sarcomere anatomy and shortening [86]. Two options exist to 'enhance' muscle cross-sectional area in engineered myocardium: 1) decrease its size at the expense of extracellular matrix components and 2) increase the amount of myocytes or myocyte volume per

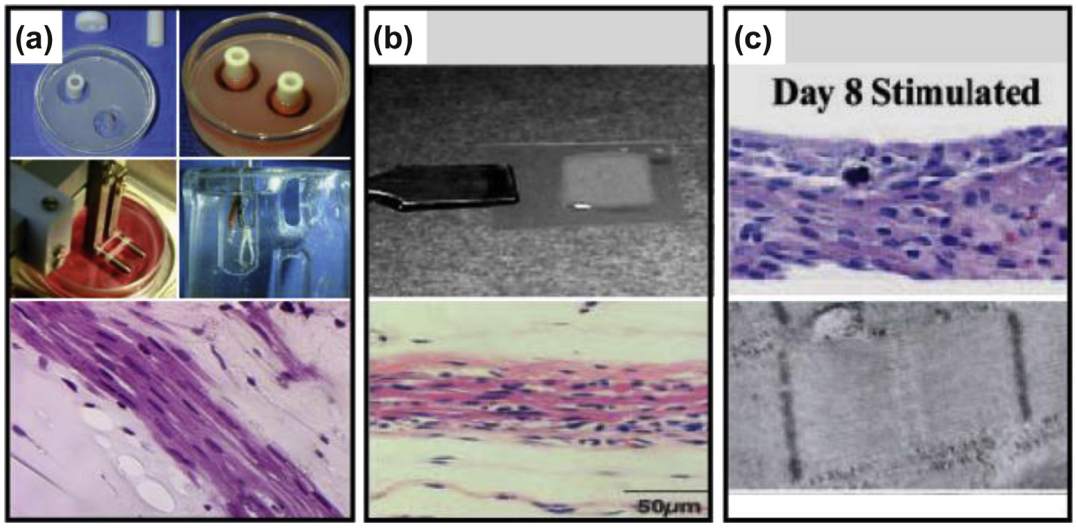

FIGURE 84.2

Current approaches to myocardial tissue engineering. (a) Macroscopic view of the setup to cast, culture, phasically stretch, and measure the contractile force of engineered heart tissue (EHT) based on collagen I/Matrigel hydrogels [62]. Cardiac tissue structure (hematoxglin & eosin, h & E, stained paraffin section). (b) Macroscopic (top) and microscopic view (H&E stain bottom) of stacked monolayers forming a beating three-dimensional tissue [72]. (c) Tissue formation (H&E stain, top) and sarcomere structure (bottom) in electrically stimulated collagen I + Matrigel-based constructs (Ultrafoam sponges) seeded with neonatal rat heart cells [75].

cross-sectional area. The first can be achieved easily by miniaturizing tissue constructs; an example is the cell sheet technology with close to 100% muscle cross-sectional area at a width of 45 μm [72]. Conversely, increasing cardiomyocyte number is complicated by limited oxygen and nutrient mass transport in tissues exceeding 100 μm. An alternative approach is to generate grafts with small and thin myocytes which undergo hypertrophy if exposed to a growth supporting environment. The capacity for hypertrophic growth is clearly present in EHT *in vitro* [86] and *in vivo* [65,87]. Cardiomyocyte proliferation in tissue-engineered myocardium appears to be just as limited as it is in postnatal hearts [86].

Critical size and vascularization

The width and length of tissue-engineered constructs can be easily expanded by adapting the dimensions of the scaffold material, casting device, or growth substrate, respectively. On the contrary, thickness of avascular myocardium is limited to 50–100 μm by physical constraints (nutrient and oxygen mass transport). This may be partly overcome by tissue perfusion and environmental oxygen enrichment [78]. However, the most straight forward approach to address this natural barrier is to perfuse tissue constructs *in vitro* [88]. Another approach is to adapt the tissue design to accommodate for the limits of nutrient and oxygen mass transport. This may be achieved by designing single tissue units at dimensions that are not oxygen deprived [86], followed by their fusion into larger tissues under the condition that mass transport remains sufficient to maintain proper tissue oxygenation (Fig. 84.3). A first step in this direction was introduced with the multiloop EHT model [65,79]. Here, complex beating tissue could be assembled and employed to re-build compact new layers of myocardium *in vivo*, reaching a thickness of 0.5–1 mm when implanted onto infarcted rat hearts. Alternatively, thin cell sheets have been repeatedly implanted on top of each other, generating similarly sized myocardium [89] (Fig. 84.4). In both cases, intense *in vivo* vascularization was observed. This process may have been facilitated by the presence of capillary-like structures in EHT *in vitro* [65,90]. However, rebuilding human heart sized muscle replacements (LV wall thickness mean: 9 mm) will likely require addition measures, such as for example attaching large caliber in and outflow vessels [91]. An elegant method of '*in situ* tissue engineering' cultured Matrigel-embedded cardiac myocytes in a silicone chamber that had been implanted

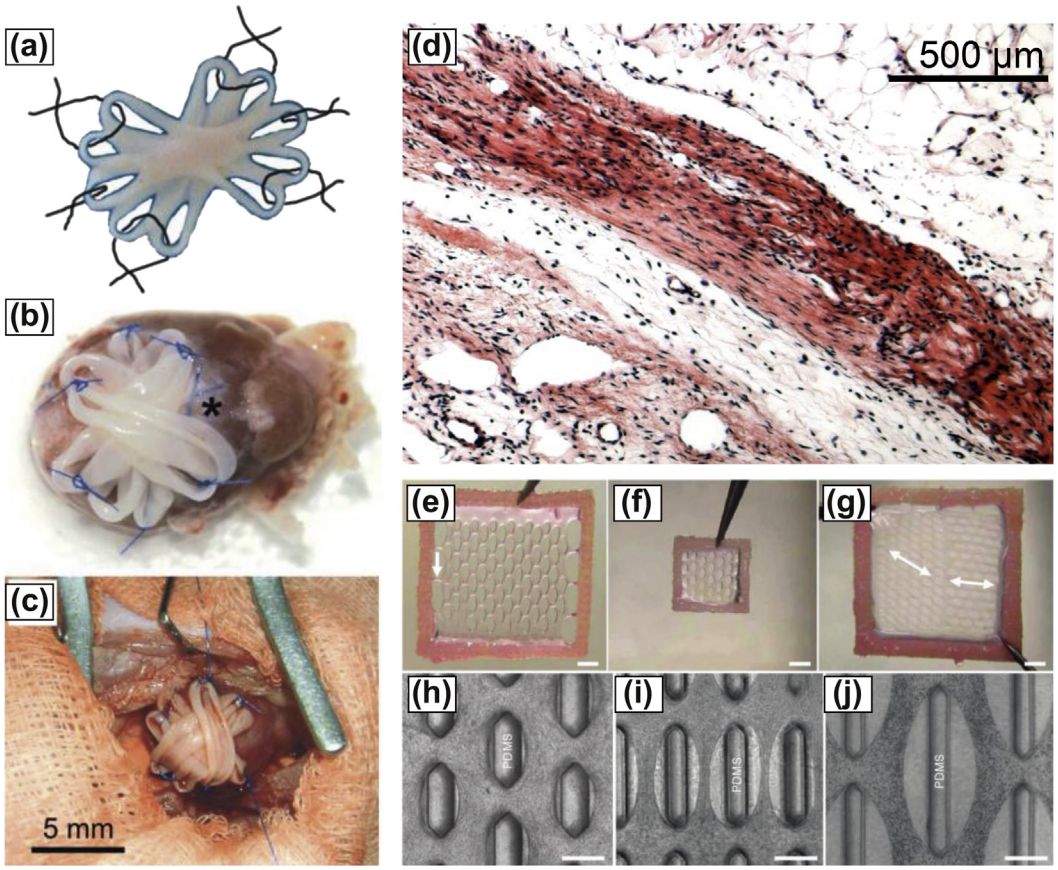

FIGURE 84.3

Strategies for cardiac repair using hydrogel-based tissue grafts of principally unlimited size. (a—d) Engineered heart tissue (EHT) approach. Several EHTs are fused *in vitro* to form multiloop EHT. Shown is one multiloop EHT with sutures before implantation. (b) Rat heart with multiloop EHTs sutured onto an infarcted rat heart *ex vivo*. (c) Rat heart situs showing a heart with a multiloop EHT fixed with six single-knot sutures placed in healthy myocardium just adjacent to the infarct scar through a left lateral thoracotomy. (d) Formation of new myocardium 4 weeks after implantation of a multiloop EHT on an infarcted rat heart [65]. (e—j) Method to generate large engineered muscle tissues by casting a cell-fibrin/Matrigel mixture onto poly(dimethyl-siloxane) (PDMS) molds with mesoscopic posts [63].

onto an isolated A/V loop of the epigastric blood vessels in rats [92] (Fig. 84.4). This approach generates a pulsatile cardiac tissue with perfusing artery and vein that can be surgically connected to carotid blood vessels and transplanted onto the heart [93]. Though not directly transferrable to humans, this study may point towards the right direction.

Electrical integration and improvement of function after implantation

Available data suggest that engineered sandwich constructs [94] and EHTs [65] find electrical contact with the host myocardium after implantation. This is in accordance with careful studies of isolated cardiac myocytes injected directly into the myocardium [95]. Studying electrical integration in engineered myocardium is particularly difficult in epicardial grafts. Here, motion artifacts and false positive signals from underlying native myocardium have to be controlled for. New developments in genetically encoded sensors, such as the calcium sensor GCaMP3, appear particularly useful in studies of cell integration [96]. Histological demonstration of gap junction formation is certainly not enough to unambiguously demonstrate graft-host coupling, also because cardiomyocyte integration may be via electrotonic contact or even via fibroblasts [97]. Electrical integration of tissue-engineered grafts into host myocardium appears essential, but, if incomplete, carries the risk of re-entry circuit formation. Hence, it was surprising to observe no evidence for enhanced arrhythmia in rats with EHT [65] or cell

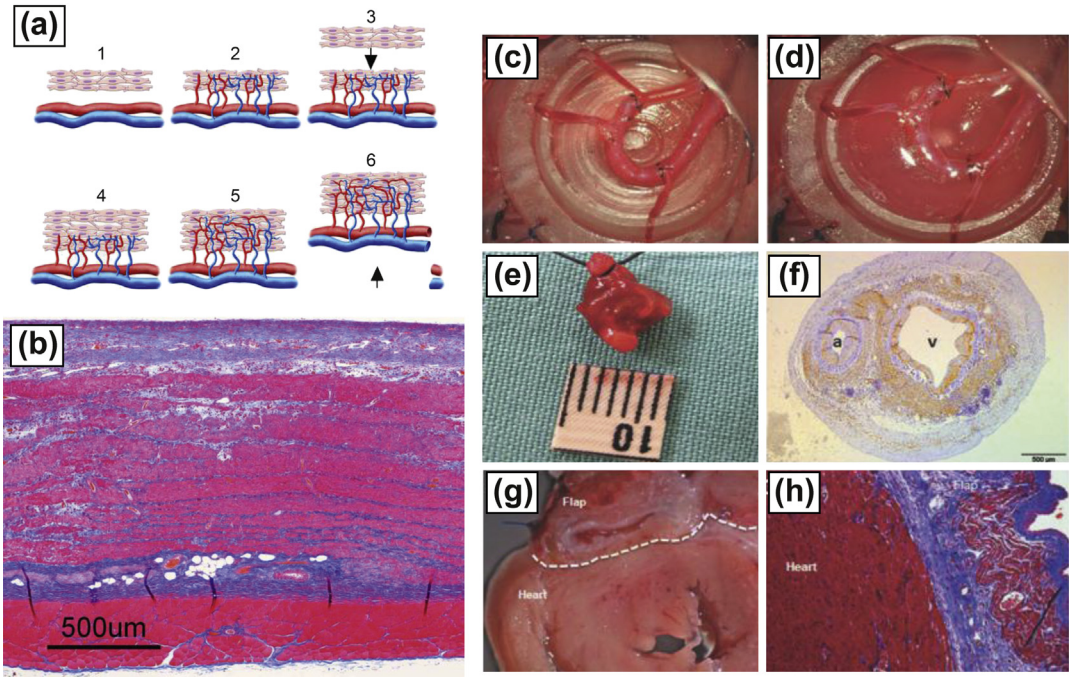

FIGURE 84.4

Strategies to generate vascularized cardiac tissue grafts. (a, b) Cell sheet polysurgery. Three cell sheets are layered on top of each other *in vitro* and implanted into a host animal over a surgically accessible artery and vein (1). This leads to vessel ingrowth (2) and allows implantation of a second multilayered graft (3), which is then also vascularized (4, 5). Finally, the vascularized construct (of principally unlimited thickness) is fully perfused by host vessels and can be surgically resected (6) and transplanted onto the heart. Adapted from 89. (c—h) *In vivo* bioreactor technique. An arterio-venous (A/V) loop is isolated from the femoral blood vessels of adult rats and surgically fixed in a polycarbonate chamber (c). A cardiac cell-matrigel mix is poured into the chamber (d). During a period of weeks a cardiac tissue flap develops that is perfused (e) and shows desmin-positive cardiac muscle tissue (f). Cardiac flaps with a long vessel pedicle are sutured to the carotid artery and vein and fixed on the heart of a second rat (g). Histology shows the formation of compact cardiac muscle tissue four weeks after implantation (h). *(Adapted from [93].)*

sheet grafts [94]. This may be explained by the comparably low spontaneous beating frequency of rat EHT grafts (2 Hz) compared to the high endogenous heart rate in rat (~5 Hz) and the mismatch in current load between graft and recipient heart muscle. Interestingly, a recent study studying the effect of isolated human ESC-derived cardiomyocyte injected in infarcted guinea-pig hearts suggested anti-arrhythmic rather than pro-arrhythmic effects [96].

Electrical coupling is clearly a prerequisite for functional myocardial support by tissue-engineered heart patches. In a large-scale study in rats in which multiloop EHTs were implanted above the infarct scar 14 days after ligation of the left anterior descending (LAD) coronary artery (Fig. 84.2) we could provide proof-of-concept for tissue-engineered heart repair by electrically integrated patches. Enhanced myocardial performance was observed especially in animals with a very poor LV function prior to implantation (fractional area shortening <30%; [65]). Despite these encouraging results, it should be noted that even the implantation of a relatively large (surface approximately 1 cm^2) and thick (Ø 1—4 mm) patch with nominally 12 million heart cells for a 1 g rat heart did not restore normal function, emphasizing the need for further improvement.

Considerations for clinical applications

True force-generating EHT grafts have not entered the clinic, mainly because an autologous and safe approach to generate heart muscle *in vitro* is not at hand yet. A case report on the implantation of autologous myoblast sheets in a patient with end-stage dilated cardiomyopathy observed contractile recovery associated with the procedure [5]. However, the negative

outcome of a clinical trial on myoblast injections [98] and the solid evidence that skeletal myoblasts do not acquire a cardiac myocyte phenotype make it unlikely that the reported benefit in this case was related to the generation of new contractile mass. An important step towards clinical application has recently been reported from a study in rhesus monkeys [99]. Allogeneic (rhesus) embryonic stem cell-derived cardiac progenitor cells were cultured on autologous adipocyte cell sheets and implanted onto experimentally infarcted rhesus monkey hearts under immunosuppression. Implanted cells formed cardiac myocytes, and no teratomas were observed in the five animals studied.

Clinical application of tissue-engineered myocardial patches requires a means to scale-up the procedure under the strict regulations of good manufacturing (GMP). This is primarily a biotechnological hurdle. In essence all cellular and extracellular components of EHTs are available at GMP-grade. Translation of the presently available technologies would also be facilitated by automation of the production process. The cell sheet stacking technology has already been automated and attempts to combine this with means to vascularize the cell sheet stacks are underway in the Okano/Shimizu group. Another important issue to consider is transplant immunology. Autologous approaches would clearly be preferred, but their realization appears unrealistic, mainly because personalized tissue-engineered products in heart repair would require generation and quality control of individual cardiogenic cell lines and heart muscle products. Induced pluripotent stem cells could in principle be used as autologous cell source, but its development under GMP-practice (integration free, [epi-]genetic stability ensured, quality controlled directed differentiation, quality controlled engineered heart muscle) in a timely manner appears unrealistic. Thus, the challenge will be to develop procedures to allocate off-the-shelf allogeneic products by making use of stem cell banking and tissue-engineering process optimization.

BOTTLENECKS

The critical bottlenecks in tissue engineering of heart valves, vessels, and myocardial patches differ. It appears that the main hurdle for tissue-engineered heart valves is the relatively good performance of conventional prostheses and the disastrous consequences of any failure. Decellularized allograft valves may have advantages over standard homografts, but more data are needed. The main challenge in vascular tissue engineering is the generation of small to medium size vessels with sufficient burst strength and long-term stability. Very encouraging progress has been made with tissue-engineered blood vessels, both with the sheet-based technique and smooth muscle cell-populated scaffolds that are decellularized before implantation. The former have already been tested successfully in patients [3,71], the latter in non-human primates and dogs [48]. Recent efforts successfully simplified the procedures and made them available 'off-the-shelf' [16,104]. This is the first time that true tissue-engineered cardiovascular products (in a stricter sense than decellularized valves) entered the clinic. Myocardial tissue patches are still far from this stage. On the one hand, they require less mechanical strength than vessels and valves, because they will likely be, with the exception of congenital malformations, applied as an add-on to an infarcted or failing heart. On the other hand, engineered myocardium, in contrast to valves and blood vessels, definitely requires functioning cells. The fact that cardiac myocytes do not (sufficiently) proliferate, impeded the progress in this field for long. However, much has changed today. The following issues require particular attention in heart muscle engineering.

Cell source

Having been a major hurdle for clinical realization of myocardial tissue-engineering concepts until recently, the exciting progress in stem cell technologies clearly changed the picture today. Human cardiomyocytes are available from embryonic and induced pluripotent stem cells at reliable quantity and quality [100]. Direct programming of fibroblasts may be

an alternative strategy to generate myocytes [101] but the low efficiency of this procedure currently precludes its application in myocardial tissue engineering. Whether adult stem cells can ever be stimulated to provide the necessary amount of therapeutic cardiomyocytes for *in vitro* tissue engineering appears questionable. It is very likely that human embryonic stem cell-derived cardiomyocytes will reach clinical application in cardiac tissue engineering first, similar to other pluripotent stem cell-based approaches [102,103]. In fact, GMP-compliant ESC-derived cardiomyocytes have already been generated by Geron. Given the clear unmet need for better heart failure therapeutics it is palpable that this biotechnological know-how will be exploited for clinical testing in the near future.

Size/vascularization

Engineered myocardium will have to be adjusted in size to reach optimal therapeutic results. During a normal myocardial infarction roughly 1 billion myocytes are lost. To have a meaningful therapeutic impact, it seems that grafts capable of replacing at least 20% of a given defect need to be engineered at a centimeter scale thickness. Whether this will require pre-vascularization of grafts or be sufficiently supported by *in vivo* vascularization to support hypertrophic growth of an engineered implant remains to be investigated.

Unwanted side effects

Demonstrating safety of tissue-engineered heart repair will be a key challenge of preclinical testing. The risk of tumor formation (especially if pluripotent stem cells will be applied) and induction of arrhythmias will have to be addressed in appropriate large animals such as the pig or non-human primate for a meaningful duration (6–12 months). Whether these studies need to be powered for established clinical endpoints such as mortality appears questionable. More likely, meaningful surrogate parameters with predictive value will have to be defined prior to first clinical studies. Here it becomes a matter of using the right model that ideally simulates human physiology and responsiveness to the respective intervention. This problem has been especially evident in large animal valve trials. Sheep tend to develop massive fibrosis, which can be misleading in two respects – overestimation of the risk of valve degeneration, but also underestimation of mechanical instability because the fibrosis has in fact stabilizing effects on the valve. Pigs show a high level of calcification. Eventually, non-human primates seem to be an ideal test bed. Raising the necessary resources for these large animal trials via traditional academic funding schemes is a challenge, but efforts are undertaken worldwide.

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CHAPTER 85

Tissue-Engineered Organs

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INTRODUCTION

Human organs are exposed to a variety of possible injuries from the time the fetus develops. Individuals may suffer from congenital disorders, cancer, trauma, infection, inflammation, iatrogenic injuries, or other conditions that may lead to organ damage or loss and necessitate eventual reconstruction or replacement. Whenever there is deficient organ function, replacement may be achieved with artificial organs or organ transplantation. These are flawed therapies, however, as artificial organs are not able to replace all physiologic functions and cannot fully integrate functionally into body systems, while organ transplantation is marred by donor shortages and immunologic rejection, and requires chronic and morbid medical therapy. In most cases, the replacement of lost or deficient tissues with functionally equivalent autologous tissues would improve the outcome for patients immensely. Tissue-engineering therapies have been developed in order to achieve this goal.

The development of functional organ systems from the building blocks of cells, scaffolding, and growth factors is a Herculean task, however, as evidenced by the few successful tissue-engineering technologies currently in clinical use. There have been successes, however, as evidenced by functioning autologous bioengineered tissues that have been implanted in humans — including trachea, blood vessels, pulmonary valves, endothelialized vascular stents, and urinary bladder [1]. An examination of the building blocks of tissue-engineering strategies, as well as the successful applications of these techniques in human clinical applications, provides an insight into the difficulties facing scientists as they seek to develop more complex bioengineered organs.

TISSUE ENGINEERING: STRATEGIES FOR TISSUE RECONSTITUTION

The field of regenerative medicine encompasses various areas of technology, such as tissue engineering, stem cells, and cloning. One of the major components of regenerative medicine, tissue engineering, follows the principles of cell transplantation, materials science and engineering towards the development of biological substitutes that can restore and maintain normal function. Tissue-engineering strategies generally fall into three categories: the use of acellular matrices (where matrices are used alone and which depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth), the use of simple cells, and the use of matrices combined with cells. The main obstacles that must be overcome for the clinical application of these strategies are: localizing reliable, immunocompatible cell sources; developing biocompatible and functional scaffolding; and providing growth factors and vascularization for bioengineered organs.

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Acellular tissue matrices are usually prepared by manufacturing artificial scaffolds, or by removing cellular components from tissues via mechanical and chemical manipulation to produce collagen-rich matrices [2–5]. These matrices tend to slowly degrade on implantation and are generally replaced by the extracellular matrix (ECM) proteins that are secreted by the ingrowing cells. Cells can be used in combination with scaffolds or injected as simple cell therapy, either with carriers (such as hydrogels), or alone [90].

CELL SOURCES

When cells are used for tissue engineering, a small piece of donor tissue must be harvested and dissociated into individual cells. These cells are either implanted directly into the host, or are expanded in culture, attached to a support matrix, and then reimplanted into the host after expansion. The source of donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous. Ideally, both structural and functional tissue replacement will occur with minimal complications. The most preferred cells to use are autologous cells, where a biopsy of tissue is obtained from the host, the cells are dissociated and expanded in culture, and the expanded cells are implanted into the same host [5–17]. The use of autologous cells, although it may cause an inflammatory response, avoids rejection, and thus the deleterious side effects of immunosuppressive medications can be avoided.

One of the limitations of applying cell-based regenerative medicine techniques towards organ replacement has been the inherent difficulty of growing specific cell types in large quantities. Even when some organs, such as the liver, have a high regenerative capacity *in vivo*, cell growth and expansion *in vitro* may be difficult. By studying the privileged sites for committed precursor cells in specific organs, as well as exploring the conditions that promote differentiation, one may be able to overcome the obstacles that could lead to cell expansion *in vitro.* For example, urothelial cells could be grown in the laboratory setting in the past, but only with limited expansion. Several protocols were developed over the last two decades which identified the undifferentiated cells, and kept them undifferentiated during their growth phase [8,18–20]. Using these methods of cell culture, it is now possible to expand a urothelial strain from a single specimen which initially covers a surface area of 1 cm^2 to one covering a surface area of 4202 m^2 (the equivalent area of one football field) within eight weeks [8]). These studies indicated that it should be possible to collect autologous bladder cells from human patients, expand them in culture, and return them to the human donor in sufficient quantities for reconstructive purposes [8,18,21–25]. Major advances have been achieved within the last decade on the possible expansion of a variety of primary human cells, with specific techniques that make the use of autologous cells possible for clinical application.

A recent discovery may well influence cell sourcing for regenerative medicine strategies. A new type of interstitial cell, termed telocytes, has been isolated, and these cells have been found to be crucial to the process of evolution of progenitor cells toward mature development. Their architecture (tracks for migrations) and paracrine secretions (including microRNA) provide the environment for progenitor cells maturation. These cells have been found in many organs including myocardium, and have been demonstrated to be crucial to neo-angiogenesis following myocardialischemia. These cells and their signals may hold clues to creating more appropriate milieus for precursor cell development and angiogenesis [26].

An area of concern in the field of tissue engineering in the past was the source of cells for regeneration. The concept of creating engineered constructs involves initially obtaining cells for expansion from the diseased organ. One study recently showed that cultured neuropathic bladder smooth muscle cells possess and maintain different characteristics than normal smooth muscle cells *in vitro*, as demonstrated by growth assays, contractility and adherence tests *in vitro* [27]. However, when neuropathic smooth muscle cells were cultured *in vitro*, and seeded onto matrices and implanted *in vivo*, the tissue-engineered constructs showed the same

properties as the tissues engineered with normal cells [28]. It is known that genetically normal progenitor cells, which are the reservoirs for new cell formation, are programmed to give rise to normal tissue, regardless of whether the niche resides in either normal or diseased tissues. The stem cell niche and its role in normal tissue regeneration remains a fertile area of ongoing investigation.

In circumstances where source cell gene expression may be pathologically altered as demonstrated by Hodges et al. [28a], gene modifying techniques have been aggressively explored — including epigenetic therapies, and gene transfer techniques (using viral vectors or polymer-based gene delivery). Although the precise control of new gene expression remains complex, the use of gene delivery in combination with varying cell populations may expand the cell sources available for regeneration [26].

ALTERNATE CELL SOURCES

Stem cells

Most current strategies for tissue engineering depend upon a sample of autologous cells from the diseased organ of the host. However, for many patients with extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous human cells cannot be expanded from a particular organ, such as the pancreas. In these situations, pluripotent human embryonic stem cells are envisioned as a viable source of cells, as they can serve as an alternative source of cells from which the desired tissue can be derived [29].

Human embryonic stem cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated, but pluripotent state (self-renew), and the ability to differentiate into many specialized cell types [30]. They can be isolated by immunosurgery from the inner cell mass of the embryo during the blastocyst stage (five days post-fertilization), and are usually grown on feeder layers consisting of mouse embryonic fibroblasts or human feeder cells [31]. More recent reports have shown that these cells can be grown without the use of a feeder layer [32], and thus avoid the exposure of these human cells to mouse viruses and proteins. These cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages when grown using current published protocols [33,34].

Human embryonic stem cells have been shown to differentiate into cells from all three embryonic germ layers *in vitro*. Skin and neurons have been formed, indicating ectodermal differentiation [35—37]. Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation [38—40]. And pancreatic cells have been formed, indicating endodermal differentiation [41]. In addition, as further evidence of their pluripotency, embryonic stem cells can form embryoid bodies, which are cell aggregations that contain all three embryonic germ layers, while in culture, and can form teratomas *in vivo* [42].

Adult stem cells have the advantage of avoiding the ethical issues associated with embryonic cells, and also they do not transdifferentiate spontaneously to a malignant phenotype. The use of adult stem cells is limited for clinical therapy due to the difficulties encountered with cell expansion into large quantities.

Fetal stem cells derived from amniotic fluid and placentas have been recently described and represent a novel source of stem cells. The cells express markers consistent with human embryonic stem cells, such as OCT4 and SSEA-4, but do not form teratomas, and do not spontaneously transdifferentiate to a malignant phenotype. The cells are multipotent and are able to differentiate into all three germ layers. In addition, the cells have a high replicative potential and could be stored for future self-use, without the risks of rejection, and without ethical concerns [43].

THERAPEUTIC CLONING

While there has been tremendous interest in the field of nuclear cloning since the birth of Dolly the sheep in 1997, the first successful nuclear transfer was reported over 50 years ago by Briggs and King. Cloned frogs [44], which were the first vertebrates derived from nuclear transfer, were subsequently reported by Gurdon in 1962 [45], but the nuclei were derived from non-adult sources. In the past several years, tremendous advances in nuclear cloning technology have been reported, indicating the relative immaturity of the field. Dolly was not the first cloned mammal to be produced from adult cells; in fact, live lambs were produced in 1996 using nuclear transfer and differentiated epithelial cells derived from embryonic disks [46]. The significance of Dolly was that she was the first mammal to be derived from an adult somatic cell using nuclear transfer [47]. Since then, animals from several species have been grown using nuclear transfer technology, including cattle [48], goats [49,50], mice [51], and pigs [52].

Two types of nuclear cloning, reproductive cloning and therapeutic cloning, have been described, and a better understanding of the differences between the two types may help to alleviate some of the controversy that surrounds these technologies. Banned in most countries for human applications, reproductive cloning is used to generate an embryo that has identical genetic material to its cell source. This embryo can then be implanted into the uterus of a female to give rise to an infant that is a clone of the donor. On the other hand, therapeutic cloning is used to generate early stage embryos that are explanted in culture to produce embryonic stem cell lines whose genetic material is identical to that of its source. These autologous stem cells have the potential to become almost any type of cell in the adult body, and thus would be useful in tissue and organ replacement applications [53]. Therefore, therapeutic cloning, which has also been called somatic cell nuclear transfer, may provide an alternative source of transplantable cells. According to data from the Centers for Disease Control, it has been estimated that approximately 3,000 Americans die every day of diseases that could have been treated with stem cell-derived tissues [33]. With current allogeneic tissue transplantation protocols, rejection is a frequent complication because of immunologic incompatibility, and immunosuppressive drugs are usually administered to treat and hopefully prevent host-versus-graft disease [54]. The use of transplantable tissue and organs derived from therapeutic cloning may potentially lead to the avoidance of immune responses that typically are associated with transplantation of non-autologous tissues [43].

While promising, somatic cell nuclear transfer technology has certain limitations that require further improvements before therapeutic cloning can be applied widely in replacement therapy. Currently, the efficiency of the overall cloning process is low. The majority of embryos derived from animal cloning do not survive after implantation [53,55,56]. In practical terms, multiple nuclear transfers must be performed in order to produce one live offspring for animal cloning applications. The potential for cloned embryos to grow into live offspring is between 0.5 to 18% for sheep, bovine, pigs, and mice [57]. However, greater success (80%) has been reported in cattle [58], which may be in part due to the availability of advanced bovine supporting technologies, such as *in vitro* embryo production and embryo transfer, which have been developed for this species for agricultural purposes. To improve cloning efficiencies, further improvements are required in the multiple complex steps of nuclear transfer, such as enucleation and reconstruction, activation of oocytes, and cell cycle synchronization between donor cells and recipient oocytes, that will more readily produce viable sources of cells.

Furthermore, common abnormalities have been found in newborn clones if they survive to birth, including enlarged size with an enlarged placenta ('large-offspring syndrome') [59], respiratory distress and defects of the kidney, liver, heart, and brain [60], obesity [61], and premature death [62]. These may be related to the epigenetics of the cloned cells, which involve the reversible modifications of the DNA or chromatin, while the original DNA (genetic) sequences remain intact. Faulty epigenetic reprogramming in clones, where the DNA

methylation patterns, histone modifications, and the overall chromatin structure of the somatic nuclei are not being reprogrammed to an embryonic pattern of expression, may explain the above abnormalities [63]. Reactivation of key embryonic genes at the blastocyst stage is usually not present in embryos cloned from somatic cells, while embryos cloned from embryos consistently express early embryonic genes [64,65]. Proper epigenetic reprogramming to an embryonic state may help to improve the cloning efficiency and reduce the incidence of abnormal cloned cells.

BIOMATERIALS

For cell-based tissue engineering, once cells have been cultured and expanded, they are seeded onto a scaffold synthesized using the appropriate biomaterial. In tissue engineering, biomaterials replicate the biologic and mechanical function of the native ECM found in tissues in the body by serving as an artificial ECM. As a result, biomaterials provide a three-dimensional space for the cells to form into new tissues with appropriate structure and function, and also can allow for the delivery of cells and appropriate bioactive factors (e.g., cell-adhesion peptides, growth factors), to desired sites in the body [66]. As the majority of mammalian cell types are anchorage-dependent and will die if no cell-adhesion substrate is available, biomaterials provide a cell-adhesion substrate that can deliver cells to specific sites in the body with high loading efficiency. Biomaterials can also provide mechanical support against *in vivo* forces such that the predefined three-dimensional structure is maintained during tissue development. Furthermore, bioactive signals, such as cell-adhesion peptides and growth factors, can be loaded along with cells to help regulate cellular function.

The ideal biomaterial should be biocompatible in that it is biodegradable and bioresorbable to support the replacement of normal tissue without inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection and/or necrosis. In addition, the degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate that keeps the concentration of these degradation products in the tissues at a tolerable level [67]. Furthermore, the biomaterial should provide an environment in which appropriate regulation of cell behavior (e.g., adhesion, proliferation, migration, and differentiation) can occur such that functional tissue can form. Cell behavior in the newly formed tissue has been shown to be regulated by multiple interactions of the cells with their microenvironment, including interactions with cell-adhesion ligands [68] and with soluble growth factors. In addition, biomaterials provide temporary mechanical support that allows the tissue to grow in three dimensions while the cells undergo spatial tissue reorganization. The properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, while in late development, the properly chosen biomaterial should have begun degradation such that it does not hinder further tissue growth [66].

Generally, three classes of biomaterials have been utilized for engineering tissues: naturally-derived materials (e.g., collagen and alginate), acellular tissue matrices (e.g., bladder submucosa and small intestinal submucosa), and synthetic polymers [e.g., polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA)]. These classes of biomaterials have been tested in respect to their biocompatibility [69,70]. Naturally-derived materials and acellular tissue matrices have the potential advantage of biological recognition. However, synthetic polymers can be produced reproducibly on a large scale with controlled properties of their strength, degradation rate, and microstructure.

Collagen is the most abundant and ubiquitous structural protein in the body and may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction. Collagen implants degrade through a sequential attack by lysosomal enzymes. Controlling the density of the implant and the extent of intermolecular crosslinking can

regulate the *in vivo* resorption rate. The lower the density, the greater the interstitial space and generally the larger the pores for cell infiltration, leading to a higher rate of implant degradation. Collagen contains cell-adhesion domain sequences (e.g., RGD) that exhibit specific cellular interactions. This may assist to retain the phenotype and activity of many types of cells, including fibroblasts [71] and chondrocytes [72].

Alginate, a polysaccharide isolated from seaweed, has been used as an injectable cell delivery vehicle [73] and a cell immobilization matrix [74] owing to its gentle gelling properties in the presence of divalent ions such as calcium. Alginate is relatively biocompatible and approved by the food and drug administration (FDA) for human use as wound dressing material. Alginate is a family of copolymers of D-mannuronate and L-guluronate. The physical and mechanical properties of alginate gel are strongly correlated with the proportion and length of poly-guluronate block in the alginate chains [73].

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues [2,4,5,74]. The matrices are often prepared by mechanical and chemical manipulation of a segment of tissue. The matrices slowly degrade upon implantation and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or ingrown cells.

Polyesters of naturally occurring α-hydroxy acids, including PGA, PLA, and PLGA, are widely used in tissue engineering. These polymers have gained FDA approval for human use in a variety of applications, including sutures. The ester bonds in these polymers are hydrolytically labile, and these polymers degrade by non-enzymatic hydrolysis. The degradation products of PGA, PLA, and PLGA are non-toxic, natural metabolites and are eventually eliminated from the body in the form of carbon dioxide and water. The degradation rate of these polymers can be tailored from several weeks to several years by altering crystallinity, initial molecular weight, and the copolymer ratio of lactic to glycolic acid. Since these polymers are thermoplastics, they can be easily formed into a three-dimensional scaffold with a desired microstructure, gross shape and dimension by various techniques, including molding, extrusion [75], solvent casting, phase separation techniques, and gas foaming techniques [76]. Many applications in tissue engineering often require a scaffold with high porosity and ratio of surface area to volume. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(ortho-esters), can also be used to fabricate scaffolds for tissue engineering with controlled properties [77].

A recently developed polymer, Polyhedraloligomeric silsesquioxane/poly(carbonateurea) urethane (POSS-PCU) is a highly biocompatible nanocomposite which has demonstrated many of the ideal characteristics for tissue engineering numerous implants [1].

Other advances in biomaterial engineering include the development of conducting polymers which may find roles in neural tissue engineering, and ionic polymer metal composites that can induce movement with appropriate stimulation and therefore may serve roles in the development of artificial muscles or dynamic sensors [1].

GROWTH FACTORS

A key component to scaffold function is the presence of the appropriate growth factors for cell growth and maturation. Growth factors may be produced by cells themselves (autocrine), or the surrounding environment (paracrine). Scientists have employed various strategies to enhance desirable growth factor production, in order to promote the cell growth and maturation needed for particular organ systems. Some investigators have attempted to influence cell growth factor production with gene therapy, by incorporating genes for growth factor transcription in the transplanted cells. Others have sought to simply inject the growth factors into the cells milieu, but this method often results in rapid dissipation of the agents due to diffusion [78].

A novel method of growth factor use in tissue engineering involves the incorporation of growth factors into carriers or cell scaffolds. The goals of these techniques are to provide localized and controlled release of growth factors necessary for cell development. Extensive research is underway to define the exact combination of growth factors needed for specific organ growth and maturation, with an emphasis on the use of these factors to induce more rapid cell development and differentiation, appropriate cell function and orientation, and ECM development and vascularization [79].

In addition to the influence of growth factors, the simple topography of a scaffold has been demonstrated to greatly affect cell growth. For example, the shape of grooves in scaffolds can influence cell proliferation, cell alignment, and assist in cell differentiation [1].

VASCULARIZATION

One of the restrictions of the engineering of tissues is that cells cannot be implanted in volumes exceeding 3 mm^3 because of the limitations of nutrition and gas exchange [80]. To achieve the goals of engineering large complex tissues, and possibly internal organs, vascularization of the regenerating cells is essential.

Three approaches have been used for the vascularization of bioengineered tissue:

1) Incorporation of angiogenic factors in the bioengineered tissue;
2) Seeding EC with other cell types in the bioengineered tissue;
3) Prevascularization of the matrix prior to cell seeding.

Angiogenic growth factors may be incorporated into the bioengineered tissue prior to implantation, in order to attract host capillaries and to enhance neovascularization of the implanted tissue. There are many obstacles to overcome before large entire tissue-engineered solid organs are produced. Recent developments in angiogenesis research may provide important knowledge and essential materials to accomplish this goal.

The modern strategies of neo-organ vascularization have investigated the aforementioned use of growth factors and cytokines such as vascular endothelial growth factor (VEGF), the use of progenitor cells, or biomaterials, or fabricated vessel conduits, or combinations of those techniques. Because tissue engineering is often required in the setting of severe tissue damage (such as following radiation for tumor therapy) and immediate oxygenation of a large construct may be needed (where neoangiogenesis may not be possible or rapid enough) the *in vivo* creation of arteriovenous loops have been favored, and the most efficacious vascularization of solid organs has been found to be accomplished with the anastamosis of prevascularized constructs in combination with the local and controlled release of angiogenic growth factors [21].

CLINICAL APPLICATIONS

The assimilation of all these complex technologies into functional bioengineered organs is a complex task. By examining the early attempts at the application of tissue engineering for clinical use, the difficulties and challenges facing scientists in the future become more clearly delineated. The application of bioengineered organs for human use has been addressed in a logical fashion, beginning with the most simple organs system (e.g., skin) and extending to hollow organs (e.g., bladder) and finally complex solid organs (e.g., kidneys).

Skin

The concept of the clinical application of skin tissue engineering had its origins in Massachusetts around 1980, when Yannas and Bell developed the technologies necessary for the growth of skin-like structures from single cells. Bell was able to create dermal skin tissue by culturing human neonatal foreskin fibroblasts in collagen gels [81]. Yannas and associates expanded this

approach, and were able to engineer skin by applying the modern principles of biomaterial scaffolding and molecular cues to influence the growth of skin cells [82,83]. These efforts resulted in several commercially available bioengineered skin replacement therapies. Yannas and co-workers developed 'Integra', a collagen template for dermal regeneration. This was followed shortly by Bell's development of a full-thickness skin substitute with keratinized epidermis and dermis, Apligraft. The most recent development in the skin substitute market is Dermagraft, or a composite of dermal fibroblasts and collagen developed by culturing neonatal foreskin fibroblasts on PLGA scaffolds [84].

Although all these products function well, their clinical use has not been widespread. The failure of any of these excellent technologies to dominate the skin replacement market emphasizes the somewhat ignored factors of tissue engineering. Mainly, for a technology to be widely accepted as an organ replacement technology, it must function better than all available options, be easy to use, and not be cost prohibitive. Indeed, a bioengineered organ that functions better than available therapies, may never be accepted into widespread clinical use if it is difficult to use, or far too expensive.

Bladder

Currently, gastrointestinal segments are commonly used as tissues for bladder replacement or repair [85,86]. Because of the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative reconstructive procedures for bladder replacement or repair such as the use of tissue expansion, seromuscular grafts, matrices for tissue regeneration, and tissue engineering with cell transplantation [85].

Cell-seeded allogeneic acellular bladder matrices are one of the few strategies of bioengineered bladder replacement with human clinical applications. The early work with this technology was performed in dogs, where a group of experimental canines underwent a trigone-sparing cystectomy with closure of the trigone without a reconstructive procedure, or reconstruction with a non-seeded bladder-shaped biodegradable scaffold, or reconstruction using a bladder-shaped biodegradable scaffold that delivered seeded autologous urothelial cells and smooth muscle cells [15].

The results were promising, with the cystectomy only and non-seeded controls demonstrating decreased capacities and compliance as compared to preoperative values, while the cell-seeded tissue-engineered bladders showed increased capacity and compliance, with histology depicting normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle [15].

Preliminary clinical trials for the application of this technology have been performed and have produced promising results. Recently published data demonstrated that engineered autologous bladder tissue may be used to safely augment the bladder in children with end-stage bladder disease secondary to myelomeningocele [87]. In this study, seven patients aged 4–19, with high pressure or poorly compliant bladders were selected as candidates for autologous augmentation cystoplasty. Bladder biopsies were obtained from each patient and smooth muscle and urothelial cells were cultured, expanded, and seeded on a composite scaffolds. Approximately seven weeks after the biopsy, the autologously generated neobladders were used for bladder reconstruction with or without omental wrapping. At a mean follow-up of 46 months, the increase in bladder volume and compliance was greatest in the engineered bladders coated in the omental wrap. Bowel function returned promptly after surgery, there were no metabolic complications, stone formation or mucous production, and renal function was preserved. In addition, engineered bladder biopsies demonstrated normal structural architecture and phenotype. Thus, this study provided good evidence in support of the use of engineered bladder tissue (created with autologous cells seeded on a composite scaffold, and wrapped in omentum following implantation), for patients in need of cystoplasty.

Kidney

Solid organs are the most challenging to reconstruct by tissue-engineering techniques because of their complex structure and function. Due to these complexities, some scientists approach the replacement of solid organ function, such as liver or kidney, with bioartificial approaches, combining the function of the organs predominate cells, with an artificial structure and organization.

An example of this strategy is extracorporeal renal replacement. Although dialysis is currently the most prevalent form of renal replacement therapy, the relatively high morbidity and mortality have spurred investigators to seek alternative solutions involving *ex vivo* systems. In an attempt to assess the viability and physiologic functionality of a cell-seeded device to replace the filtration, transport, metabolic, and endocrinologic functions of the kidney in acutely uremic dogs, a combination of a synthetic hemofiltration device and a renal tubular cell therapy device containing porcine renal tubules in an extracorporeal perfusion circuit was introduced. It was shown that levels of potassium and blood urea nitrogen (BUN) were controlled during treatment with the device. The fractional reabsorption of sodium and water was possible. Active transport of potassium, bicarbonate, and glucose and a gradual ability to excrete ammonia was observed. These results showed the technologic feasibility of an extracorporeal assist device that is reinforced by the use of proximal tubular cells [88].

Using similar techniques, the development of a tissue-engineered bioartificial kidney consisting of a conventional hemofiltration cartridge in series with a renal tubule assist device containing human renal proximal tubule cells provides was used in patients with acute renal failure (ARF) in the intensive care unit. The initial clinical experience with the bioartificial kidney and the renal tubule assist device suggests that renal tubule cell therapy may provide a dynamic and individualized treatment program as assessed by acute physiologic and biochemical indices [89].

The ultimate goal of tissue engineering, however, is the growth of fully functional and fully integrated replacement organs. Thus, another approach towards the achievement of improved renal function involves the augmentation renal tissue with kidney cell expansion *in vitro* and subsequent autologous transplantation. The feasibility of achieving renal cell growth, expansion, and *in vivo* reconstitution with the use of tissue-engineering techniques has been explored.

Most recently, an attempt was made to harness the reconstitution of renal epithelial cells for the generation of functional nephron units. Renal cells were harvested and expanded in culture. The cells were seeded onto a tubular device constructed from a polycarbonate membrane, connected at one end with a Silastic catheter that terminated into a reservoir. The device was implanted in athymic mice. Histologic examination of the implanted devices over time revealed extensive vascularization with formation of glomeruli and highly organized tubule-like structures. Immunocytochemical staining confirmed the renal phenotype. Yellow fluid was collected from inside the implant, and the fluid retrieved was consistent with the makeup of dilute urine in its creatinine and uric acid concentrations [90]. Further studies have been performed showing the formation of renal structures in cows using nuclear transfer techniques [91]. Challenges await this technology, including the expansion of this system to larger, three-dimensional structures.

CONCLUSION

Tissue-engineering efforts are currently being undertaken for almost every major type of tissue and organ within the body. Most of the effort expended to engineer these organs has occurred within the last decade. Tissue-engineering techniques require a cell culture facility designed for human application. Personnel who have mastered the techniques of cell harvest, culture,

and expansion as well as polymer design are essential for the successful application of this technology. Before these engineering techniques can be applied to humans, however, further studies need to be performed in many tissues.

In addition to the challenges mentioned above, there are numerous other hurdles that face the tissue engineers of tomorrow. For example, physiologically accurate bioreactors must be developed and refined if complex organs are to be grown and functionalized prior to implantation in humans. Also, a refinement in the approaches to tissue engineering must be achieved, as scientists explore whether pre-engineered, functional bioengineered replacement organs must be produced to repair tissue function, or whether the goal of tissue engineering should be to use simple biomaterials, cells, or growth factors to induce the repair or regrowth of these damaged organs.

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PART 22

Regulation, Commercialization and Ethics

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CHAPTER 86

The Regulatory Process from Concept to Market

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INTRODUCTION

The US Food and Drug Administration (FDA) is responsible for regulatory oversight for a wide range of products, including food, medical products, products for veterinary use, and most recently, tobacco products. The FDA is comprised of seven centers, three of which, the Center for Drug Evaluation and Research (CDER), the Center for Devices and Radiological Health (CDRH), and the Center for Biologics Evaluation and Research (CBER), are charged with oversight of medical products. The Office of Cellular, Tissue, and Gene Therapies (OCTGT) in CBER, regulates a wide range of products, including: gene therapies, cell-based products, tumor vaccines, human tissues for transplantation, xenotransplantation products, certain combination products, and certain medical devices. This chapter discusses issues relevant to the development of products regulated by OCTGT, specifically, cell-based products or therapies.

Most of the products under OCTGT purview are regulated as biologics, which primarily include cellular and gene therapy products. Therefore, this chapter mainly discusses the Investigational New Drug/Biologics Licensing Application (IND/BLA) pathway. However, since biologics can be combined with or delivered by medical devices, some device-related issues will also be covered within these specific contexts.

REGULATORY BACKGROUND

In order to understand the regulatory process, it is helpful to have some background knowledge of the relationship between laws, regulations, and guidances. Laws, which are enacted by Congress and signed by the President, provide the legal mandate for FDA's

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regulatory oversight. In many cases FDA publishes regulations ('rules') to implement a law's legislative mandates.[a] Rules can be modified by FDA over time to allow for changes in science or technology as long as the revised rule remains responsive to the legislative mandates. As an example, FDA recently published a revised rule regarding sterility testing for biological products [6]. This new rule provides manufacturers of biological products with greater flexibility and encourages use of the most appropriate and state of the art test methods.

Unlike laws and regulations, which are both legally enforceable by FDA, guidance documents represent the Agency's current thinking on specific topics and are not legally enforceable. Guidance documents are intended to provide general advice to interested stakeholders and product developers (sponsors) on how to comply with certain laws and regulations. FDA issues guidances that provide advice on topics ranging from overarching regulatory process to the Agency's scientific expectations for meeting requirements for specific product classes. For example, if FDA recognizes a need for developers of a specific product type, or for a specific clinical need, FDA may provide guidances that explain its thinking on the applications of the regulations to those specific areas.

Over the last decade FDA has published numerous guidance documents that are relevant to the development of cellular and cell-based products. These include guidance on cell manufacturing characterization for cell therapy products [1], and on potency [5], as well as guidance documents that address Agency cross-cutting issues, such as evidence of effectiveness [8]. Investigators may also wish to refer to other resources, such as OCTGT Learn, a web-based educational series that provides outreach to OCTGT stakeholders on a broad range of topics [29], or the CDRH Device Advice [32] webpage, which provides links to extensive regulatory information including CDRH Learn. References to these and other resources, including guidance documents and draft guidance documents,[b] can be found at the end of this chapter.

EARLY-STAGE DEVELOPMENT

In order to successfully develop a cell-based therapy for marketing approval, the development program must address certain key issues: a consistent, controlled manufacturing process incorporating characterization of the product's core attributes; preclinical testing incorporating proof-of-concept (POC) and toxicology studies that provide scientific rationale and support the proposed use of the product; clinical evidence of effectiveness; and clinical evidence of an acceptable safety profile weighed against the benefit of the therapy. Forward-looking consideration to each of these key areas at an early stage may improve the overall efficiency of product development.

Chemistry, manufacturing, and controls (CMC)

There are a number of key CMC issues to consider early in the development of a cell-based product. For example, if the cells to be delivered were not derived from the recipient (e.g., cells collected from an allogeneic donor), donor screening and testing will be necessary (21 CFR Part 1271) [17,18]. It is important to give early consideration to this issue, since incomplete or insufficient information often necessitates additional product safety testing.

[a] The Government Printing Office annually publishes all current regulations (rules) in the Code of Federal Regulations (CFR) (in print and electronically). The CFR is divided into 50 titles or subject areas, and Title 21 pertains to food and drugs. Specific parts of CFR are referred to throughout this chapter and the Appendix to this chapter provides a summary of the parts of the Title 21 that are most closely related to material in this chapter.
[b] Draft guidances are published for comment and are not for implementation until finalized. Stakeholders should contact the relevant product office or division for the most up-to-date Agency perspective on an issue discussed in draft guidance.

The strategy for manufacture of a cellular product from a characterized source of cells can, in some cases, mean establishment of a two-tiered cell bank, the Master Cell Bank (MCB), and the Working Cell Bank (WCB), which is derived from the MCB [4]. Early consideration of a cell banking program capable of supporting aspects of both preclinical and clinical development is important. In the event it is necessary to establish new cell banks during product development, plans should be in place to demonstrate their comparability to the previous cell banks used for product manufacture.

Comprehensive cell characterization is a cornerstone in the development of cell-based products. A multi-parametric analytical testing approach designed to assess distinguishing attributes of a cell-based product will help meet regulatory expectations for demonstration of product identity, purity, and potency. Characterization of the cellular product serves to inform development of an overall testing strategy and establishment of appropriate acceptance criteria to ensure product quality. Careful efforts devoted to characterization during the initial stages of premarket development may aid in detection of sub-populations of cells in the final product that could present safety concerns.

Early attention to details associated with the design of a robust manufacturing process is recommended. Consideration should be given to the feasibility of reproducibly generating cells with the desired attributes in sufficient numbers to support overall product development. This includes assessing the quality and accessibility of reagents critical to execution of the manufacturing process. It should be noted that unanticipated changes in critical reagents are likely to have an impact on product quality.

In certain instances, a cell-based product will be regulated as a combination product, as a cellular component is physically or chemically combined with a drug and/or device (21 CFR Part 3), often as a 'cell-scaffold' product. For such cases, sponsors need to design a testing strategy that adequately tests the final product and the individual components [14].

Pharmacology/Toxicology

Prior to initiation of a clinical trial, a sponsor will need to provide adequate data generated from pharmacological and toxicological studies to establish that it is reasonably safe to conduct the proposed clinical investigation. The results of these studies provide data critical to:

1) Establishing the scientific rationale and biological plausibility of the proposed approach (i.e., demonstration of POC);
2) Identifying and characterizing potential local and systemic toxicities, including the time frame for onset (i.e., acute vs. long-term), incidence, severity, and duration;
3) Determining a possible dose-response relationship for guiding dosing for the planned clinical trial;
4) Supporting subject eligibility criteria; and
5) Identifying physiologic parameters to help guide appropriate clinical monitoring.

Multiple *in vitro* and *in vivo* studies, spanning several iterative prototypes of the product and delivery device, may be necessary to translate a cell-based product from the bench to the initial clinical trial. During this process, the incorporation of forward-looking features into the design of early discovery phase animal studies may maximize the relevance and interpretability of collected data and facilitate translation to the clinic. For example, if safety endpoints are incorporated into discovery phase studies, it may be possible to identify potential safety concerns early in product development, which can then be managed (such as through changes to product design or manufacturing) and investigated further in future preclinical studies. Similarly, archiving relevant biospecimens during preclinical development allows for the opportunity for subsequent analysis if unanticipated adverse events arise in later studies,

which may help identify the cause of an observed toxicity. In turn, this may inform the design of appropriate follow-on investigations.

For cell-based products, early investigations in discovery phase studies into cellular distribution and the rate of resorption/degradation of the construct will be beneficial. As the product degrades or the injured tissue undergoes remodeling, structural properties, donor and host cell activity (such as migratory activity, proliferation, phenotype), and the rate of tissue in-growth will likely change. An understanding of these *in vivo* parameters and their impact early in product development will help identify appropriate endpoints, outcomes measurements, and study durations for later-stage definitive preclinical studies. For example, if the product is designed to provide mechanical support or structure, an assessment of biomechanical properties and function at multiple time points during degradation or remodeling may be particularly important. In some instances it may not be possible to conduct complete functionality testing of a product prior to implantation, as it may undergo remodeling *in vivo* with accompanying changes in biomechanical performance. This product characteristic would then be an important consideration when designing the POC and safety studies.

Some additional early considerations for preclinical development may include choosing a biologically-relevant animal model, random assignment of animals to groups, the use of appropriate control groups (such as implantation of the scaffold alone), assessment of effects of any changes to product manufacturing on *in vivo* activity, and characterization of both the final product and the individual components.

Clinical

Development of novel cell-based therapies is an iterative process. Therefore, even well-considered investigational plans are likely to need revision as additional data are acquired. However, early thought to the tentative design of the clinical studies can provide a framework for the overall development program, including preclinical studies. Preclinical studies usually mimic the clinical situation as closely as possible; therefore, integrating the preclinical and clinical programs may help improve efficiency. For example, if a preclinical animal study uses a specific delivery procedure, and then it is decided that the Phase 1 trial will use a different delivery procedure, additional preclinical studies may be necessary to support the safety of the new clinical procedure.

Phase 1 trials generally have a primary objective of assessing safety. However, co-primary or secondary objectives usually include bioactivity assessments. Such preliminary activity data can help guide the subsequent development program.

When selecting the study population for a Phase 1 trial, sponsors should consider the target indication, interpretability issues, and the risk of the study procedure. A product which can potentially cause long-term adverse effects is usually felt to have an unacceptable risk when administered to normal healthy volunteers. Therefore, Phase 1 biologics trials usually enroll a study population of subjects who have a specific disorder.

In the absence of preliminary evidence of safety for a single dose and data regarding the time course for bioactivity of the biologic, repeat dosing may represent an unreasonable risk. Therefore, a single dose regimen is appropriate for most first-in-man trials. Additionally, prior to the collection of preliminary safety data for the product, a first-in-man study should avoid concurrent administration of the product to multiple subjects. Staggered administration to sequential subjects provides an inter-subject interval to monitor for acute and sub-acute adverse events (AEs). This approach may prevent multiple subjects from experiencing an AE related to the product.

The safety monitoring plan should include assessments that can capture early, intermediate, and delayed AEs that may be expected, based on pre-clinical and clinical data, as well as on theoretical concerns.

Stopping rules are criteria, usually based on a number or frequency of specific AEs, that if triggered would temporarily halt the study, pending a safety review. Stopping rules should be considered for all studies, and may be required for higher risk studies.

Selection of the number of subjects that will be enrolled into a Phase 1 trial should consider the power of the sample size to rule out AEs that occur at a clinically meaningful rate. In general, the number of subjects in Phase 1 trials reviewed by OCTGT is less than twenty. Enrolling a relatively small number of subjects avoids exposing a large number of subjects to risk, while allowing for collection of preliminary evidence of safety.

FDA/SPONSOR MEETINGS

OCTGT holds informal and formal meetings with sponsors prior to and during product development. Informal meetings generally occur via phone, while formal meetings are conducted via phone or face-to-face. There are several different types of formal meetings that can occur between OCTGT and sponsors. These meetings often represent critical points in the regulatory process, and each is subject to different procedures.

Pre-submission meetings provide sponsors the opportunity to obtain non-binding feedback from FDA on specific questions prior to submission of an investigational (IND) or marketing (BLA) application. Such meetings may help sponsors to avoid missteps or delays that may be costly in terms of time and financial resources.

A meeting package that includes targeted, thoughtful, and clearly articulated questions, as well as a detailed summary of information, facilitates a productive meeting. Information about what should be included in meeting packages, meeting timelines, and meeting procedures can be found in FDA guidances [16,20,22], the OCTGT Learn webinar series, or by contacting the Branch Chief or Team Lead of the Regulatory Management Staff in OCTGT.

SUBMITTING AN IND

An IND is required (21 CFR 312) prior to clinical testing of a cell-based product that has not been approved by the FDA. A complete IND package consists of all required forms and sections, as specified in 21 CFR 312.23(a) and itemized in the checklist on page 2 of Form FDA 1571. A more detailed description of the key information that should be submitted with the original IND application is provided below.

Required FDA forms

Forms 1571, 1572, and 3674 should be included in an IND submission. These forms can be found on the FDA website, along with more detailed instructions for completing the forms [25,27].

Form 1571 is a formal contract indicating that a sponsor will adhere to the principles of informed consent, obtain Institutional review boards (IRB) review of the IND, and comply with the IND regulations. Form 1571 also contains basic administrative information regarding the sponsor, investigational drug and proposed clinical trial (e.g., indications(s), phase of investigation, data monitoring), and includes a checklist of submission components. A signed Form 1571 is required for all original IND submissions.

Form 1572, the Statement of Investigator, is an agreement that an investigator will provide certain information to the sponsor and will comply with FDA regulations. This form serves the dual purpose of enabling the sponsor to establish and document the qualification of the investigator and clinical site, and informing the investigator of his/her obligations. Although it is the IND sponsor's responsibility to obtain a signed Form 1572 from each investigator (21CFR 312.53(c)), the FDA does not require that Form 1572 be submitted to the Agency.

However, since Form 1572 collects all the information that must be submitted to the FDA as per 312.23(a)(6)(iii)(b) [31], many sponsors choose to submit Form 1572 along with the curriculum vitae (CV) of the investigator(s) [26].

Form 3674, 'Certification of Compliance with Requirements of ClinicalTrials.gov Data Bank', ensures compliance with the requirement for registering and reporting results from clinical trials of human drugs (including biological products) and devices to the clinical trials data-bank [21].

IND contents

In addition to the forms described above, all IND submissions must include a table of contents, an introductory statement, and a general investigational plan. An Investigator's Brochure (IB) is required unless the IND is submitted by a sponsor-investigator (21 CFR 312.23). A well-organized IND includes distinct, stand-alone sections for the clinical protocol, product/CMC information, preclinical pharmacology/toxicology information, and other sections described below.

CLINICAL PROTOCOL

An IND submission should contain a clinical protocol for each planned study. The complexity of the protocols will vary with the phase and size of the planned clinical investigation, however, some key elements include: the study objectives, selection of the study population, dosing, safety monitoring plan, and sample size.

CMC SECTION

The CMC portion of the IND contains information about manufacturing, including details about the starting material and reagents used in the manufacturing process [1,2]. For processes that use materials and reagents of human or animal origin, it is important to provide documentation of compliance with 9 CFR 113.53. The CMC section should describe the safety and quality testing that will be performed on the final product prior to release for administration to subjects. If the manufacturing process is complex and involves multiple steps executed over an extended period of time (e.g., days to weeks), the CMC section should include details of the in-processing testing performed at key stages of the manufacturing process. This section should also include data regarding product stability and shelf-life and other controls, such as information regarding the containers, labels and tracking information.

For cellular-device combination products, where the device components may be scaffolds/matrices or delivery devices, the IND should contain detailed source, manufacturing, and testing information for those components. This information should include device composition, physicochemical/mechanical testing, and specifications. Information regarding sterilization and stability/shelf-life are also important. If degradable biomaterials are used as scaffolds or for encapsulation, detailed information regarding the profile/kinetics (e.g., molecular weight, diffusion/mechanical properties, degradation by-products) should be provided. In general, the type of information that should be provided in the IND for the device component is generally similar to what should be provided in an Investigational Device Exemption (IDE) application (21 CFR Part 812). Therefore, relevant CDRH guidances may provide helpful information about what to include in an IND for a combination product [33].

PHARMACOLOGY AND TOXICOLOGY DATA

The FDA determines whether the pharmacology/toxicology data submitted with the IND provide sufficient evidence that the investigational product is reasonably safe for initial testing

in humans (21 CFR 312.23 (a)(8)). For each preclinical study intended to support the safety and rationale of a proposed clinical trial, a complete study report should be provided which includes:

1) a prospectively designed protocol,
2) A detailed description of the study performed (i.e., description of animal model, control and test articles used, dose levels, detailed procedures for test article administration and collection of all study parameters),
3) Results for all parameters evaluated (i.e., individual animal data as well as summarized and tabulated results),
4) An analysis and interpretation of the resulting data, and
5) A statement of compliance with Good Laboratory Practice (GLP) regulations (21 CFR Part 58).

PREVIOUS HUMAN EXPERIENCE

The IND should include detailed summaries of any available safety and efficacy data from previous clinical experience with the study agent or related products. The FDA will consider this safety and efficacy data, along with the comparability of the study products, the study populations, and the delivery procedures, when reviewing the IND.

ADDITIONAL INFORMATION

All other pertinent information can be submitted in this section. This could include copies of relevant publications, plans for assessing safety and effectiveness in a pediatric population, certificates of analysis obtained from manufacturers/suppliers of non-clinical grade reagents, and copies of meeting minutes from pre-IND interactions. Also in cases in which information for the product has been wholly or partially submitted to the FDA in previous pre-market submission(s) and/or master file(s), cross-reference authorization may be provided in this section of the IND. A letter of cross-reference grants FDA permission to review specific information contained in these files to support other premarket or marketing applications. The letter of cross-reference authorization is provided by the sponsor/holder of the referenced IND(s) and/or master file(s).

FDA review of an original IND application

When a sponsor submits an original IND, the FDA reviews the submission within the next 30 calendar days. Unless the FDA imposes a clinical hold, the clinical studies described in the original IND submission may be initiated at the conclusion of the 30 day review period. A clinical hold is an order by the FDA to delay a proposed clinical investigation or to suspend an ongoing study. When FDA review of an IND results in a clinical hold, within 30 days of being notified that the IND is on hold, the sponsor receives a clinical hold letter. The clinical hold letter summarizes the deficiencies that caused the clinical hold.

The IND regulations outline the general reasons for which an IND can be placed on clinical hold (21 CFR 312.42). A retrospective analysis performed by FDA staff evaluated clinical hold letters issued over a three year period. This analysis identified some common reasons for clinical hold of INDs regulated by OCTGT including CMC deficiencies (donor screening and testing, MCB qualification), insufficient information to assess the risks to the subjects, and issues with the proposed study population or procedures (e.g., an unacceptable risk of performing the investigational procedures in the proposed study population) [38].

LATER-PHASE DEVELOPMENT TOPICS

Compliance with current good manufacturing practice

Similar to other aspects of product development, the Current Good Manufacturing Practice (CGMP) requirements become more tightly controlled over the course of the product lifecycle. For Phase 1 studies, investigational cell-based products are excluded from complying with CGMP regulations (21 CFR Parts 210 and 211). However, the statutory GMP requirement in the Federal Food, Drug and Cosmetic Act (section 501(a)(2)(B)) is applicable for manufacture of clinical product during all phases of clinical study, including Phase 1, to ensure consistent product performance. In addition, CGMPs appropriate for Phase 1 investigational products should be in place for production of clinical lots to ensure product safety. These include sterility assurance, quality oversight, and facilities control. Adequate documentation and records management should also be in place for all phases of clinical study. FDA has issued a guidance document regarding CGMP requirements for Phase 1 studies [3]. Complete CGMP regulations should be followed for Phase 2 and 3 clinical studies of cell-based products.

Because CGMP applies to both the manufacturing process and the facilities, it is important to note that many elements of product manufacturing and characterization and CGMP compliance cannot be separated and should develop concurrently. Examples of CGMP activities that may develop over time as clinical development progresses include process improvements and process validation, as well as analytical methods development and validation. Process controls and acceptance criteria should be further refined prior to Phase 3 based on knowledge of the manufacturing process and critical product attributes related to safety and efficacy. Sponsors can request a meeting with CBER's Office of Compliance and Biologics Quality (OCBQ), specifically with the Division of Manufacturing and Product Quality (DMPQ), to obtain specific advice on facility design, manufacturing controls, and process validation.

Product readiness for Phase 3

To initiate Phase 3 clinical studies that are intended to demonstrate clinical efficacy, it is necessary to have in place a consistent, reproducible, and adequately controlled manufacturing process. Standard operating procedures that direct each stage of manufacturing should be finalized prior to Phase 3. Critical analytical assays for process intermediate and final product evaluation should have already been identified, developed and implemented. Acceptance criteria for these assays should be identified and appropriately narrowed to reflect the range of test result values accumulated through previous manufacturing experience. Any manufacturing changes introduced at this point in product development will usually be conservative in their character. One such example would be modification of procedures to boost production capacity or a change in suppliers of a key reagent. Final formulation, storage, handling and shipping conditions should also be established prior to Phase 3.

Potency assay

By Phase 3, an assay or compilation of assays developed to measure product potency should be identified and incorporated into the panel of product tests (21 CFR 600.3(s)). The potency assay provides information on the consistency of the manufacturing process, attests to the comparability of products manufactured from different starting materials, is useful in assessing the impact of changes made to the manufacturing process on final product attributes, and provides evidence of product stability under storage and shipping conditions.

The appropriate assessment of potency for each product will be determined based on attributes specific to the product. These include molecular, biochemical, immunologic, phenotypic, physical and biological properties. Accordingly, the FDA assesses the adequacy of a specific potency assay on a case-by-case basis.

Cell-based products are characterized by dynamic biological properties that present significant challenges to the development of a reliable, quantitative potency assay. As such, development of a potency test for these products is frequently done in a step-wise, incremental approach that allows for its modification as new information about the product characteristics, including mechanism of action, are discovered during the process of clinical development. However, even though establishing a potency assay may not be a reason for clinical hold prior to Phase 3 clinical testing, it is important to emphasize the role of the potency assay. In conjunction with other lot release tests, the potency assay is intended to demonstrate that only those manufactured product lots which meet pre-defined specifications or acceptance criteria are administered throughout the entire lifecycle of product development [5].

In many cases, a single biological or analytical assay may not provide an adequate measure of potency and it may be more appropriate to use a panel of complementary assays (i.e., potency assay matrix) that assess different product attributes associated with quality, consistency and stability to demonstrate product potency. The potency assay matrix could include testing for relevant non-biological attributes such as mechanical properties that are important for product function.

Pharmacology and toxicology

Preclinical studies supportive of the initial safety of a proposed clinical program will have been completed prior to initiation of a Phase 3 trial. However, additional animal studies may be necessary in certain circumstances. For example, depending on the product type, target population, and any concerns that arose during earlier phases of testing, reproductive/developmental toxicity studies may be needed to support licensure. These studies can usually be conducted concurrently with Phase 3 trials [7]. In other instances, additional preclinical studies may be necessary prior to initiation of later-phase trials, such as following any significant changes to the dose level(s), dosing regimen, or target population. Similarly, if there is a modification to the manufacturing of the product, the comparability of the later-phase product to the product evaluated in earlier-phase clinical trials may be unclear. In this case, bridging preclinical studies may be necessary to establish comparability.

After clinical development has begun, additional preclinical studies are sometimes warranted to provide insight into the *in vivo* pharmacology and mechanism of action of the product. For example, preclinical data may aid in the identification of an appropriate potency assay for Phase 3.

Phase 3 clinical development

By the time the Phase 3 clinical studies are planned, it is important for sponsors to understand some of the key issues that inform the design of Phase 3 trials. Early-phase trials offer an opportunity to optimize study procedures in terms of product delivery, dosing, and concomitant treatments. Early-phase clinical trials can also provide an understanding of the appropriate study population, the treatment effect size for the Phase 3 primary endpoint, the time frame for product bioactivity, and the durability of response.

When submitting a Phase 3 protocol, the FDA recommends that sponsors consider submitting a request for a Special Protocol Assessment (SPA) [9]. Concurrence on a protocol under the SPA program is an agreement between the FDA and a sponsor on the design and size of a Phase 3 trial that is intended to provide evidence of effectiveness.

MEDICAL DEVICES

An IDE application is required for a clinical investigation of an investigational product that meets the legal definition of a medical device, or of a combination device which has a primary mode of action that is consistent with that of a medical device, (i.e., the structure or function of man is affected, but not through a chemical action or metabolism within or on the body) [30]. Information about the required contents of an IDE can be found in 21 CFR 812.20. CDRH is one of the three FDA Centers that regulate medical products for human use. Specifically, CDRH determines the appropriateness of initiating commercial distribution of many diagnostic and therapeutic medical devices as well as combination products.

A risk-based approach determines the level of information for premarket review of medical devices. A Premarket Notification (510(k)) application seeks to demonstrate that a Class II medical device is substantially equivalent to another device already being marketed within the US by presenting data on device design, preclinical testing and possibly clinical studies (21 CFR Part 807) [35]. When insufficient experience exists to predict the clinical performance of a specific product based on comparisons with similar devices, sponsors submit a Premarket Approval (PMA) application that describes device composition and manufacture as well as the results of preclinical and clinical studies (21 CFR Part 814) [34]. Many of the cellular and cell-based products reviewed by CDRH are combination products regulated as Class III devices under the Medical Device Authorities. A limited number of these products have also entered commercial distribution after approval of Humanitarian Device Exemption (HDE) applications, because these Class III medical devices were designed to treat limited patient populations (i.e., less than 4,000 cases per year) (21 CFR Part 814 Subpart H).

CBER and CDRH often collaborate in the premarket review of combination products that contain both device and biological components. Regarding the device component (e.g., a scaffold), insight into issues of product manufacture, biocompatibility, physicochemical and mechanical testing may be obtained by review of FDA guidance documents and the published summaries of 510(k) and PMA applications. CDRH has published guidance documents that address such issues as approaches for evaluating the biological responses to biomaterials [13] and material sourcing issues, physiochemical analyses and preclinical tests that may be appropriate for scaffolding materials [15]. Although the latter document focuses on a particular type of product, both are informative as to the types of issues that may need to be addressed. Review of relevant CDRH-recognized standards may also be helpful [36]. A common misconception when developing tissue-engineering products is the importance of using 'FDA approved biomaterials'. This misunderstanding may arise, because CDRH uses both sponsor-derived test data and published scientific literature, (when sufficient physicochemical data exist to demonstrate the similarity of previous and proposed biomaterials), to determine the safety of a product. Thus, one should understand that FDA does not 'approve biomaterials', because the clinical behavior of a product can be profoundly affected by other components in the final product. When weighing the value of previously published data (and/or the need for additional studies), one should consider the intended use, route of administration, and composition of the previous product.

OTHER REGULATORY TOPICS

Clinical research involving children

FDA regulations in 21 CFR Part 50 Subpart D outline special protections for children, which are in addition to the safeguards for all clinical trial subjects that are contained in 21 CFR 312. In general, initial studies of cell-based products should be conducted in an adult population prior to investigations in a pediatric population. However, in some situations, first-in-human studies may be ethical in a pediatric population.

The regulations require IRBs to review clinical investigations involving children and to make an independent determination regarding the degree of risk to the subject. For pediatric studies that involve more than minimal risk, which is most likely to be the case for first-in-man pediatric trials of cell-based products, the considerations for IRB approval are outlined in 21 CFR 50.52 [10].

Expanded access to investigational drugs for treatment use

FDA's expanded access regulations (21 CFR 312 Subpart I) allow access to investigational drugs for the primary purpose of diagnosing, monitoring, or treating a patient's disease or condition, rather than for generating scientific data intended to characterize the drug [24]. The intent of these regulations is to facilitate availability of these products to patients with serious diseases or conditions when no comparable or satisfactory alternative therapy exists. The regulations describe the criteria that must be met to authorize expanded access.

Charging for investigational drugs under an IND

The IND regulations allow sponsors to charge for the administration of investigational products in clinical trials, and to charge for investigational products made available under FDA's expanded access regulations (21 CFR 312.8) [24]. These regulations specify general criteria for authorizing charging, specific criteria for charging for an investigational drug in a clinical trial, and specific criteria for charging under the expanded access provisions, and clarify what costs can be recovered.

Responsibilities of sponsors and investigators

Sponsors and investigators are responsible for specific activities to ensure that the rights, safety, and welfare of subjects are protected, to ensure the quality of the clinical trial, and to meet regulatory requirements (21 CFR 312 Subpart D). The FDA regulations (21 CFR 312.3) divide clinical trial responsibilities among these two parties: sponsors are responsible for communicating with FDA through the IND and managing the clinical trials; clinical investigators conduct the studies and administer the investigational product to subjects.

SPONSOR RESPONSIBILITIES

Sponsors have several responsibilities for managing the clinical trials under an IND. Sponsors must select investigators who are qualified by training and experience to administer the investigational product to study subjects and conduct safety and efficacy monitoring. Each study site should have an investigator who signs Form FDA 1572, listing the name, address, and the locations where the study will be conducted, all sub-investigators, and the IRB responsible for review of the study (21 CFR Part 56). The investigator is not required to be a physician, but it is reasonable to assume that a physician will be part of the study team. The FDA expects that the sponsor will evaluate potential clinical sites to determine that the facilities and resources are adequate to conduct the study [23].

Sponsors must provide investigators with the protocol, the IB, and any other instructions necessary to conduct the study. As the study progresses, the sponsor must communicate any protocol revisions and new information about the safety of the investigational product to the investigators. Sponsors must also monitor the progress of all investigations conducted under the IND to ensure they are conducted in accordance with the general investigational plan and protocol [11]. If an investigator is not complying with the protocol, the sponsor must either obtain an assurance of compliance from the investigator or discontinue shipments of the product to the investigator.

IND safety reporting is another sponsor responsibility (21 CFR Part 312.32). Sponsors must promptly ($\leq$15 calendar days) notify FDA and all investigators receiving investigational product in an IND safety report of potential serious risks. Sponsors must also promptly

(≤7 calendar days) notify FDA of any unexpected fatal or life-threatening suspected adverse reaction. The investigator shall also notify the IRB (21 CFR 312.66) of all unanticipated problems involving risk to human subjects or others.

Sponsors must maintain adequate records of financial interest and records of the receipt, shipment, use, or other disposition of investigational product. All records related to the IND must be retained for two years after a marketing application is approved, or for two years after shipment and delivery of the investigational product was discontinued and FDA was notified. Sponsors must permit access for FDA to inspect the study records. A sponsor may transfer any or all of the sponsor's IND obligations to contractors, who then assume the obligations of the sponsor for the designated activities.

CLINICAL INVESTIGATOR RESPONSIBILITIES

Clinical investigators are responsible for protecting the rights, safety, and welfare of the subjects under the investigator's care. Investigators are required to conduct the study in accordance with the protocol, for example ensuring that subjects meet the eligibility criteria and that all follow-up assessments are performed as scheduled. They must personally supervise the administration of the investigational product and the conduct of the study. The investigator may delegate certain duties to the sub-investigators that are under his/her supervision, but he/she cannot transfer his/her regulatory obligations to others. Investigators must obtain a review of the study by the IRB that oversees their clinical site and must obtain the informed consent of study subjects prior to enrolling them into a study.

Investigators must limit the use of the investigational product to only the subjects enrolled in the study, and must maintain records of the storage and use of the product. Investigators must report AEs and progress reports to the IRB and to the sponsor so both are aware of new information about the safety of the investigational product. Investigators must also submit financial disclosure forms to the sponsor and update them when required. Investigators must prepare and maintain adequate and accurate case histories for each subject, and must retain all study records for two years after a marketing application is approved, or for two years after the investigation is discontinued and FDA is notified. Investigators should plan for how they will maintain these records for several years, especially if they are participating in early-phase studies. When the study at a clinical site has been completed, the investigator must submit a final report to the sponsor and must allow access for FDA to inspect the study records.

SPONSOR-INVESTIGATOR RESPONSIBILITIES

A sponsor-investigator is an individual who is the IND sponsor and is the only clinical investigator for a clinical study of the product he/she is developing. Sponsor-investigators are required to fulfill all of the responsibilities of sponsors and all the responsibilities of investigators, with the exception of the requirement for an IB. Sponsor-investigators must consider how to monitor the clinical trial he/she is personally conducting. Some options include review of the study by someone unaffiliated with the research, such as a colleague in another department, from an institution's research office, or by a contract research organization.

Clinical research conducted outside of the United States

A sponsor who is conducting a foreign clinical study may choose, but is not required, to conduct the foreign clinical study under an IND. When a foreign clinical study is conducted under an IND, all FDA IND requirements must be met unless a waiver is obtained. FDA may accept a well-designed, well-conducted, non-IND foreign study as support for an IND or application for marketing approval if the study was conducted in accordance with good clinical practice (GCP) and if FDA is able to validate the data from the study through an onsite inspection, if necessary (21 CFR 312.120). Regarding the ability of data from a non-IND

foreign study to support a marketing application, one consideration is the applicability of the data to the US population [12].

Use of standards

For FDA, standards describe how manufacturers might meet regulatory requirements defined in FDA regulations. Standards used for regulatory purposes may be 'physical standards' or 'reference materials' that are highly characterized reagents distributed nationally and internationally to assure consistent quality and safety of a product. Standards may also be in the form of documents that describe standard nomenclature, test methods, recommended practices and guides, specifications, and performance standards. For tissue-engineered medical products (TEMPs), the use of standards may help to facilitate product design and testing and lead to improved time to market. Standards Development Organizations (SDOs) that are actively engaged in developing standards for TEMPs products are ASTM International (formerly known as American Society for Testing and Materials) and International Organization for Standardization (ISO). FDA staff actively engages in standards development activities with SDOs by participating in workshops and meetings, and by commenting on standards documents [19,28].

FDA international regulatory activities

FDA has a history of productive working relationships with international regulatory authorities around the globe. Such interactions help facilitate FDA's goals to safeguard global public health and facilitate the availability of safe and effective products. FDA's global efforts for products regulated by OCTGT have focused on activities that foster the independent development of national guidelines and regulations that reflect a shared regulatory perspective internationally. International activities for regenerative medicine and cell-based products range from formal arrangements with international regulatory authorities, such as the Advanced Therapy Medicinal Product Cluster and Parallel Scientific Advice with the European Medicines Agency, to ad hoc product-specific discussion with international regulatory partners. FDA also participates in regulatory convergence activities with international groups such as the Regulators Forum Cell Therapy Convergence Group (of the International Conference on Harmonization) and the Asia-Pacific Economic Cooperation (APEC) Life Sciences Innovation Forum [37].

The role of cell-based products in medical product testing

A major priority of the FDA's strategic plan for advancing regulatory science includes efforts to modernize toxicology to enhance product safety. This emphasis recognizes the need to improve the predictive value of preclinical studies for all medical products, and the potential role for cell and tissue-based assays (a subset of bioengineered tissue products) in achieving this goal [31]. Implicit in this goal is the need to improve manufacturing processes, to increase the understanding of the biology, and clinical applicability of cell and tissue-based assays. These knowledge gaps are similar to those that exist for bioengineered tissue products that are being developed for therapeutic indications. Although the markets and regulatory considerations for *in vitro* assays to be used in development of traditional pharmaceutical products, and investigational bioengineered tissue products are distinct in many ways, the scientific challenges facing the widespread development of both are similar, and therefore the potential is that both may benefit from increased cross-talk between these specialized areas.

CONCLUSION

Federal regulations address many aspects of drug development, including CMC, preclinical development, and clinical trials. Efficient drug development depends on an understanding of these regulations and the regulatory process. OCTGT collaborates with sponsors to address the challenges that arise during the development of cellular and cell-based products.

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APPENDIX: CFR CITATIONS RELEVANT TO CELLULAR AND CELL-BASED PRODUCT DEVELOPMENT[d]

CFR Citation	Summary of Regulation
9 CFR 113.53	Requirements for ingredients of animal origin used for production of biologics
21 CFR Part 3	Product jurisdiction
21 CFR Part 50	Protection of human subjects
21 CFR Part 50 Subpart B	Informed consent (IC) of human subjects
21 CFR Part 50 Subpart D	Additional safeguards for children in clinical investigations
21 CFR Part 56	Institutional review boards (IRB)
21 CFR Part 58	Good Laboratory Practice (GLP) for nonclinical laboratory studies
21 CFR Parts 210 and 211	Current Good Manufacturing Practice (CGMP) for finished pharmaceuticals
21 CFR Part 312	Investigational new drug application (IND)
21 CFR Part 312.8	Charging for investigational drugs under an IND
21 CFR Part 312.23	IND content and format
21 CFR Part 312.32	IND safety reporting
21 CFR Part 312.42	Clinical holds and requests for modification
21 CFR Part 312 Subpart D	Responsibilities of sponsors and investigators
21 CFR Park 312.120	Foreign clinical studies not conducted under an IND
21 CFR Part 312 Subpart I	Expanded access to investigational drugs for treatment use
21 CFR Part 600	Biological products
21 CFR Part 600.80	Postmarketing reporting of adverse experiences
21 CFR Part 601	Biologics licensing
21 CFR Part 601.2	Applications for biologics licenses; procedures for filing
21 CFR Part 610	General biological products standards
21 CFR Part 610.12	Sterility
21 CFR Part 800	General medical devices
21 CFR Part 801 or 809	Labeling

[d] Complete electronic CFR available at http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&tpl=%2Findex.tpl

Appendix—*Cont'd*

CFR Citation	Summary of Regulation
21 CFR Part 812	Investigational device exemptions (IDEs)
21 CFR Part 807	Establishment registration and device listing for manufacturers and initial importers of devices
21 CFR Part 814	Premarket approval (PMA) of medical devices
21 CFR Part 814 Subpart H	Humanitarian Use Devices (HDE)
21 CFR Part 820	Quality System Regulation (QSR)
21 CFR Part 1271	Human cells, tissues and cellular and tissue-based products (HCT/Ps)

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CHAPTER 87

Business Issues

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INTRODUCTION

Imagine a world of medicine where replacement or repair of body parts could be accomplished using tissues grown in a laboratory. Or better yet, the body could be induced to repair and regenerate itself — to harness the body's natural healing processes, and activate these processes when and where they are critically needed. A recent headline announced that 'The First Person to Live to 150 has Already Been Born'; a headline which could all too easily be dismissed as being overly boastful. The question to ask ourselves, however, is should we be so quick to write off such a possibility? Whether or not the first sesquicentenarian is already among us aside, the progress in preclinical and clinical regenerative medicine programs, particularly stem cells, has picked up considerable pace and is being continually primed by a growing pipeline of promising research and therapeutic programs aimed at repair and regeneration. In the last 120 years, as a consequence of lifestyle and non-regenerative advances in medicine, we have seen an increase in average life expectancy from 43 years to 85 years. As we achieve the ability to extend or replace tissues and organs — to begin to realize the aspirational goals of tissue engineering, and regenerative medicine in general — would doubling life expectancy over the next 150 year period in fact be a possibility? And if so, what are the societal and commercial implications?

The past thirty or so years have seen the emergence of an area of medicine called tissue engineering and regenerative medicine. This discipline is a truly multidisciplinary field, involving scientists, engineers, and physicians working to construct biological substitutes which can replace (or help regenerate) diseased and injured tissues. It promises to revolutionize the ways we approach health and quality of life for tens of millions of people worldwide. The field has already produced notable examples of actual therapies, including skin replacement and cartilage repair. The pump is primed, but companies, payers and policy makers will need to assess long-range cost benefit trade-offs for engineered tissues and cell therapies when compared to traditional therapies. Understanding the interplay giving rise to opposing pressures — one from an expanding elderly population to improve health in old age, and the other a growing concern for containing healthcare costs — will be critical to tissue and cell therapy companies as they plan for and address concerns about how to patients can afford innovation in this important area of treatment.

THE AGING POPULATION

To consider the implications that further enhancing longevity through tissue engineering may have on society and medicine, one needs to start by understanding current trends and projected impacts. The age structure of the overall population is projected to change greatly

Principles of Tissue Engineering. http://dx.doi.org/10.1016/B978-0-12-398358-9.00087-2

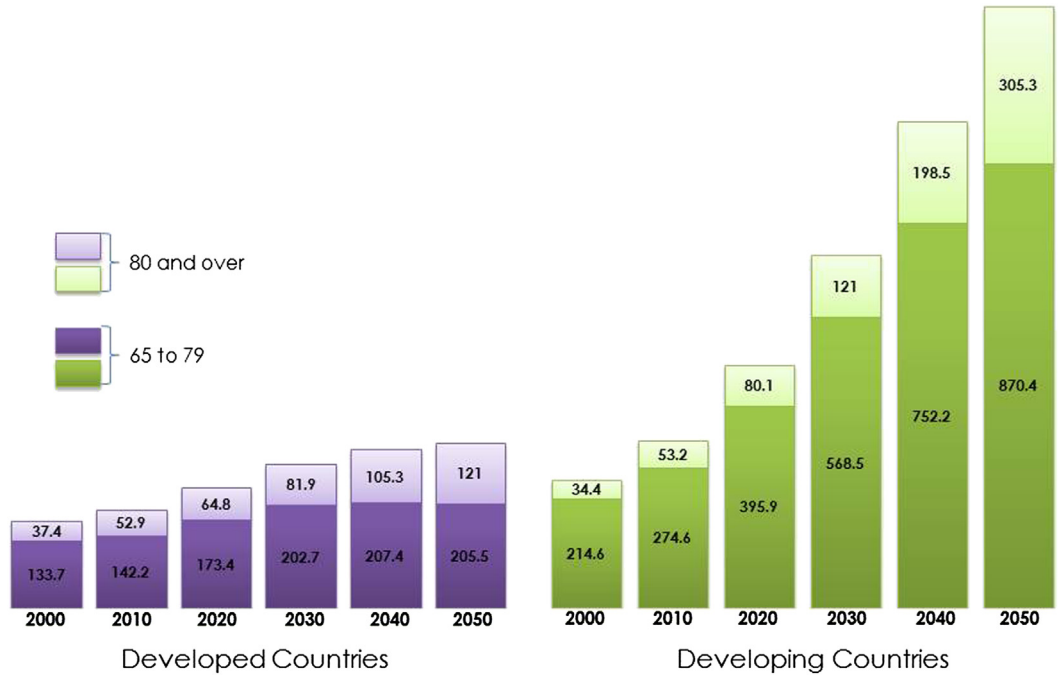

FIGURE 87.1

Population aged 65 and over for developed and developing countries (in millions). *(Source: U.S. Cenus Bureau Publication "65+ in the United States". P23-209).*

over the next forty years. The world's population is aging, primarily as a function of declining fertility coupled with increasing life expectancy. Aging is occurring not only in high-income countries, but also in middle- and low-income countries. In fact, between now and 2050 the world is projected to experience rapid growth in its older population. Fig. 87.1 shows the projected growth of two groups of older people — those aged 65—79 and those 80 years and older. The rapid growth of the over-80 group is related to increases in life expectancy related to improving medical care and nutrition which have occurred during the century.

People live longer now than at any time in the past; with advances in medicine promising to extend life expectancy even further. US life expectancy at birth rose from 47.3 years in 1900 to 76.9 years in 2000. Greater longevity, combined with relatively low fertility rates, has rapidly increased the proportion of the oldest old among the total older population. In 1900, only 4.0 percent of all older people were aged 85 and older; by 2000, that proportion had grown to 12.1 percent [1].

In 2050, the number of people over the age of 65 living in (now) developed countries is projected to exceed 325 million, with more than 1.5 billion persons in this over-65 category when including the developing countries in the equation — a nearly tripling of the number of over-65 people living in all these countries today. In the United States, as an example, the number of Americans aged 65 and older is projected to be 88.5 million, more than double the population of 40.2 million in 2010. The baby boomers are largely responsible for this increase in the older population, as they recently began crossing into this category in 2011. By 2050, the US population is projected to grow by 439 million, an increase of 42 percent relative to the 2010 census numbers of 310 million. The nation will also become more racially and ethnically diverse. The population is also expected to become much older, increasing from 15 percent today to nearly 20 percent of the US population being aged 65 and older by 2030, and continuing to increase to about 22 percent by 2050. Those over the age of 85 accounted for 5.8 million Americans in the 2010 census, and are expected to reach 8.7 million by 2030 and then 19 million by 2050. In terms of overall population, this 'oldest' old population will

increase to 4.3 percent of the population by 2050 — representing only 1.8 percent of the population in the 2010 census.

Much of the advances in longevity early in the 20th century arose from improvements in socioeconomic and living conditions and a decrease in infectious disease deaths. Gains during the latter part of that century came from periodic breakthroughs in public health and biomedical research that have led to new treatments for, and a later onset of, chronic diseases [2]. If improvements in treating and even preventing onset of chronic diseases can be sustained and further enhanced, then the age structure of the older population will be even more positively affected than the above projections suggest. This aging of the population will have wide-ranging implications. The projected growth of an older population will present challenges to policy makers and programs, such as Social Security and Medicare in the United States. The health status of the aging population is essential not only to those who comprise this age group, but also to the broader population because of the impacts on social and economic systems. As the older population grows not only in size, but more importantly in their proportion of the total population, the potential implications for families, businesses and healthcare providers will become increasingly significant.

Chronic diseases and impairments, which are among the leading causes of disability in older people, can negatively impact quality of life, lead to a decline in independent living, and impose an economic burden. About 80 percent of seniors have at least one chronic health condition and 50 percent have at least two [3]. Indeed, of the roughly 150,000 people who die each day, about two thirds die of age-related causes. In industrialized nations, the proportion is much higher, reaching 90% [4].

Concern is growing that medical advances leading to longevity will in turn lead to an older population who have a higher incidence of functional and cognitive impairment. With increases in life expectancy and a simultaneous rise in the number of people with chronic diseases and disability, researchers are focusing on facilitating both longer life and disability-free healthy life. Focusing on the quality as well as length of life, the World Health Organization (WHO) has introduced estimates of 'healthy life expectancy' (HALE), which is defined as the average number of years of life free from disability, physical performance limitations or impairments, other disabilities, or social handicaps [5]. Using various measurements and methods of analysis, including HALE, recent studies conclude that in addition to living longer, the current generation of older people is healthier and less disabled than the preceding ones [6–8]. But as Fig. 87.2 illustrates, as life expectancy increases so does the incidence rate of chronic and degenerative health conditions, threatening to create a wider gap between HALE and total life expectancy.

Currently, the vast majority of treatments for chronic and/or life-threatening diseases are palliative. Others delay disease progression and the onset of complications associated with the underlying illness. Only a very limited number of therapies available today are capable of curing or significantly changing the course of disease. The result is a healthcare system burdened by costly treatments for an aging, increasingly ailing population, with few solutions for containing rising costs. Fig. 87.3 demonstrates the predicted impact that our aging population is expected to have on the cost of healthcare in less than twenty years time. The demographics shift in aged populations towards older adults in the United States alone will likely add more than one trillion dollars to the direct cost of healthcare, and multiples of that amount for the indirect costs associated with accommodating the needs of older Americans. According to a June 2009 report from the Council of Economic Advisers (CEA) to the White House, healthcare expenditures in the United States are currently about 18 percent of GDP, and this share is projected to rise sharply. If healthcare costs continue to grow at historical rates, the share of GDP devoted to healthcare in the United States is projected to reach 34 percent by 2040. Viewing the distribution of the cost across all working Americans, Fig. 87.4 shows the potential impact on total compensation when the projected rising cost of healthcare is factored into predicted salaries.

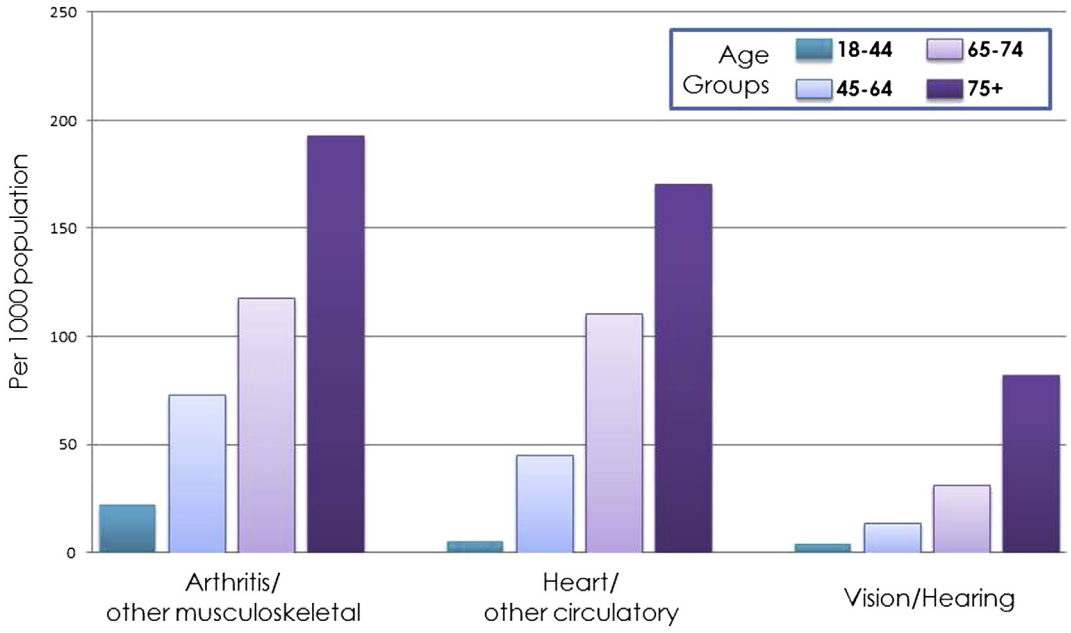

FIGURE 87.2
Exemplary chronic health conditions. *(Source: U.S. Cenus Bureau Publication "65+ in the United States". P23-209).*

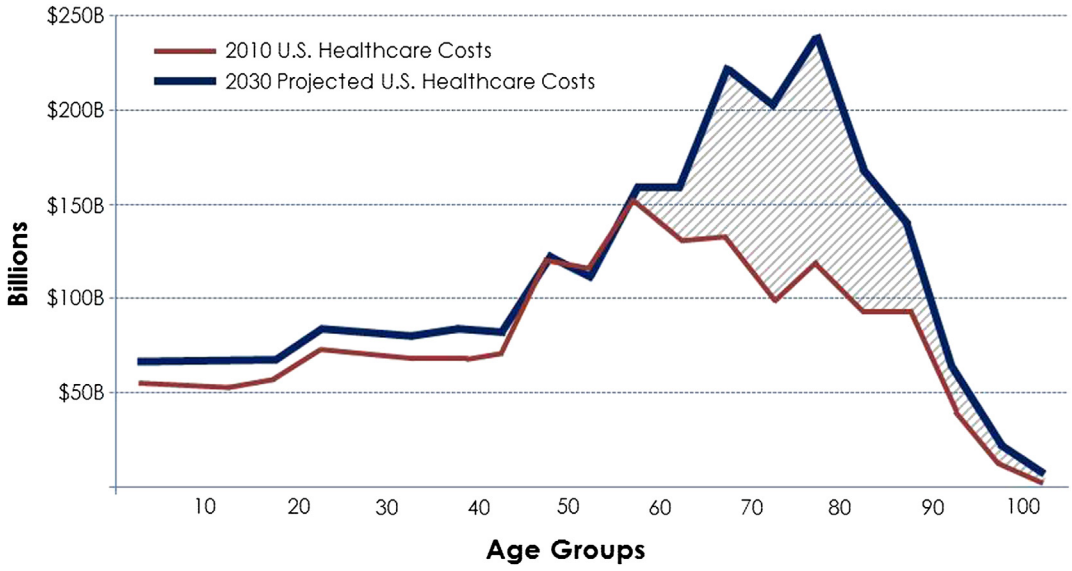

FIGURE 87.3
Impact of aging on US healthcare costs. *(Source: Alliance for Regenerative Medicine 2012 Annual Industry Report).*

RISE OF REGENERATIVE MEDICINE

The best way to address the escalating economics of healthcare includes developing more effective treatments, and even cures, for the most burdensome diseases – diabetes, neurodegenerative disorders, stroke, macular degeneration and cardiovascular disease, for

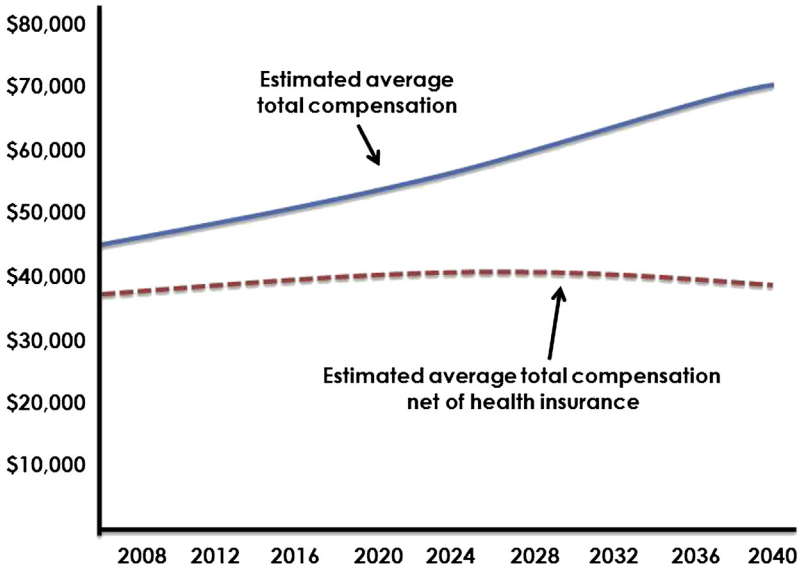

FIGURE 87.4
Projected annual compensation and compensation less health insurance premiums. *(Source: Office of the President, Council of Economic Advisors "The Economic Case for Healthcare Reform" June 2009).*

example — to facilitate longer, healthier, and more productive lives. Regenerative technologies, such as cell and tissue-based therapies, have the power to be a critical component in reducing the otherwise predicted increase in healthcare costs resulting from aging. A more effective, sustainable healthcare system is a potential as a consequence of the innovations occurring in regenerative medicine. However, it will require the concerted efforts of patients, government and private insurance companies, doctors, life science companies, private investors, and governments. Regenerative medicine promises to completely change the way we think about disease, aging, and even the practice of medicine itself — and, as the promise goes, to help bend down the ascending curve of projected healthcare costs.

Regenerative medicine, and particularly cell and tissue therapies, has already had some clinical impact. Hundreds of thousands of patients have already had bone marrow or hematopoietic stem cell transplants, with more than 50,000 new transplants being carried out each year [9]. A more recent category of approved products include tissue-engineered skin, with more than 250,000 patients having now received artificial skin grafts [10]. While the number of patients treated with cell therapies is still low by the standards of conventional drug- or biologics-based treatments, there is a robust pipeline of new therapies in clinical or preclinical testing.

However, the realization of the power of cell therapies, and with it the emergence of this growing area of medicine, is not only dependent on resolving the science but also on a number of business factors unique to cells as therapeutic agents. Regulatory oversight, clinical end-points, drug labeling, pricing and reimbursement, manufacturing, cold chain, and essential infrastructure are some of the many components from the business perspective which typically have unique and far reaching consequences in the path from the laboratory bench to patient treatment. Laminated onto these business issues can be layers of political, legal and ethical sensitivities which need to be considered. Regenerative medicine is uniquely capable of altering the fundamental mechanisms of disease; however, to realize its potential we must think differently about therapeutic development and commit to investing in these trans-formative technologies. A more effective, sustainable healthcare system is possible through regenerative medicine, but it will require the combined efforts of patients, payers, healthcare providers, biotech and pharmaceutical companies, private investors and governments working together.

The reality is that not every new company or technology in this space can or will succeed. Not every new invention relating to regenerative medicine is necessarily commercially tractable. The technology may be too complex or high touch to be used in an FDA-regulated commercial setting, or too costly, particularly where reimbursement by insurance entities or the government needs to be considered. Unfortunately, great science does not always translate into great business. The history of the life science industry is littered with many examples of brilliant research and breakthroughs unable to be translated into a viable commercial product. Understanding what are often the gating issues, and how to manage through those issues, including recognizing the 'go/no go' decision points, can greatly increase the likelihood of success. Realizing the promise of regenerative medicine from the perspective of the life science company will require understanding the regulatory policies in the major markets, appreciating the impact of final manufacturing, final formulation and the cold chain of the distribution, as well as developing strategic business and reimbursement plans. Added to this equation for success is the inevitable complexity of intellectual property issues.

PRODUCT DEVELOPMENT

Tissue engineering encompasses an array of technologies in biology, chemistry and physics into materials, devices, systems, and a variety of therapeutic approaches — including cell-based therapies — to augment, repair, replace or regenerate organs and tissues (see Fig. 87.5), with the end goal often being curative by targeting the root cause of disease. This rapidly evolving, interdisciplinary field in healthcare is transforming the practice of medicine, medical innovation and the production of medical devices and therapies.

Living cells are incorporated into regenerative medicines to achieve a variety of positive effects, such as: replacing damaged or diseased cells and/or tissue; stimulating healing and regeneration in diseased tissue; and delivering small molecule therapies to targeted areas. However, by their very nature, cell and tissue therapies face fundamentally different development and regulatory pathways to market when compared to traditional small molecule-based drugs or protein therapeutics. Alongside the complexity of the agent being administered to patients come added layers of safety and efficacy concerns. Cell therapies face the challenge of manufacturing a living product alongside associated difficulties in their storage and delivery. Depending on the solutions, companies may need to decide whether to focus on manufacturing a product or becoming a service-based entity, each of which requires a fundamentally different business strategy. As of 2012, the regenerative medicine industry to include more than 700 companies ranging from divisions of global pharmaceutical companies

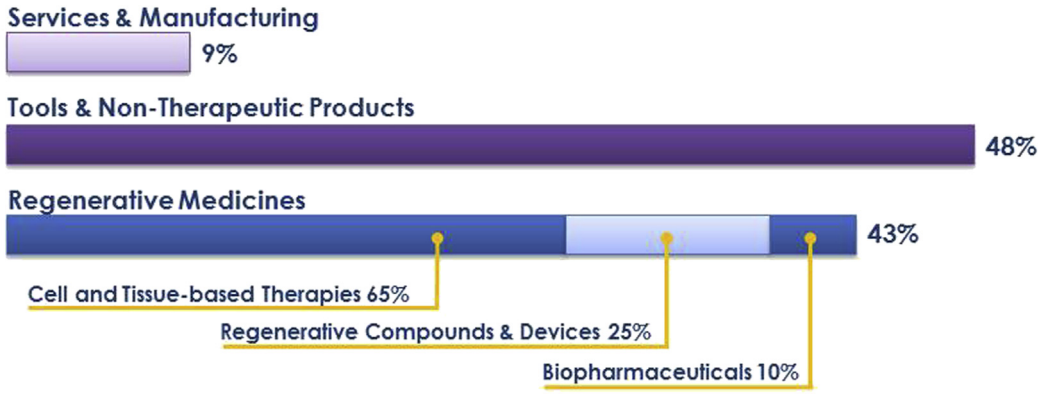

FIGURE 87.5
Regenerative medicine industry sectors. *(Source: Alliance for Regenerative Medicine 2012 Annual Industry Report).*

to smaller life science companies focused solely on regenerative medicine technologies [11]. The product pipeline of these groups includes hundreds of cell-based therapies, small molecules, biologics, tissue-engineered cells and materials and implantable devices. Other companies work toward providing research tools such as equipment, consumables, software, cells as drug discovery or toxicity testing tools as well as clinical tools, bioprocessing tools and platforms that include equipment, consumables, reagents and storage systems. The field also incorporates a variety of service companies specializing in clinical trial management, manufacturing, engineering and financing among others.

An important concern for emerging life science in the regenerative medicine field relates to developing a compelling business model. It is important to understand the consequences arising from, for example, the choice of technology by a company with how it relates to the practical realities of commercialization, particularly manufacturing, distribution and adoption by healthcare providers, not to mention coding and reimbursement. There are fundamental differences in the business models arising from the choice between an allogeneic cell or tissue product (a source which can be scaled-up and universally applied to patients), versus providing autologous cells or tissues requiring the manipulation of a patient's own tissues. The choice between the allogeneic and autologous therapy routes is proving to be a fundamental one for early adopting companies with significantly different production, infrastructure, logistics, skills, and storage requirements for each approach. Autologous cell therapies already represent a significant industry, though principally as a service-based model rather than something more akin to a pharmaceutical or biologics business model. One advantage of autologous transplants is that the body recognizes the cells as self and therefore does not reject the transplanted materials. For example, bone marrow transplants have been successfully used for many years to restore immune function after chemotherapy, and therapeutic trials using patients' own stem cells to restore other tissues are already underway. The allogeneic cell transplants have greater potential for scale-up and widespread distribution, thus potentially benefiting from greater economies of scale. This model is closer to the traditional pharmaceutical model, and shares many similarities to biomanufacturing processes that pharma have already invested in heavily over the last decade. However, allogeneic therapies face a greater risk of immunological rejection as the cells are typically not HLA matched to the intended recipient patient. For these reasons, the allogeneic business model is anticipated to develop more slowly, beginning first with cells and tissues that are transplanted in immune privileged sites in the body such as the CNS or sub-retinal space, or for which the cell is itself immune privileged, as is the case with mesenchymal stem cells.

In many respects, regenerative medicine companies are no harder to start up than other life science companies. Yet establishing a successful cell therapy company poses some unique challenges, in part because the field is only recently emerging and the regulatory oversight and reimbursement for patient therapy is still undefined in many ways. In the end, cell and tissue therapies are likely to be more expensive to develop than small molecule or protein therapeutics largely because the safety and proof-of-concept in relevant animals, if available at all, that the regulatory agencies want as part of the preclinical package. Yet those risks can be dwarfed by the concern that potential therapies will get entangled in regulatory red tape before they can even work their way through clinical trials.

Embryonic stem cells

One of the fundamental issues arising from the use of adult-sourced human stem cells for allogeneic therapies or *ex vivo* engineered tissues derives from the limited replicative capacity that adult stem cells have in culture. Very often, the adult cells will undergo only a handful of cell doublings before large percentages of the cells in culture senesce or differentiate into unwanted cell types. In contrast, a human embryonic stem cell (hESC) line represents a nearly inexhaustible supply of pluripotent cells. Derived from a single clonal embryonic stem cell, the

replicative capacity of an hESC line is enormous. However, for a long time there was a concern in the industry around whether or not the regulatory agencies would permit a differentiated cell made from an hESC line to even be tested in patients. The concern, at least as it manifests itself in the industry and among potential investors, arose from the ability of embryonic stem cells to form any tissue in the body – including tumors. The fundamental question was under what circumstances would the FDA or EMA (for instance) permit a differentiated cell product to be tested in human patients? How would they control for the risk that a cell therapy product might be contaminated with an undifferentiated cell, particularly an embryonic stem cell?

That question was finally answered in 2010 when two companies, Advanced Cell Technology and Geron each obtained FDA to conduct Phase I/II studies in human patients using cells derived from hESC lines. While Geron ultimately abandoned its cell therapy programs to focus on oncology product lines, Advanced Cell Technology moved forward in clinical trials both in the Unites States and the United Kingdom for treating various forms of macular degeneration using retinal pigment epithelial (RPE) cells derived from an hESC line. In the company's IND and IMPD filings with the US and U.K. regulatory agencies respectively, Advanced Cell Technology described a carefully controlled cell culture process which, when shifted from proliferation to differentiation was incapable of supporting growth of undifferentiated embryonic stem cells. Moreover, the company had developed a release assay for the final RPE cellular product that was several orders of magnitude more sensitive than PCR for detecting contamination with undifferentiated cells. Putting these two manufacturing steps together, along safety data from hundreds of animals injected with the human RPE cells, the company was able to establish an acceptable reduced risk profile to be permitted to test the RPE cells in human patients.

In July 2011, Advanced Cell Technology treated its first two patients with hESC-derived RPE cells. One patient suffered from Stargardt's disease, a juvenile onset form of macular degeneration, and the other patient suffered from dry Age-Related Macular Degeneration (dry AMD), the most prevalent form of AMD and the leading cause of blindness in people over the age of 65. In 2012, the company published its preliminary findings on these first two patients, reporting that there were no adverse events associated with the RPE cells, and demonstrating both anatomical and functional evidence that the injected cells engrafted, persisted and were apparently recapitulating the function of the missing native RPE cells [12].

Induced pluripotent stem cells

As described in greater detail in earlier chapters, terminally differentiated somatic cells can be reprogrammed to generate induced pluripotent stem (iPS) cells by the enforced expression of a few embryonic transcription factors. The resulting iPS cells are morphologically and phenotypically similar to embryonic stem (ES) cells and thus offer exciting possibilities in stem cell research and regenerative medicine. The first iPS cells were produced in 2006 from mouse cells and subsequently in 2007 from human cells [13,14]. The most widely used set of reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, was identified initially by screening 24 pre-selected factors in mouse embryonic fibroblasts (MEFs) by Takahashi and Yamanaka. Forced expression of these factors initiates phenotypic and molecular changes in the targeted somatic cell, eventually leading to the reactivation of endogenous pluripotency genes and acquisition of pluripotency [15]. The ability to restore pluripotency to adult cells through the induction of a small number of reprogramming factors promises to create powerful new opportunities for modeling human diseases, and perhaps more excitingly, offers hope for patient-specific cell therapies.

However, the challenge now is to better define differences in the epigenetics and gene expression of the resulting cells and subsequently improve the reprogramming methods in order to make human iPS cells a truly tractable alternative to human ES cells. While at first

approximation ES cells and iPS cells appeared equivalent and suggested interchangeability, recent studies indicate that significant variations can exist that markedly affect the epigenetic and functional properties of the iPS cells. Through different mechanisms and kinetics, these two reprogramming methods reset genomic methylation, an epigenetic modification of DNA that influences gene expression, leading various groups to hypothesize that the resulting pluripotent stem cells might have different properties. Indeed, a number of reports have indicated that iPS cells often have some DNA methylation patterns which are characteristic of the somatic cell origin prior to induction of pluripotency. This 'epigenetic memory' can favor differentiation of the resulting iPS cell back along lineages related to the original parent cell, and often also results in restriction of alternative cell fates. Indeed, techniques such as high-resolution methylation analyses demonstrated that differences in methylation patterns could consistently distinguish between iPS and ES cells [16] and produced variety in the level of expression of gene programs. Even subtle changes in expressions levels for just a handful of genes have given rise to rejection of iPS cells in mouse transplantation models, indicating that some iPS cells are immunogenic and raising concerns about their therapeutic use [17].

In the coming years, the efficiency of generating iPS cells and the understanding of the mechanisms of cell programming and reprogramming is likely to improve. However, there are ongoing concerns over safety presenting a significant hurdle before we will see significant progress towards therapies. At this point, considering just the regulatory issues alone, the differences between iPS and ES cells becomes the emphasis in calculating the remaining steps necessary to sufficiently characterize the properties and differentiation capabilities of iPS cells as sources of potential commercial therapeutic products. Such studies will be crucial for determining the suitable iPS cell types for future stem cell-based therapies for human degenerative diseases.

Direct reprogramming of differentiated cells

In the last few years a number of studies have shown that a direct route can be taken to convert one differentiated cell to another, without going through an embryonic-like undifferentiated state. Direct reprogramming of differentiated cells has already been demonstrated, as explained earlier and the ability of certain genetic factors to dominantly specify cell fate has been known for some years. However, based on recent advances, this technology is likely to progress significantly over the next five years. Direct reprogramming has a number of major advantages including the potential to produce therapies based on small molecules and/or biologics for *in vivo* reprogramming. This method would also produce cell therapies without the need to use a pluripotent cell stage, thus greatly reducing the risk of rogue cells leading to uncontrolled cell growth or inappropriate differentiation into an unwanted cell type.

Small molecule-induced differentiation

Small molecules can also induce differentiation and have advantages in terms of the ability of the clinician to control dosage. For example small molecules have been used to generate iPS cells by acting as substitutes for genetic reprogramming factors. Such approaches offer the longer-term potential to activate dormant stem cells in the adult body, and proof-of-concept for this has been most recently demonstrated through the use of a small naturally occurring molecule, thymosin-β4, to stimulate cell mediated repair of a damaged mouse heart [18]. Other small groups also are making inroads with chemically induced pluripotency, for instance, showing that specific DNA-binding hairpin pyrrole-imidazole polyamides (PIPs) could be conjugated with chromatin modifying histone deacetylase inhibitors like SAHA to epigenetically activate certain pluripotent genes in mouse fibroblasts [19].

REIMBURSEMENT

At the end of the day, life science companies developing new drugs and treatments, including those in the regenerative medicine space, must justify their existence to the shareholders who invested and therefore funded the translation of a therapy from animal models to an authorized therapeutic product. That justification is largely based on the ability of the developing company to sell the product into the market place. Accordingly, before deciding to invest in developing a new regenerative medicine product, especially a cellular or tissue product, the company needs to carefully consider the sunk cost to get to an approved product and the resulting cost-of-goods once approved and weigh that against the likelihood of private insurance companies (private payers) and governmental payers (such as Medicare) being willing to reimburse the company for treatment of patients with the approved product, and if so, at what amount. Reimbursement is a multipronged process that requires evaluation of several components: coding, coverage and payment.

It is the responsibility of companies to demonstrate clinical and economic value for new regenerative medicine therapies. The field of establishing cost-benefit trade-offs associated with new treatments is known as pharmacoeconomics. A pharmacoeconomic study evaluates the cost (expressed in monetary terms) and effects (expressed in terms of monetary value, efficacy or enhanced quality of life) of a pharmaceutical product. There are several types of pharmacoeconomic evaluation: cost-minimization analysis, cost-benefit analysis, cost-effectiveness analysis and cost-utility analysis. Pharmacoeconomic studies serve to guide optimal healthcare resource allocation, in a standardized and scientifically grounded manner. In this regard, pharmacoeconomic analysis will determine whether regenerative medicine approaches are clinically and economically effective, capturing such factors as the downstream costs associated with managing disease, assessing direct, and in some cases indirect, costs associated morbidity and mortality in the patient population. This analysis includes assessing the downstream impact of a new treatment on patient quality of life and comparing it with the incremental cost of treatment over time. For example, living with reduced vision due to ischemic retinopathy, or an amputated limb due to a diabetic ulcer would each significantly reduce the quality of life value for a diabetic patient. When appropriate data is available, it is possible to develop a reliable set of outcomes and economic scenarios, consider trade-offs and make better decisions regarding the value of a novel therapy.

Emphasis on economic justification for patient care is evident across all aspects of healthcare, from reimbursement decisions by government and private insurance entities, to payers demanding data that support treatment pathways, to large payer systems that collect and analyze their own data. Companies are incorporating pharmacoeconomic assessment tools and decision analysis processes earlier in product development. More frequently pivotal clinical studies are designed to capture cost data as well as clinical outcomes. Every advanced cellular therapy developed today will undergo some level of pharmacoeconomic analysis.

Obtaining a medical procedure code is important for reimbursement purposes, and this process begins prior to FDA approval. The Healthcare Common Procedure Coding System (HCPCS), developed and maintained by the Centers for Medicare & Medicaid Services (CMS), is the major medical procedure code system used for billing in the United States. Medicare is the largest single payer of healthcare services in the United States, with almost 50 million beneficiaries and a budget in excess of $425 billion in FY2012. While Congress has the authority to change Medicare benefit categories, CMS makes the decision whether to approve new treatments and these decisions also influence private health plans' coverage decisions, because of Medicare's size. CMS decisions are based on the statutory requirement to cover treatments that are 'reasonable and necessary' from a clinical perspective; neither comparative effectiveness nor cost effectiveness is currently an explicit criterion.

Medical providers submit claims to public and private insurance payers to receive payment for their services. This information is conveyed to payers through codes — numeric and

alphanumeric characters which represent a specific service, procedure or product provided to a patient. Two code sets are used. Current Procedural Terminology (CPT) codes are maintained by the American Medical Association (AMA) and are used to report office visits, surgeries and other services reported by physicians. Changes to CPT codes (additions, revisions or deletions) are made annually by the AMA. HCPCS Level II codes are alphanumeric codes maintained by CMS. These codes are typically used to identify supplies, products and services not included in CPT, such as drugs and durable medical equipment. Changes to HCPCS codes are made quarterly by CMS.

To assess a new technology's clinical effectiveness and safety, CMS and private payers undertake technology assessments. For example, the assessment may determine if a technology achieves particular goals, which may include:

1) The technology must have received final approval from the appropriate governmental regulatory bodies, such as FDA;
2) The scientific evidence must allow conclusions to be drawn concerning the technology's effect on health outcomes;
3) The technology's beneficial effects must outweigh any harmful effects;
4) The technology must be as beneficial as any established alternatives; and
5) The health improvement must be attainable outside investigational settings.

That being said, even if a product has a code, it is not always reimbursed. Some insurance payers, including Medicare, have separate coverage determinations that indicate when a service is or is not covered. Coverage determinations describe whether specific services, treatment procedures, and technologies can be reimbursed and under what conditions. Most frequently, Medicare coverage determinations are made by local insurance contractors through local coverage determinations (LCD) and address coverage, coding, and billing guidance. Medicare also issues National Coverage Determinations (NCD) for an item or service to be applied on a national basis.

For Medicare, services and products are reimbursed through payment systems determined by the site-of-service. The following payment systems are relevant to regenerative medicine: outpatient prospective payment system (OPPS) for outpatient hospital, ambulatory surgery center services; physician fee schedule (PFS) for physician office services; and the inpatient payment prospective system (IPPS) for hospital inpatient services. Each payment system is unique and reimbursement strategies will need to be developed with site-of-service in mind.

Often insurers will require companies to demonstrate the cost effectiveness of their products, especially relative to the costs of existing treatments for the underlying condition. In addition, payers are becoming more interested in the comparative clinical effectiveness of new products versus existing treatments. The burden is on the companies bringing forward new and innovative products to demonstrate why insurers should be paying for them. Accordingly, early in their clinical programs companies should begin to plan their reimbursement strategy by conducting an initial reimbursement analysis of their product. The following questions will help companies determine where they should focus their efforts.

- *Where will the product be administered?* The reimbursement strategy will vary based on the site-of-service. If the product is performed in different settings (physician's office, hospital outpatient, etc), it may require several different strategies based on the location where the service is performed.
- *Who is the customer?* Strategies will be different for physicians, hospitals or specialty pharmacies as each one is reimbursed through different payment systems.
- *What patient population will receive the product?* For example, if the product is intended primarily for an over age 65 population, the payers of concern might be private commercial insurance, but more likely would be Medicare and Medicaid.

- *Is the product unique or are other similar products on the market?* If similar products are on the market, it is possible that payment and codes already exist for the product.
- *Does a code exist that accurately reflects the product?* If no code exists, begin the process to secure a new code assignment. If it does exist, is the payment level appropriate for the product?
- *What clinical evidence exists?* Medicare and private payers examine clinical evidence when determining coverage. Companies should begin early with compiling clinical evidence as this will be needed for coverage and coding requests. In addition, the company should compile economic data related to the use of its product.

Emphasis on economic justification for patient care is evident across all aspects of healthcare, from reimbursement decisions by CMS, to payers demanding data that support treatment pathways, to large payer systems that collect and analyze their own data. Companies are incorporating pharmacoeconomic assessment tools and decision analysis processes earlier in product development. More frequently, pivotal clinical studies are designed to capture cost data as well as clinical outcomes. Every advanced cellular therapy developed today will undergo some level of pharmacoeconomic analysis. The following is a checklist for companies, along with timeframes, to help guide executives through the reimbursement process.

- *Conduct a reimbursement analysis and formulate a strategy.* The analysis ideally should be prepared as the company prepares for Phase II clinical trials, but if not, it should be done prior to commencing Phase III trials. The analysis should include an assessment of where and how the product will be administered, how the product will be coded, and how similar/comparative products and existing treatments are reimbursed. Based on this assessment, the company should begin formulating a reimbursement strategy.
- *Gather health economics and comparative clinical effectiveness data.* During Phase II and Phase III trials, the company should collect pharmacoeconomic data to demonstrate the cost effectiveness and clinical effectiveness of the product relative to existing treatments. More and more frequently, payers are expecting companies to demonstrate the 'value' of their product prior to reimbursing it.
- *Secure a meeting with CMS (Medicare), private insurers, or both.* This will accomplish two goals. First, it will educate payers about the technology and its clinical and economic value. This is especially important for new technologies such as in regenerative medicine. Second, it will give the company an opportunity to ask questions and get a better understanding of the data needs that payers have when making coverage and payment decisions. This information can be used when designing your Phase II and III trials.

CONCLUSION

Regenerative medicine is not just a future hope, it is a reality today. Cell-based therapies and products are on the market now and many more are in advanced stages of being tested in patients. These products provide insight into what the future holds in terms of patient health and economic impact. Regenerative medicine is a multidisciplinary field, and to increase the likelihood of success, it is incumbent on those involved to draw on the expertise of a wide range of fields and stakeholders. Collaboration between academia, industry and clinicians is a vital component for the future success of the regenerative medicine field. This rapidly emerging field of medicine, particularly cell-based therapies, has the potential to deliver dramatic clinical benefits and address important unmet medical needs. It is possible to translate clinical improvement into a robust assessment of the economic benefit derived from superior clinical outcomes.

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CHAPTER 88

Ethical Issues

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INTRODUCTION

Case study: The small black box holds a perfectly shaped ear. The scientist at the front of the room explains how it was made. A scaffold of nanoparticles supports fibroblast cells that grew over the form, and the ear now looks and feels actual: it can be transplanted to tissue. For burn patients, this represents an enormous chance and change. It is the prototype of a new genre of medicine, one that uses powerful technologies and methods of bioengineering and cellular biology to transform the matter of the world.

The ear in the box is not a freak example of a new technique. It is, in fact, one of a number of new devices that utilize the convergent technologies of several different fields of science and engineering to create tissue that can mimic the structure and function of the natural world. Other examples include the creation of skin grafts, corneas, bone, cartilage, and, in some pilot studies, bladders. Based on the new technologies of genomics, informatics, nanoscale engineering, molecular biology, and stem cell research, tissue engineering can be said to alter the concept of medicine itself. Instead of treating ailing tissues or organs with drugs intended to repair their structure or function, tissue engineering aims at replacing the diseased or injured or aging tissues of the body with new ones entirely, made from component parts of the material of the world, both naturally occurring and synthetic.

Such an advance heralds a remarkable ability to heal, a long-awaited solution to several intractable problems, and a serious alternative to cadaveric or living-donor whole organ transplants, which have long been an ethically challenged sector of medicine [1,2]. Yet such a remarkable construction of the human body asks a great deal of any social world into which it is introduced, for it is the body that is the place of the self, the location of the acts of the sacred, and the sensory arbiter of the real. In fact, tissue engineering queries two of the very aspects of our humanity that we consider distinctive: our integral embodiment and our finitude. If we are indeed a collection of replaceable and adaptable parts, some people reason, what is it that separates us from any other engineered machine? If we can engineer, for example, a synthetic and improved lymphatic system, might we improve our chances to adapt to and overcome infectious disease? What other capacities for healing or alteration of our bodies might be prudent? How do we ensure that such changes are indeed ethical?

It is this query that has greeted the new biotechnologies of the body, one based, this chapter argues, in social reactions largely shaped by culture both ancient and contemporary. We then ask: What are the ethical challenges to the field of tissue engineering? Does tissue engineering raise new ethical issues, or is it a description of one of the modalities enabled by the convergence of other technologies that have been understood to be individually ethically freighted?

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In this chapter, I suggest using an established ethical framework that was suggested in 1999 by a committee of the American Association for the Advancement of Science on inheritable genetic germline modification and used far more widely by the field of bioethics to assess new technologies. We then review the responses given to new technologies in the past from a variety of sources in bioethics, philosophy, and theology. Finally, we reflect on how the legal and regulatory structure for tissue engineering impacts on our reflections on ethical norms [3].

Research evaluation (adapted from the AAAS Working Group on Human Inheritable Genetic Modifications 1998–2000 [3])

- Are there reasons, in principle, why performing the basic research should be impermissible?
- What contextual factors should be taken into account, and do any of these prevent the development and use of the technology?
- What purposes, techniques, or applications would be permissible and under what circumstances?
- On what procedures and structures, involving what policies, should decisions on appropriate techniques and uses be based?

ARE THERE REASONS, IN PRINCIPLE, WHY PERFORMING THE BASIC RESEARCH SHOULD BE IMPERMISSIBLE?

Principled reasons for objections to basic research are extremely difficult to conceive in research that is, by its very nature, intended to be translational and clinical. Yet ethical objections to the manipulation, replacement, and engineering of human tissue can be seen as part of a long continuum of dissent about medical technology that began to assume full voice in the 1970s, when successful genetic manipulation of bacterial genomes became possible [4].

All new technology raises new challenges — in particular, technology that refashions the embodied self, becomes a part of the 'self' and the identity of the subject, and seems to raise the deepest anxieties. Even tissue engineering, an emerging field with clear targets, clinical successes, and patient needs, will raise familiar concerns.

First among these is the argument that humans possess an essential nature and live within an essential natural order that cannot be altered without harm. For C. S. Lewis [5], this is expressed as a concern that the very acts of rational science — dissection, analysis, and quantification — are a violation of the sacred integrity that lies behind all of nature:

> Now I take it that when we understand a thing analytically, and then dominate and use it for our own convenience, we reduce it to the level of 'nature', we suspend our judgments of value about it, ignore its final cause (if any), and treat it in terms of quantity. This repression of elements in what would otherwise be our total reaction to it is sometimes very noticeable and even painful: Something has to be overcome before we can cut up a dead man or a live animal in a dissecting room.

For Lewis, the understanding of the body as replaceable is disturbing:

> 'The real objection', he says, 'is that if man chooses to treat himself as raw material, raw material he will be, not raw material to be manipulated by himself as he fondly imagined, but by mere appetite' (p. 274).

(Lewis imagines that new transformative technology will be manipulated by 'controllers' who will eventually transform man into mere matter.)

Callahan [6] echoes Lewis' concern, both in the sense that limits need to be placed on what is decent to do to nature and in the sense that such action is a part of a larger danger — that power

in the hands of medicine to heal is really power in the hands of the elite, or the state, to manipulate and control. He argues:

> The word No perfectly sums up what I mean by a limit — a boundary point beyond which one should not go. . . . There are at least two reasons why a science of technological limits is needed. First, limits need to be set to the boundless hopes and expectations, constantly escalating, which technology has engendered. Advanced technology has promised transcendence of the human condition. That is a false promise, incapable of fulfillment. . . . Second . . . limits (are) necessary in order that the social pathologies resulting from technologies can be controlled. . . . [W]hile it can and does care, save, and free, it can also become the vehicle for the introduction of new repressions in society.

These objections, made over 30 years ago, are still made (despite, one may note with some irony, 30 years of medicine that have indeed seen rapid and successful advances, without their being used by the state for repression and without any fundamental change in the capacities for intellectual and spiritual self-possession). Nevertheless, the powerful arguments of opposition to the manipulation and replacement of tissues and organs continue, with some people worried that perfection itself is sought when healing is the goal. Such critics, many from the disability community, raise principled objections to the use of tissue engineering if the goal is to alter the disability. Activists in the deaf community, for example, defend their disability as a culture and a language exchange, not as a loss of function. Others are concerned that our society's focus on 'fixing things' will allow a devaluation of the persons that currently bear the broken bodies and parts. Adrienne Asche suggests that there is a 'troubling side to every cure, that those of us who are uncured are seen as less valuable, perhaps even expendable'. For Gerald McKenney [7], a community needs to embrace brokenness and to: 'deny that the worth of one's life is determined to how closely one conforms to societal standards of bodily perfection'. McKinney is also concerned that if medicine is successful, it will create a social and economic system that: 'virtually demands that we be independent of the need to care for others'.

Duty and healing: natural makers in a broken world

While the opposition to medical technology has indeed been persistent, it has not been unchallenged. For many, the response lies in the nature of brokenness and the human duty to respond to the need of the suffering other [8,9]. The principle at stake in the assessment of tissue engineering as an ethical act is not how its use might potentially violate an abstract community in the future, but the actual problem of what one must do as a moral being when one's neighbor is in need. In this important sense, the duty to heal cannot be overridden by a 'sense' of discomfort (as Lewis notes in the earlier quote).

It is the nature, goal, and meaning of science to address the human condition in all its yearning and capacity for defeat and failure. In this sense, there is no principle objection to the science of tissue engineering, and in fact there may well be a strong moral imperative to develop the technology.

Joseph Fletcher [10] noted, in speaking of an earlier generation of medical technology and answering the critics of science, wrote:

> 'The belief that God is at work directly or indirectly in all natural phenomena is a form of animism or simple pantheism. If we took it really seriously, all science, including medicine, would die away because we would be afraid to 'dissect God' or tamper with His activity'. 'Every widening and deepening of our knowledge of reality and of our control of its forces are the ingredients of both freedom and responsibility'.

McKinney, Childress, and Lewis use an argument that is rooted in Christian moral theology: that since human persons are fallen creatures in a fallen world, we cannot really be counted on

to know the right and the good. God, who is transcendent, from this fallenness, has set us in this place, not essentially to alter it toward our own transonic, but to find its meaning and purpose. Yet the traditions of other faiths differ. For Jewish and Islamic theorists, the world is morally neutral. Human persons may — and will — fail in their aspirations but can be trusted to have the capacity for moral behavior and moral yearnings. Finding meaning in suffering is not the core task. The task is to alleviate suffering, which is understood as chaotic, meaningless, and agonistic. Hence, many of the core objects in principle are rooted in religious constructions and understandings.

To make is to know: notes on an old problem about knowledge

The classic debates of the 1970s are not the only set of problems engendered in the history of ethical responses to the technological gesture at the heart of tissue engineering. At stake as well is the special kind of knowledge that such making implies. For Aristotle and the Hellenists, useful knowledge, 'practical wisdom', was phronesis. Phronesis implied actually doing an act; making, in order to know. The act of making, not the act of perception or contemplation alone, was how wisdom and, indeed, rationality and power were achieved. Hence, making new tissue is a somewhat different moral gesture than curing the body by altering it with drugs that essentially allow the body to heal itself.

Second, the use of technology within the body of the patient is a different matter than the use of technology essentially to enhance the body of the practitioner. For all earlier technology, the thing that was changed or enhanced was the sense perceptions of the doctor. Stethoscopes and otoscopes allowed the sounds of the body to be more audible. X-rays, CT scans, and MRIs allow the inner vistas of the body to be revealed. EEGs and EKGs allow the electrical currents that animate the central and peripheral nervous systems to be charted in quantifiable units. Microscopes allow invasive bacteria to be seen at the microscopic and, increasingly, molecular levels. These earlier technologies extended the reach of what Bacon increasingly trusted and that the Greeks did not: the perception and observation of the phenomena of the world and the perception of the outcome of its deliberate perturbation.

Bacon's method presupposes a double empirical and rational starting point. True knowledge is acquired if we proceed from lower certainty to higher liberty and from lower liberty to higher certainty. The rule of certainty and liberty in Bacon converges… For Bacon, making knows and knowing is making (cf. Bacon IV [1901], 109—110). Following the maxim 'command nature . . . by obeying her' (Sessions [11]; cf. Gaukroger [12]), the exclusion of superstition, imposture, error, and confusion are obligatory. Bacon introduces variations into 'the maker's knowledge tradition' when the discovery of the forms of a given nature provide him with the task of developing his method for acquiring factual and proven knowledge (Stanford Encyclopedia of Philosophy website, 2003).

Thus, the world is known by understanding the parts of the world and, from that, theorizing (knowing), by induction, to principles or axioms or laws of nature, physics, and chemistry. In contemporary science, knowing is done largely by 'unmaking', by deconstruction of the component parts in ways scientists of Bacon's era were unable to imagine. Many of these 'unmaking' techniques, such as the splicing of alternative DNA and the manipulation of cellular structures, allow a sense of inherent interchangeability, as if the real and the person were merely a set of Lego parts awaiting clever recombination.

What is a thing? The perils of deconstruction

Making actual tissue in mimesis of the real tissue of the actual body extends the Baconian act in radical ways. Here, the experimental perturbation is the unmaking of tissue and the remaking of tissue, only in more controllable form. This cannot help but excite concern about the nearly infinite possibilities for technological shaping of the self. Heidegger asks: 'What is a

thing?' and in so reflecting, understands a thing as an object separate from the self. But what of a made thing, an object that becomes the self?

The technology of the alteration of the patient is distinctive. Devices for altering the functioning of the body that become a part of the body and are actually a tissue of the body are a step beyond the idea of a device held within the body. This is important in any ethical assessment of the technology, because the patient's consent and participation are needed for the final act of the technology to be completed. Such an event only happens in a specific context, for technologies, patients, and practitioners operate in a social, religious, and economic context. Here we turn to the second ethical consideration.

WHAT CONTEXTUAL FACTORS SHOULD BE TAKEN INTO ACCOUNT, AND DO ANY OF THESE PREVENT THE DEVELOPMENT AND USE OF THE TECHNOLOGY?

Tissue engineering is a complex procedure still in experimental stages. Yet to be an ethical technology, it must be directed toward accessibility, just distribution, and efficacy. Hence, the troubling context of widespread healthcare disparity is a problem not only for this advanced technology but for all newly emerging technologies. Emergence into an unjust world asks certain moral questions of new technological advances. First among these is the query about burdensomeness versus benefit in a context in which the vast majority of the world's people suffer from easily treatable infectious diseases such as tuberculosis, malaria, AIDS, and infant diarrhea. How can tissue engineering be justly promoted in the face of other, pressing needs?

This objection can typically be met by noting that it would be deeply inappropriate to withhold medical knowledge until the world is entirely perfected, and that applications not only will be increasingly available to the poor (as in vaccines, once rare) but also that the very process of research has typically uncovered new and useful ways to understand disease.

The goal of tissue engineering is the widespread use of the technique. Unlike solid-organ transplants, which would always require significant resources far outside the capacities of developing-world clinics, tissue replacement, stem cell therapies, and other transportable therapies are designed for widespread use. The possibility to create a method for allografts that uses the patient's own cells and the possibility for allologous cells to provide an 'off-the-shelf' source of tissue may provide the basis for access (but only if research priorities are discussed in advance of design), a process, as we describe later, that will need careful support and monitoring. The question of how to achieve this and how to enable a more just use of each technology has not yet been solved.

A second contextual factor for tissue engineering is that all human tissue is marked by its genomic identity. It is the very nature of cells, what allows them to copy and reproduce, to carry identifying markers linked to some person somewhere. In the past, such use has raised serious objections. Such tissue can be traced and known, which may have implications for the person who is the source of the tissue, raising significant new issues in genetic privacy for the donor.

Further, whose is the tissue that is derived from the cells of a particular body? Who should have the rights to, and a fair share of, the profits derived from its use? In the seminal case in the field, Moore v. the Regents of the University of California [17], the issue of ownership was addressed. In this case, Mr. Moore had his T cell lymphocytes taken from his spleen during the course of treatment for hairy-cell leukemia, cells that proved effective in deriving resistant cell cultures. Patented after manipulation to make a new 'product', the cells were indeed profitable. Mr. Moore's complaint was that he was not informed of, much less a part of, the scientific enterprise and the lucrative payout for his cells. The case was decided in favor of the

research labs. But in the ensuing decades, alert patients with unique cell types or unusual cancers sought for research have been selling their materials as personal possessions to the lab that wishes to procure them. Ownership is limited, however, by the common law of the United States and the European Union which constrains this ability to claim tissue as property. The goals of such restraints were put into place to prohibit the buying and selling of human tissue and organs, for fear, given the desperation of the poor, that selling the bodies of the poor would become permissible and lead to their exploitation. Thus, the entire process that allows for the derivation of tissue sources needs to be noted. The current context for tissue donation is a mixed system. Organs, tissues such as blood, corneas, and marrow are donated or exchanged without compensation. Gametes, however, are another matter entirely. Because the use of human sperm and eggs emerged in the context of fertility treatment and because this treatment was largely conducted in stand-alone, private clinics that functioned without public oversight or regulation, the marketplace standards prevailed. What originally began as a compassionate exchange of gametes between family members when an infertile couple could not conceive quickly changed into a robust marketplace in human gametes. As of this writing, international standards prohibit the use of marketplace incentives for gametes or embryos (International Society for Stem Cell Research, 2006).

A final context for the debate about the ethics of tissue engineering in general is the special case of human stem cells to make tissues. Because some applications of tissue engineering use stem cells as a part of the method of treatment [13], the debate about the ethics of the use of human stem cells is directly adjacent to this technology. For many, the origins of tissue matter a great deal. For some Christians, many Roman Catholics, and some Hindu sects, the destruction of the human embryo, even at the blastocyst stage, is tantamount to killing. For these faith traditions, the derivation of stem cells from embryos is always impermissible. For many other faith traditions, such as Judaism, Islam, Jainism, Buddhism, Confucian philosophy, and Daoism, the use of these cells is permissible within certain constraints, as we will see later. For all faith traditions, however, the manipulation of adult somatic cells in their precursor form is completely sanctioned. Precursor cells are not as flexible as pluripotent cells, and it is that very pluripotency and immortality that are important in tissue engineering. These factors raise concern. Yet the contextual factors alone do not prohibit entirely the use of this technology, for justice in distribution, the possibility of the loss of genetic privacy, and the controversy over stem cell research when pluripotent embryonic cells are used affect many aspects of the new techniques in medicine. Hence, we turn to the third major issue.

WHAT PURPOSES, TECHNIQUES, OR APPLICATIONS WOULD BE PERMISSIBLE AND UNDER WHAT CIRCUMSTANCES?

Many of the salient, justifying arguments for the use of tissue engineering hinge on the telos, or goal, of the treatment: If the goal is to cure or treat human disease, then the benefits will outweigh the burdens of the work — controversy, cost, and difficulty. Clearly, then, tissue engineering ought not to be used in a trivial or wasteful fashion. Human tissue is understood by many as deserving a special sort of 'respect' (Nelson, 2002) [14].

This proviso may not be so simple, for a core problem in genetic engineering has been the use of the technique for 'enhancement' of human characteristics or traits. The initial ethical discussions about therapeutic uses of medicine versus cosmetic ones imagined ethical bright lines that would define the boundary between the use of such technology to restore 'species normal functions' [15] for each tissue and for the person as a whole. Yet medical practice has long gone beyond these lines, using surgery, for example, for cosmetic purposes. Will it be possible to restrict tissue replacement to burn victims, spinal cord injury, and diabetics? How can such a distinction be made?

Some tissue replacement therapies, such as the use of skin grafts for full-face transplant, may also raise questions about the nature of identity. Indeed, the notion of a full-face transplant alerted us to the depth of resistance to identity — altering tissue replacements. (Could persons use any face? What if persons in need of facial transplants wished to change ethnicity? Should faces 'match', and why?) Like many other aspects of this technology, this tension about identity was not new, only heightened. For example, the first years of organ transplant raised the same issues for recipients of hearts, a key aspect of identity in many cultures. If the face is our key determinant of the self in modernity and, even more so, if the brain is such, then how are we to understand the use of tissue engineering to transform identity?

Hence, linked inexorably to this technology are larger considerations of the use of tissue engineering for neuroscience, both for therapy and for enhancement. The applications of tissue transplant in Parkinson's disease are important. Yet will there be concern about this use of the neurons of a stranger in the brain of the self? Of all the possible uses of tissue engineering, the ones that may alter consciousness and memory are the most troubling. (What capacities or memories could neurons store?) Here, the need for restrictions on applications may be the clearest, yet it is not clear who ought to decide and who ought to ensure that the restrictions on unethical applications are maintained. By what criteria will such limits be set?

New research possibilities also offer applications to engineer gametes for use and storage. Engineered follicles may now be saved, frozen, matured, and used in animal models to create the possibility of human fertility after cancer chemotherapy or other environmental risk (Woodruff et al., 2006).

With this, as with all such technology, there will have to be careful attention to how the market may drive technology toward specific research goals rather than others or to whether research goals will be framed only by the values of profit and efficacy and not by ones of more general interest: compassion, healing, and solidarity. The powerful applications and the potential for widespread use itself create the possibility for serious conflicts of interest, for serious market forces may be the core drivers of technology, especially in an aging population with increasing needs for all manner of new tissues and organs. This turns us to the consideration of our final set of issues.

ON WHAT PROCEDURES AND STRUCTURES, INVOLVING WHAT POLICIES, SHOULD DECISIONS ON APPROPRIATE TECHNIQUES AND USES BE BASED?

Much of the first reviews of the ethical issues in tissue engineering have in fact focused on the issues of policy:

Safety, patents, and gating. Products and drugs are typically controlled via four levels of restraints. The first is elaborate premarket gating, first involving animal models and then typically done for pharmaceuticals in a decade-long series of tests, phased to test the drug on an increasing but controllable number of human subjects. Such trials must be gender balanced, and subjects must give full, informed consent and be able to leave the trial at any time (which could be difficult in cases of implanted tissues).

The next gating is the system of intellectual property. Patents and licensing control the use of the products, and even the replication of the experiments. The next gating is that of financial backing. To perform the enormous clinical trials, to do premarket investigation, and, of course, actually to make and sell the product require a production apparatus, which must be assembled and supported. Finally, each drug or device must be approved for use by the insurers. As David Smith (2004) notes, tissue engineering faces a gauntlet

of issues and a 'new order of magnitude in interactions and science patents'. Additionally, notes Smith, the 'things' engineered are hybrids of two jurisdictions, that of drugs and that of devices.

Are genetically engineered insulin cells a drug, like insulin, a device, like a stent, or a biologic? Unlike stents, which are entirely synthetic, tissue engineering uses actual human cells, only manipulated in *de nova* ways.

Standards will need to be set for safety, efficacy, and fair use. Standards for clinical use, standards for clinical trial, and standards for tissue stability and purity will be needed for the research and application to be safe.

Getting informed consent in this case will present significant challenges. Patients in need of organs, for example, are particularly desperate, and their consent may be deeply affected by their utter lack of options. Eight percent of the medical system is already devoted to organ transplantation, and the lack of organs is an overwhelming problem for nearly half of the patients hoping for transplants [16]. Yet the first year of the use of engineered tissue will be experimental and will need to be conducted under the strongest possible set of NIH guidelines. How the first trials of engineered tissue are conducted will set the tone and the future for all subsequent use.

The question of policy and the regulation of policy are manifested in many of the first documents that evaluate the ethical and legal implications of tissue engineering. While, as Smith notes, the United States faces a complex regulatory system, the European Union has regulated such research products as medical products, and these will fall under the regulatory gaze of the European Medical Evaluation Agency (EMEA). In both the United States and the EU, the synthetic nature of tissue engineering, the very *de novo* quality of the work and the uneasy greeting that met genetically modified food has created serious political opposition. Policies need to be crafted with transparency and full public participation, for such research needs not only public funding but public understanding of complex theory and practice of tissue engineering — what promises it can hold and what cautions need to be applied prior to use. Policymakers will need to attend to calls for justice in distribution, as was noted earlier, and will need to set in place structures for regulation.

How can new technologies best be regulated? I contend that a full array of regulatory structures can be employed. First among these are local committees, IRBS, and local review boards. The National Academies have played a large role in policy writing for both recombinant DNA and for stem cell research and, in both instances, called for special, national, ongoing oversight on such research. It would be prudent to reflect on the need for such a process for tissue engineering, for established structures largely address issues involving the use of donated tissue, not engineered tissue. Structures that protect human subjects also need strong enforcement, as noted earlier, both for donors and for recipients of tissues.

But regulation, government oversight, and market forces can only go so far in shaping just research goals and commitments. The goal of ethics is to develop moral agents who are aware of a constancy of duty toward subject and to humanity, who not only follow rules correctly but who, given the chance and grace to work at the frontiers of science, act with courage and decency in their research.

CONCLUSION

Tissue engineering suggests that an old dream — the replacement of human body parts — may be realized. While any sober and reflective scientist understands the long way to success of this idea, the science described in this volume clearly suggests that our society is on the road to the enactment of the possibility.

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EPILOGUE

The emergence of the field of Tissue Engineering and Regenerative Medicine tracks with the publication of new editions of this textbook, now in its fourth iteration. Like any active field in science, ongoing evolution in ideas, discovery, and innovation will never allow a final chapter. The 2012 Nobel Prize in Physiology and Medicine was awarded for stem cell work resulting in the induced pluripotential stem cell, a stunning and important achievement not mentioned in the third edition. Likewise, a new scaffolding approach involving decellularization of tissues and organs with preservation of vascular architecture is now being studied world wide but had not been published prior to the last edition. The important lesson is that the field is healthy and active because of the engagement of creative and passionate young investigators who have a desire to make a difference in the lives of the many humans who desperately need our help.

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