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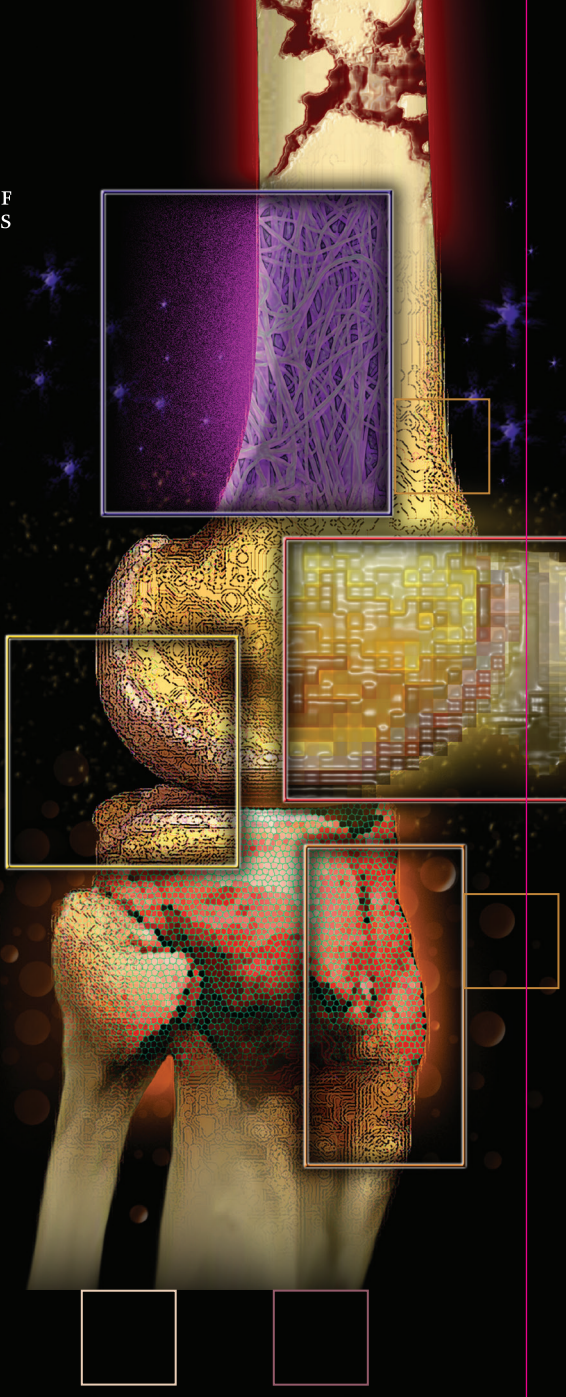

Bone Graft Substitutes and Bone Regenerative Engineering

2nd Edition

Editors
Cato T. Laurencin, M.D., Ph. D.
Tao Jiang, Ph.D., MBA

Cato T. Laurencin M.D., Ph.D. and Tao Jiang Ph.D., MBA

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Dedication

To my wife Cynthia and my children Ti, Michaela, and Victoria

—Cato T. Laurencin

To my wife Hua and my children Richard and Suri, with much love and thanks

—Tao Jiang

THIS PUBLICATION, *Bone Graft Substitutes and Bone Regenerative Engineering: 2nd Edition,* was sponsored by Committee F04 on Medical and Surgical Materials and Devices. This is Monograph 6 in ASTM International's monograph series.

Contents

Foreword

We are truly delighted to write the foreword for *Bone Graft Substitutes and Bone Regenerative Engineering*. Edited by Professor Cato Laurencin and Professor Tao Jiang, the book exemplifies the use of Convergence in the design of new technologies for bone repair and regeneration. Over the past several decades, bone grafting has been a common task for orthopedic surgeons and each year millions of patients receive bone grafts worldwide. As the first generation bone grafting products, autologous bone grafts have been considered the gold standard; however, these grafts are severely limited by supply. Since the late 1980s, Dr. Laurencin and others pioneered a new area of research called bone tissue engineering (BTE). BTE has gradually emerged as a promising alternative to bone autografts in treatment scenarios. As such, several engineered products such as MicroFuse® have been inspired by technologies that originated in Dr. Laurencin's laboratory. Nevertheless, various challenges in bone tissue engineering still exist, such as the need for advanced biomaterials, appropriate and reliable cell sources, and a thorough understanding of regenerative biology and tissue morphogenesis as new technologies influencing regeneration. Therefore, many believe that Regenerative Engineering, a new field described by Dr. Laurencin with a focus on Convergence integrating biology, biomedical science, physics and engineering, serves as the future of tissue engineering. In the past decades, the Raymond and Beverly Sackler Foundation has supported and endowed numerous programs that embrace the concept of Convergence in scientific research. We are happy to have supported the creation of the Raymond and Beverly Sackler Center for Biomedical, Biological, Physical and Engineering Sciences at the University of Connecticut Health Center directed by Dr. Laurencin to support his efforts in defining the new field of Regenerative Engineering.

This timely book provides a well-rounded and articulate summary of the present status of using allogeneic, xenogenic, and synthetic bone graft substitutes to reconstruct bone tissues. Specifically, fifteen concise and comprehensive chapters are prepared by experts in their fields from both academia and industry. These chapters encompass numerous topics discussing the use of a wide selection of bone graft substitutes ranging from bone allografts and xenografts derived from human and animal tissues to synthetic biomaterials, cells, and growth factors. While many of these bone graft substitutes have experienced great clinical successes and have helped improve patients' health, challenges still remain to reconstruct functional bone tissue mimicking natural bone morphogenesis. This is precisely where bone Regenerative Engineering has a niche and a significant role to play. In this regard, the book also includes a number of chapters discussing frontier paradigms such as advanced materials, stem cells,

nanobiotechnology, and developmental biology aiming to regenerate bone tissue in a more natural and effective way. Convergent technologies integrating the aforementioned disciplines promise to continue extending research frontiers, pushing bone regeneration therapies towards a more personalized approach that can truly benefit individual patients.

This book presents an outstanding view of the subject. It will appeal to a broad audience including researchers, clinicians, and regulatory specialists in both academia and industry and will serve as a valuable resource to these professionals. We believe that this book will be a welcome addition to personal collections, libraries, and classrooms throughout the world.

Raymond R. Sackler, MD, OBE
Founder and President,
Raymond and Beverly Sackler Foundation

Evan Vosburgh, MD
Executive Director,
Raymond and Beverly Sackler Foundation

Preface

In 2003, the American Society for Testing and Materials (ASTM International) published a landmark book entitled *Bone Graft Substitutes*. The book was a direct result of a workshop cosponsored by ASTM International and American Academy of Orthopaedic Surgeons (AAOS) that one of us chaired (CTL). Since the publication of the book, more than a decade has passed and the field of bone grafting has seen many dramatic changes. While the use of both autogenous and allogeneic bone grafts still remains a viable and important choice to surgeons, new technologies and strategies have gradually emerged and attracted great interests from both academia and industry. Therefore, we feel that it is necessary to publish a new book that updates the trends involved with this important field.

Among the new technologies and strategies for bone regeneration, advanced materials, nanotechnology, stem cell science, and bone developmental biology are central pieces of significant research and development interests and have added to the toolbox available to biomaterials scientists and engineers to regenerate bone tissues. The convergence of these disciplines has opened a new field that we define as *Regenerative Engineering*. We believe that regenerative engineering is an invaluable tool and will ultimately help researchers and surgeons better regenerate individual musculoskeletal tissues and more complex organs. Successful regeneration of tissue and organ systems will rely on a multidisciplinary strategy integrating the biological principles involved in cell and developmental biology with advanced biomaterials, nanofabrication, biomechanics, and tissue engineering. To reflect on the importance of the concept of convergence, we renamed the book *Bone Graft Substitutes and Bone Regenerative Engineering* to capture the excitement of this new field. The chapters of the book are written by well-known researchers in academia, surgeons, industry leaders, and regulatory specialists. We believe that this book will be of value to people who work in all fields involving bone.

We want to acknowledge the efforts of all the contributing authors in completing this exciting project. A number of individuals who contributed to the first edition of the book have been very enthusiastic about this new book. As such, Dr. Barbara Boyan, Dr. Joseph Lane, Dr. Mark Borden, Dr. Dhirendra Katti, Dr. Yusuf Khan, Dr. Treena Arinzeh, and Dr. Sergio Gadaleta have been extremely supportive and contributed to this new work. We also want to thank Dr. Saadiq El-Amin, Dr. Steven Gitelis, Dr. Syam Nukavarapu, Dr. Jeffery Hollinger, Dr. Peter Ma, Dr. Thomas Webster, and Dr. Yunzhi Peter Yang for their fine contributions to the book and their unique perspectives on a variety of important areas on bone repair and regeneration. The publication of this book would be impossible without the assistance from numerous people at ASTM

International. We would like to thank Dr. Michael Mitchell (Editor-in-Chief of the Journal of Testing and Evaluation), Ms. Kathy Dernoga, Ms. Heather Blasco, and Ms. Monica Siperko for their diligence, professionalism, and tremendous support in all phases during the production of the book. Finally, we thank the Raymond and Beverly Sackler Foundation for their tremendous support in our efforts to define the new field of Regenerative Engineering.

Cato T. Laurencin, M.D., Ph.D.
Tao Jiang, Ph.D., M.B.A.

List of Contributors

Celeste Abjornson, Ph.D.
Director, Spinal Research
Weill Cornell Medical College
Hospital for Special Surgery
New York, NY

Pedro Alvarez, Ph.D.
Department of Biomedical Engineering
Carnegie Mellon University,
Pittsburgh, PA

Treena Livingston Arinzeh, Ph.D.
Professor, Department of Biomedical Engineering
Director of the Graduate Program
New Jersey Institute of Technology
Newark, NJ

Aditya Arora, B.E.
Department of Biological Sciences and Bioengineering
Indian Institute of Technology Kanpur
Kanpur, India

Arijit Bhattacharjee, M.S.
Department of Biological Sciences and Bioengineering
Indian Institute of Technology Kanpur
Kanpur, India

Mark Borden, Ph.D.
President/Chief Executive Officer
Synergy Biomedical
Collegeville, PA

Barbara D. Boyan, Ph.D.
Dean, School of Engineering
Professor and Goodwin Chair in Tissue Engineering
Virginia Commonwealth University
Richmond, VA

David J. Cohen, M.D.
Research Associate
Department of Biomedical Engineering
Virginia Commonwealth University
Richmond, VA

Brian J. Cole, M.D., M.B.A.
Professor
Department of Orthopaedics
Department of Anatomy and Cell Biology
Section Head, Cartilage Restoration Center at Rush
Rush University Medical Center
Chicago, IL

Sita M. Damaraju, B.S.
Department of Biomedical Engineering
New Jersey Institute of Technology
Newark, NJ

Ming Dang, M.S.
Macromolecular Science and Engineering Center
University of Michigan
Ann Arbor, MI

Bruce Doll, D.D.S.
Navy Medicine Research and Development, U.S. Navy
Frederick, MD

Amy Donovan, B.S.
Department of Biomedical Engineering
Carnegie Mellon University,
Pittsburgh, PA

Saadiq F. El-Amin III, M.D., Ph.D.
Director of Shoulder and Sports Medicine
Assistant Professor, Division of Orthopaedic Surgery
Adjunct Assistant Professor Medical Microbiology, Immunology and Cell Biology
Adjunct Assistant Professor Electric and Computer Engineering/Biomedical Engineering Program
Southern Illinois University
Springfield, IL

Taj Eubanks, M.D.
Division of Orthopaedics and Rehabilitation, Department of Surgery
School of Medicine
Southern Illinois University
Springfield, IL

Yale Fillingham, M.D.
Resident
Rush University Medical Center
Chicago, IL

Rachel M. Frank, M.D.
Resident
Rush University Medical Center
Chicago, IL

Sergio J. Gadaleta, Ph.D.
Vice President, Regulatory Affairs and Regulatory Compliance
Becton Dickinson
Franklin Lakes, NJ

Steven Gitelis, M.D.
Professor and Vice Chairman, Department of Orthopaedic Surgery
Director, Section of Orthopaedic Oncology
Associate Chief Medical Officer for Surgery, Chief of Surgery
Rush University Medical Center
Associate Dean of Surgical Services at Rush Medical College
Chicago, IL

Khadidiatou Guiro, M.S.
Department of Biomedical Engineering
New Jersey Institute of Technology
Newark, NJ

Ashim Gupta, Ph.D.
Division of Orthopaedics and Rehabilitation, Department of Surgery
Department of Medical Microbiology, Immunology & Cell Biology
Southern Illinois University
Springfield, IL

Joshua D. Harris, M.D.
Rush University Medical Center
Chicago, IL

Jeffrey O. Hollinger, Ph.D.
Professor, Biological Sciences and Biomedical Engineering
Director, Bone Tissue Engineering Center
Carnegie Mellon University,
Pittsburgh, PA

Kristen Hussey, B.S.
Rush University Medical Center
Chicago, IL

Tao Jiang, Ph.D., MBA
Assistant Professor
Institute for Regenerative Engineering
Department of Medicine
University of Connecticut Health Center
Farmington, CT

Dhirendra S. Katti, Ph.D.
Professor
Department of Biological Sciences and Bioengineering
Indian Institute of Technology Kanpur
Kanpur, India

Yusuf Khan, Ph.D.
Assistant Professor
Department of Orthopaedic Surgery
University of Connecticut Health Center
Farmington, CT

Joseph M. Lane, M.D.
Assistant Dean, Hospital for Special Surgery, Weill Cornell Medical College and affiliated hospitals
Professor of Orthopaedic Surgery, Weill Cornell Medical College
Adjunct Professor, Department of Orthopaedic Surgery, Columbia University, College of Physicians & Surgeons
Chief, Metabolic Bone Disease Service
Attending Orthopaedic Surgeon, Hospital for Special Surgery
New York, NY

Cato T. Laurencin, M.D., Ph.D.
University Professor
Albert and Wilda Van Dusen Distinguished Professor of Orthopaedic Surgery
Professor of Chemical and Biomolecular Engineering
Professor of Materials Science and Engineering
University of Connecticut
Farmington, CT

Peter X. Ma, Ph.D.
Richard H. Kingery Endowed Collegiate Professor
Professor, Department of Biologic and Materials Sciences in the School of Dentistry
Professor, Department of Biomedical Engineering
Professor, Macromolecular Science and Engineering Center
Professor, Department of Materials Science and Engineering in the College of Engineering
University of Michigan
Ann Arbor, MI

Benjamin J. Main
Department of Medical Microbiology, Immunology & Cell Biology
Southern Illinois University
Springfield, IL

Ángel E. Mercado-Pagán, Ph.D.
Postdoctoral Research Fellow
Department of Orthopaedic Surgery
Stanford University
Stanford, CA

Paiyz E. Mikael, M.S.
Department of Materials Science and Engineering
University of Connecticut
Storrs, CT

Siyu Ni, Ph.D.
Postdoctoral Research Fellow
Department of Chemical Engineering
Northeastern University
Boston, MA

Syam P. Nukavarapu, Ph.D.
Assistant Professor
Institute for Regenerative Engineering
Department of Orthopaedic Surgery
University of Connecticut Health Center
Farmington, CT

Tamara Pylawka, M.D.
Division of Orthopaedics and Rehabilitation, Department of Surgery
School of Medicine
Southern Illinois University
Springfield, IL

Jennifer I. Rulka, B.S.
Department of Biochemistry and Molecular Biology
University of Massachusetts Amherst
Amherst, MA

Zvi Schwartz, D.M.D., Ph.D.
Associate Dean for Strategic Initiatives
Professor, Department of Biomedical Engineering
Virginia Commonwealth University
Richmond, VA

Jae Min Shin, B.S.
Department of Biologic and Materials Sciences
University of Michigan
Ann Arbor, MI

Arun Shrivats, Ph.D.
Department of Biomedical Engineering
Carnegie Mellon University,
Pittsburgh, PA

Linlin Sun, M.S.
Department of Chemical Engineering, Bioengineering Program
Northeastern University
Boston, MA

James Veronick, M.S.
Department of Biomedical Engineering
University of Connecticut
Storrs, CT

Thomas J. Webster, Ph.D.
Chair and Professor of Chemical Engineering
Northeastern University
Boston, MA

Ross Wilkins, M.D.
Colorado Limb Consultants
Denver, CO

Yunzhi Peter Yang, Ph.D.
Associate Professor
Department of Orthopaedic Surgery
Department of Materials Science and Engineering
Stanford University
Stanford, CA

Chapter 1 | Bone Graft Substitutes: Past, Present, and Future

Cato T. Laurencin,[1,2,3,4,5,6] Yusuf Khan,[1,2,3,4,5] James Veronick,[1,2,4]

INTRODUCTION

The field of medicine as a formal discipline has been traced by many to Imhotep and his descriptions of ailments and treatments found written on papyrus and translated in the mid-1800s by Edwin Smith [**1**]. Among the medical descriptions included in Imhotep's writings are cervical dislocations, skull fractures, and compound fractures [**1**]. Indeed, mummies found in Egyptian tombs have been found with crude braces constructed from wood planks and linen straps on their limbs representing some of the earliest accounts of orthopedics [**2**]. The use of autografts, allografts, and bone graft substitutes also has interesting origins. The use of each graft type dates back several hundred years to apparently crude yet inspired methods and theories, which nonetheless set the stage for what we today consider state of the art. The following is a brief history of each graft subgroup.

Autografts were first used as far back as the early 1800s when, after a trephination (i.e., the practice of drilling holes in the skull to release pressure), Walther repaired the defect by refilling the hole with the original bone plug [**3**]. This repair resulted in good healing and informally began the practice of autografting. In the late 1800s, more reports of autografting emerged: Seydel used tibial periosteal flaps to close a cranial defect and Bergmann used a fibular graft to close a tibial defect [**4**]. By the early 1920s, more than 1600 autograft procedures had been documented [**4**]. However, early structural limitations of cancellous autograft tissue delayed its full emergence, which did not occur until more modern tools of external and internal fixation were available [**4**]. One of the primary reasons for the success of autografts is their ability to be

[1] Institute for Regenerative Engineering, UConn Health, Farmington, CT
[2] Raymond and Beverly Sackler Center for Biomedical, Biological, Physical and Engineering Sciences, UConn Health, Farmington, CT
[3] Department of Orthopaedic Surgery, UConn Health, Farmington, CT
[4] Department of Biomedical Engineering, University of Connecticut , Storrs, CT
[5] Department of Material Science and Engineering, Institute of Material Science, University of Connecticut, Storrs, CT
[6] Department of Chemical and Biomolecular Engineering, University of Connecticut, Storrs, CT

osteoinductive, which is due to the presence of blood, cells, growth factors, and proteins within the graft that stimulate and facilitate healing. Although it is within the past 30 years that intense attention has been paid to these growth factors as healing tools, the notion that the body's own fluids could provide stimulus for healing and bone growth dates back further than that. Early attempts at nonunion treatments involved sawing both ends of the fracture to expose fresh bone, rubbing both ends of the bone together, and splinting the wound to allow some limited motion in hopes of stimulating inflammation and thus healing [**5**]. Although early surgeons may not have realized it, this procedure may have stimulated the recruitment of growth factors and inflammatory elements. A similar approach to nonunions was described by Physick in 1802, when he repaired a fracture nonunion by running a seton, or a small bundle of fibers, through and between both ends of the fracture with the hopes of stimulating an exaggerated immune response and healing [**6**].

Allograft use has been reported as far back as the late 1800s when Macewen reported on the implantation of a tibial graft from one child to another [**7**,**8**]. In the early 1900s, cadaveric and fresh allografts were used as in the case of a transplant of cadaver cartilage to a patient and another of a fresh bone allograft from parent to child for the treatment of spina bifida [**3**,**9**]. The earliest collections of allograft tissue, or bone banks, were established in the beginning of the 20th century when Bauer refrigerated bone samples for 3 weeks and then implanted them in dogs. Allografts were prepared for storage at this point by chilling or heating, but it was soon determined that boiling the bone samples rendered them inferior in healing to autografts because the endogenous proteins and factors were undoubtedly destroyed during heating [**9**]. The big leap forward in bone banking came during World War II when new methods of bone storage preparation were studied, including freezing, freeze-drying, deproteinating, irradiating, autoclaving, demineralizing, and chemically treating the harvested bone. Initially prompted by the U.S. Navy to help combat war injuries, the expansion of bone banking continued with a new focus on civilian needs. Many of today's currently held beliefs and understandings about bone bank tissues came from the naval projects [**8**]. It was about this time that the use of fresh allograft tissue declined sharply in orthopedic procedures, giving rise to the need for better allograft treatments and bone graft substitutes in general.

Some of the first evidence for the use of bone graft substitutes, crude as it may be, has been found in prehistoric skulls with gold and silver plates and even remnants of coconut shells found in place of cranial defects [**3**]. In more recent times, several synthetic materials have been used as either bone graft substitutes or internal fixation devices. Several metals, including platinum, vitallium, tantalum, stainless steel, and titanium, have been used for joint replacements or fracture fixations. Polymers including polyethylene, silicon rubber, acrylic resins, polymethylmethacrylates, and others have been used, as have ceramics, in place of bone grafts. In their infancy, these materials were more suited for replacement rather than regeneration of bone tissue. However, the current generation of bone graft substitutes has been designed with replacement

and regeneration in mind. Materials are either designed with living tissue structures in mind or are combined with factors, proteins, and other tissues to encourage rapid and complete healing. Some of the more successful materials have been around for decades. For example, calcium sulfate, also known as gypsum or plaster of Paris, was used in the late 1800s by Dreesman to fill bone voids [**10**], and it is still used today as a bone graft substitute with very good clinical results. The newest generation of bone grafts and bone grafts substitutes, of which this book is the focus, continues a long tradition.

Between 1998 and 1999, the number of bone graft procedures in the United States climbed from 300,000 to 500,000 with the estimated cost of these procedures approaching $2.5 billion per year [**11,12**]. Also in 1998, nine of ten procedures used autograft or allograft tissue [**11**]. The autograft, tissue harvested from the patient (commonly the iliac crest but other regions as well) and implanted within the patient at another site, is the current gold standard of bone grafts because of its inherent osteoconductivity, osteogenicity, and osteoinductivity [**13**]. Osteoconductivity describes a graft that supports the attachment of new osteoblasts and osteoprogenitor cells onto its surface and has an interconnected pore system that allows these cells and others to migrate. Osteogenicity describes a graft that supports the apposition of the graft with the preexisting bone. Osteoinductivity describes a graft that can induce nondifferentiated stem cells or osteoprogenitor cells to differentiate into osteoblasts. Although autografts provide the best replacement tissue to a defect site, the harvesting procedure requires an additional surgery at the donor site, which can result in its own complications, most commonly pain and risk of infection. This donor-site morbidity occurs in approximately 20 % of all cases [**13–15**]. Supply limitations are also a problem for the autograft, further limiting its desirability. There are several categories of bone graft substitutes encompassing varied materials, material sources, and origin (natural vs. synthetic). Accordingly, a bone graft classification system, described in **Table 1.1**, has been developed that describes these groups based on their material makeup.

TABLE 1.1 Description of Classification System for Bone Graft Substitutes.

Class	Description
Allograft-based	Allograft bone used alone or in combination with other materials
Factor-based	Natural and recombinant growth factors used alone or in combination with other materials
Cell-based	Use cells to generate new tissue either alone or seeded onto a support matrix
Ceramic-based	Includes calcium phosphate, calcium sulfate, and bioactive glasses used alone or in combination
Polymer-based	Degradable and nondegradable polymers used alone and in combination with other materials

Note: Many of the currently available bone graft substitutes fall within one or more of the above-described groups.

Allograft-Based Substitutes

Before the 1980s, allograft tissue was primarily used as a substitute for autografts in large defect sites, but since then, allograft tissue use has expanded from approximately 5000–10,000 cases in 1985 to almost 150,000 in 1996 [**16**]. The coordination of donor screening and tissue processing methodologies has reduced the risk of disease transmission from allograft tissue; thus, it has become a more attractive alternative to autograft. With the increase in acceptance of allograft tissue, several products have emerged that are allograft-based but also used in combination with other materials. See Chapters 2, 4, and 5 for an in-depth discussion of allografts as bone graft substitutes.

Factor-Based Substitutes

The factors and proteins in bone regulate cellular activity by binding to receptors on cell surfaces and thereby stimulating the intracellular environment. This activity generally translates to a protein kinase that induces a series of events that result in the transcription of mRNA and ultimately into the formation of a protein to be used intra- or extracellularly. The simultaneous activity of many factors acting on a cell results in the controlled production and resorption of bone. These factors, residing in the extracellular matrix of bone, include transforming growth factor-β (TGF-β), insulin-like growth factor (IGF) (I and II), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and the bone morphogenetic proteins (BMPs). These factors have been isolated and some have been synthesized, allowing for the examination of function of the factors alone and in combination. The ability to isolate appropriate factors from bone, synthesize them in large quantities, and reapply them in concentrated amounts to accelerate bone healing has produced many possibilities for bone graft substitutes. Much work has been done and continues in the research setting, and some products have appeared on the market for clinical use.

Although growth factors have provided advantages in bone healing, they also present some distinct disadvantages, such as high manufacturing cost, risk of contamination, potential immunological response, protein instability [**17–19**], and the risk of uncontrolled bone growth or cancer [**20**]. An alternative approach to protein- or factor-based therapies is small-molecule therapy, a relatively new area of research that is growing rapidly. "Small molecules" for tissue repair are lower-molecular-weight organic compounds than their full protein counterparts (typically <1000 Da) and are capable of diffusing across cell membranes to reach intracellular targets [**21,22**]. Small molecules exhibit beneficial qualities beyond some of the limitations of protein growth factors, including being more stable, soluble, nonimmunogenic, affordable, and requiring a lower effective dose [**23**] while still affording the same beneficial effects as the full protein. See Chapters 7 and 8 for an in-depth discussion of growth factors and bone graft substitutes.

Cell-Based Substitutes

As regenerative medicine capabilities emerge, various sources of stem cells will be required to meet patient-specific demand. A few commonly studied stem cells for use in conjunction with bone graft substitutes include mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSCs), and induced pluripotent stem cells (iPSCs). To differentiate MSCs in vitro to the osteogenic lineage, stem cells are cultured in the presence of certain additives. After culture in these additives, phenotypic assays and staining can confirm the osteoblast-like cell phenotype of the stem cell [**24**]. The addition of TGF-β and BMP-2, -4, and -7 to culture media has also been used to guide the stem cells toward the osteogenic lineage. ADSCs are an attractive source of stem cells because supply limitations and ease of harvesting is less of a problem given the ready access of adipose tissue deposits found under the dermal layers. A significant breakthrough in 2006, Yamanaka et al. discovered how adults cells treated with the right factors could be engineered back to a pluripotent state capable of producing any cell in the body. These cells induced back to an earlier lineage became known as iPSCs [**25**]. With the advances in stem cell technology, the interaction between stem cells and their potential use in bone graft substitutes for clinically relevant applications are, and continue to be, evaluated and developed. Chapter 6 discusses cell-based approaches in greater depth.

Ceramic-Based Substitutes

Many of the currently available bone graft substitutes contain ceramics, including calcium sulfate, bioactive glass, and calcium phosphates. The use of ceramics, especially calcium phosphates, is motivated by the fact that the primary inorganic component of bone is calcium hydroxyapatite (HA), a subset of the calcium phosphate group. Hence, depending on the structure and porosity of the scaffold, calcium phosphates can come close to mimicking the natural matrix of bones. It is of no surprise that the most widely used bone graft substitutes contain HA-based biomaterials because of their unique properties [**26**]. Calcium phosphates are also osteoconductive, osteointegrative, and in some cases osteoinductive [**27**]. For example, MSCs cultured and seeded onto HA constructs have been shown to successfully differentiate into osteoblasts, resulting in bony tissue growth on the HA surface [**28**]. In addition to calcium phosphate composition, structure and crystallinity also play a role in how osteoblasts proliferate and differentiate when in contact with calcium phosphate and can be modified as needed during the fabrication process. Higher crystallinity HA used for in vitro culturing of rat osteoblasts caused an early increase in proliferation with a subsequent dropoff as culture time increased [**29**]. However, when rat osteoblasts were cultured on lower crystallinity HA scaffolds, which more closely mimic natural bone in overall crystallinity, proliferation was gradual yet increased as culture time increased. In addition, lower crystallinity calcium phosphates are more soluble in body fluid or in vitro analogues than higher crystallinity calcium phosphates, leading to a higher ion concentration near the scaffold [**29**] and a plate-like precipitation on the scaffold

surface, resulting in increased bone repair activity [**30**]. Although ceramics generally have many positive attributes, their use in scaffold formation often requires exposure to high temperatures, which can complicate adding biological molecules, and ceramics generally tend to have brittle failure properties, making them challenging in certain bone graft applications. To combat the brittle nature and to facilitate the addition of biological molecules, they are frequently combined with other materials to form a composite (see *Polymer-Based Substitutes*). See Chapter 10 for a detailed discussion of calcium-based ceramics as bone graft substitutes.

Polymer-Based Substitutes

The final group of bone graft substitutes are the polymer-based groups. Polymers present some options that the other groups do not. For example, there are many polymers that are potential candidates for bone graft substitutes representing different physical, mechanical, and chemical properties. These polymers used today can be loosely divided into natural polymers and synthetic polymers, which can be divided further into degradable and nondegradable.

Natural polymers, such collagen or chitosan, are derived from living sources such as animals or plants, whereas synthetic polymers do not exist in nature as such. A degradable polymer is ideally used in a tissue engineering application where the natural extracellular matrix will eventually replace the scaffold. However, the tissues surrounding the scaffold must be able to metabolize or excrete the products from the polymer's degradation, otherwise an immunological response may occur. Poly(lactide-*co*-glycolide) (PLGA) is an attractive, synthetic, degradable polymer for bone graft substitute applications because it breaks down with the addition of water to lactic acid and glycolic acid, two safe and naturally occurring metabolites in animals. Although synthetic polymers may have optimal mechanical properties and affordability, they can lead to toxicity or chronic inflammation. Natural polymers are advantageous because they can mimic the endogenous extracellular matrix and surrounding tissues can recognize and metabolize their products through common pathways. However, some natural polymers can cause immunological responses, may have variability among different supply sources, and may offer inferior mechanical properties to synthetic polymers. Hydrogels, another representation of polymeric structures, are networks of natural or synthetic hydrophilic polymer chains capable of containing over 99.9 % water by mass. Collagen hydrogels are attractive candidates for use as scaffolds in tissue engineering because cells can adhere and grow on the collagen fibers within the hydrogel, similar to the cell's natural environment. As with ceramics, the functionality of polymers can be enhanced if used in combination with other materials, such as ceramics, to form composites.

To mimic natural and physiological conditions, in many cases composites, or substances containing two or more constituent materials, are optimal for the application. From an engineering perspective, composite materials can often harness benefits beyond which each of its constituent materials would possess on its own, in essence

providing the best of both worlds. In summary, one constituent material could not perform without failure for a particular application without the other. In terms of an orthopedic example, bone tissue is a naturally occurring composite in which collagen proteins provide an elastic or flexible phase to a more rigid and stiffer calcium phosphate matrix. In the end, bone tissue has evolved to become a strong enough support system to carry the weight of the human body, yet flexible enough to endure the daily stresses and loads that act upon it with rare failure. Polymer-ceramic composites, like bone, provide the opportunity to impart the benefits of each material while counteracting their limitations. Toward this end, polymer-ceramic composite scaffolds have successfully been used in vitro and in vivo to differentiate stem cells into osteoblasts [**31**]. Chapter 9 discusses polymers as bone graft substitutes in detail.

REGENERATIVE ENGINEERING AND FUTURE WORK

Although significant advances in bone graft substitutes have been made in recent years, research progress continues to bring various technologies and theories together to produce clinical solutions for orthopedic repair. The human body is undoubtedly a highly organized and efficient machine. As more is learned about genetic and cellular pathways and questions are answered, new questions arise to replace the old ones. Advances in biomaterials such as osteoinductive ceramic-polymer composites may not only provide a superior healing potential than conventional methods but also a more affordable and available alternative, resulting in a better quality of life for more patients.

Tissue engineering has been developing over the last 25 years. However, recent advances in tissue engineering technologies have paved the way for a new perspective—regenerative engineering [**32–35**]. Regenerative engineering has been defined as "the integration of tissue engineering with advanced material science, stem cell science, and areas of developmental biology for the regeneration of complex tissues, organs, and organ systems" [**32**]. As the field of material science has progressed, new materials can be chosen to satisfy the required mechanical properties, degradation rates, and chemical functionality of the application. Advances in stem cell technology may allow patient-specific cells to be directed down the appropriate lineage on a scaffold construct to heal the proper tissues [**33**]. Lastly, a better understanding of the genetic expression of regenerative-capable animals such as newts and salamanders may give insight to the morphogenesis required to form complex human tissues. Many of the concepts introduced here are expanded in Chapter 15.

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Chapter 2 | Bone Graft Substitutes: Classifications and Orthopedic Applications

Celeste Abjornson,[1] B. Victor Yoon,[1] and Joseph M. Lane[1]

INTRODUCTION

Much has changed in the bone graft substitute arena in the past decade since the last edition of this book. The regulatory pathways have significantly changed in the United States with regards to demineralized bone matrices (DBMs), with the U.S. Food and Drug Administration (FDA) reclassifying certain versions of DBMs to be required to go through the 510(k) approval process whereas other versions remain exempt as human tissue products on the basis of the level of manipulation and additives. We have also experienced the greatest rise and fall of one specific product, BMP-2. In the mid-2000s, annual sales of BMP-2 rose as high as $900 million per year, but in light of new data and the medico-legal aspects, the market has receded approximately 20 % year over year to approximately $486 million in 2012 [1]. Lastly, an area almost nonexistent a decade ago has now gained almost 10 % of the market, cell-based matrices. These matrices are a broad category of materials defined as products with claims of stem cells or related factors.

Bone graft substitute is a general term to describe any material used to aid in the regeneration of bone, such as fracture repair, spine fusion, or defect reconstitution. There is a wide spectrum of materials used today for the purpose of grafting; however, their ultimate goal remains the same and that is to form functionally viable bone that meets the mechanical and biological needs of the site. This chapter aims to review many of the common bone grafting materials used in the estimated over 1,000,000 grafting procedures performed in the United States each year and objectively evaluate the materials. Most bone grafting procedures are in spinal procedures, accounting for 90 % of cases and the remaining 10 % split between trauma and reconstruction. There are two main divisions of bone graft substitutes—those that are naturally derived and those that are synthetically fabricated. In each case, the goal of this chapter is to describe how they are made, how they are incorporated in situ, preclinical evidence for their viability, and published clinical experience.

[1] Weill Cornell Medical College, Hospital for Special Surgery, New York, NY

Bone grafting has become a standard procedure in orthopedic surgery over the past 3 decades and has led to an increased interest in bone grafting materials. Surgeries often require grafting procedures to induce de novo bone in an area stabilized by metal devices. The most common examples are tibial plateau fractures and spinal fusions. When considering potential graft materials assuming an adequate blood supply, a successful graft needs to have at least two of the following three properties: cells, signal, and matrix. *Cells* refers to the process of osteogenesis that is defined as cellular formation of new bone. These are dedicated cells in the area of the graft such as osteoblasts or stem cells that enter the osteoblastic lineage and ultimately form new bone. The *signal*, or osteoinduction, is orchestrated by bioactive molecules, primarily low-molecular-weight members of the transforming growth factor-β (TGF-β) family that actively recruit mesenchymal cells and stimulate them to differentiate into bone forming cells for osseous repair. The *matrix* is the scaffolding that permits cell infiltration and in-growth of new host bone and is referred to as *osteoconduction*. The combination of these properties can either come from materials introduced to the site or recruited from the host.

NATURALLY DERIVED BONE GRAFTING OPTIONS

Autograft, the first known bone grafting material in modern medicine, has been documented since the early 19th century and is still considered the gold standard of grafting today. The first report was for a maxillofacial application of refilling holes in the skull with the original bone plugs after the holes were drilled to release pressure [**2**]. By the late 1800s, Bergmann reported using a fibular graft to close a tibial defect [**3**]. Also by the late 1800s, another bone grafting procedure was reported by Phelps [**4**] in which he transplanted bone tissue from an animal (dog) to a man. Although xenografts have never become commonplace, animal-derived collagen scaffolds are commonly used today.

In this section, materials of natural derivation from bone, bone marrow, bone proteins, and blood products will be reviewed. Commercially available DBM has undergone significant processing, but it is still allograft bone and will be included in this section. Other technologies to be discussed are bone marrow aspirate, platelet-rich plasma, and autograft.

Autograft

Autologous bone graft (ABG) has long been considered the standard method of grafting because it contains all three of the grafting components. It contains osteoprogenitor cells in the bone marrow, the morcelized surfaces of the bone chips act as a scaffold, and the osteoinductive factors are within the bone chips. Many papers in the past 2 decades have illustrated methods of harvesting autograft. Today, autograft is most commonly taken from the iliac crest because of the cancellous structure and cell volume. However, significant complications have been associated with the procedure [**5–8**].

Complication rates have been reported ranging from 9 % to as high as 50 % for ABG procedures [**8,9**]. Falsely considered "free" by many surgeons in comparison to commercially available options, these complications can often become quite costly over time. Other known drawbacks to this grafting method are a second site of morbidity, the increased surgery time and blood loss, and limited supply. Limited supply and quality of bone and cells in older patients is especially something to consider. Newer techniques focused on less invasive bone collection methods have markedly reduced morbidity.

When ABG is introduced into the graft site, the initial response by the surrounding tissue is similar to a convoluted fracture. Most transplanted cells die because of ischemia. Fortunately, mesenchymal cells are the most resistant to ischemia and may survive to begin differentiation and proliferation. The efficacy of the ABG is intimately linked to the survival of these cells and is thought to be the most vital component of the graft. The cell signal is necessary for an osteoclastic response to break down the fragmented bone. As the osteoclasts begin to digest the surfaces of the mineralized fragments, the collagenous and noncollagenous proteins as well as signaling molecules are exposed and signal for osteoblastic activity. Grossly simplified, the reaction to ABG is to consolidate, remove, and rebuild. Resorption initially outpaces formation. However, even with long-term follow-up, some devitalized autogenous bone will remain unremodelled.

The clinical evidence for autograft bone grafting success greatly precedes the preclinical testing. However, the 20th century provided plenty of evidence for its use. As a matter of fact, as almost all new technologies are evaluated, they are compared preclinically and clinically to autograft. Literature citations for the efficacy of autograft will be included later in the chapter when comparing to other bone graft substitutes as the studies were usually designed. However, it is important to note that not all autogenous bone is equivalent in relation osteogenic potential. An ideal location for harvesting autograft, such as the iliac crest, is often preferred to local bone for this reason.

DBM

Brief History

Similar to the history of autograft, the first reports in modern medicine of allografts came in the late 19th century. The next large milestone came during World War II when the U.S. Navy Tissue Bank was founded and tissue banking procedures were established [**10-13**]. The allografts available at that time were fresh or fresh-frozen bulk allografts. These types of allografts are not the focus of this discussion because they are not bone graft substitutes but structural bone grafts. Instead, we will focus on demineralized bone that was first described by Urist [**14**]. Although the potential of DBM was discovered almost 40 years ago, it has only been clinically available since the early 1990s. Mounting interest in this applied science has come with many early growing pains. With an increased demand by surgeons for allograft bone, the National Organ Transplant Act was passed in facilitating the development of tissue and organ donor

networks (Public Law 98-507, 1984). With an increasing availability of allografts, the focus became in preparing allografts for transplantation by a reliable process that could ensure safety. In July 1997, the FDA released industry standards for donor screening that are complimented by the American Association of Tissue Banks (AATB) requirements for screening, processing, and distribution procedures of all donors. Although commercially available for many years, in March 2004, Wright Medical received FDA 510(k) approval of Allomatrix. Allomatrix is a composite of DBM powder and calcium sulfate. The calcium sulfate alone is a device and has been marketed for many years as Osteoset. Feeling the mounting pressure, Wright Medical was the first company to go through the rigorous task of getting approval as a medical device. In 2006, the FDA reclassified DBMs with carriers to be a Class II product requiring 510(k) approval. However, DBMs that that do not have carriers are still considered human cells, tissues, and cellular and tissue-based products (HCT/Ps) and are not regulated as devices.

How It Is Processed

DBM is formed after a mild acid extraction of cadaveric bone that removes the mineral phase, leaving collagen, growth factors, and noncollagenous proteins. DBM offers the intrinsic properties of osteoconduction and osteoinduction. It is processed in various ways and made into a powder. This powder does not have the optimal characteristics desired by a surgeon; therefore, it is mixed with a carrier to provide better handling characteristics. DBM is clinically available in gels, putties, pastes, and fabrics that have been tailored to try to meet the needs of the surgical procedure.

DBM has an osteoinductive property because of retained growth factors from the original bone that remain intact after processing. These osteoinductive factors are a superfamily of polypeptide growth factors that regulate in vivo the expression and proliferation of differentiated phenotypes for many cell populations. Among these factors are the low-molecular-weight bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and a broad spectrum of proteins and factors within physiologic concentration that play a pivotal role in bone formation and repair. In addition, other noncollagenous bone signaling proteins such as osteocalcin and osteopontin are also contained within the DBM. DBM is osteoconductive because of the collagen matrix.

DBM is a form of allograft bone and, as such, it begins its processing much like other allografts. The process begins with appropriate donor screening. In accordance with FDA regulations, when a donor becomes available, full medical history of the donor and interviews with family members are required as well as an autopsy and serological screening. The risk of transmitting HIV through an appropriately screened donor is less than 1 in 1.6 million [**15**]. However, this is not nearly acceptable and has led to the need for commercial processing methods.

The goal of processing is to provide a sterile DBM graft that is free of virus and blood constituents while maintaining the biological signals. The FDA guidance

documents for processing recommend a method able to clear 3–6 logs above the maximum viral burden. The maximum viral burden is defined as the theoretical maximum of virus that could be in the bone tissue. Using clearance studies, virus is spiked into the tissue before each of the processing steps and titers are taken after the processing step. The AATB accredits processes that are able to establish a validated method and have proven viral inactivation 3–6 logs above maximum viral burden. In accredited methods, the results show that the chance of virus surviving processing is extremely low. However, most commercially available products have not received accreditation for viral inactivation. However, the demineralization process alone has recently been shown to have a significant effect on virus levels. Swenson and Arnoczky proved that a clinically accepted demineralization procedure would alter the nucleic acids of the feline leukemia virus (a retrovirus similar to HIV), inactivating the virus in infected bone and rendering it noninfectious [**16**]. There has been one reported incident of HIV-infected donor tissue that offers further support regarding the inability of HIV to be transmitted in processed DBM. In 1985, LifeNet Transplant Services of Virginia Beach supplied tissue and organs from a donor later realized to be HIV-positive. Patients that received the donor's heart and kidneys died from HIV-related complications. None of the patients that received DBM resulted in a conversion to HIV-positive, which is thought to be related to the cleaning and demineralization processes [**17**].

There are various processing methods used in products that are commercially available today, each with its own limitations. Treatment solutions, solvent concentrations, and chelating agents are all suspected of affecting the osteoinductivity of the DBM. Urist showed that hydrochloric acid, commonly used in DBM processing, mixed with alcohols produces noninductive DBM [**18**]. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) have been shown to not fully demineralize the bone and reduce DBM performance [**18**].

Other factors that must be considered in processing DBM are antibiotics, particle size, temperature, calcium content, and sterilization method. Urist proved that for various antibiotics such as erythromycin, penicillin, and streptomycin, there is no inhibitory effect on osteoinductivity [**19**]. However, Urist and others have shown that cold and hot temperature extremes during processing can detrimentally affect the osteoinductive function of DBM [**14,19,20**]. Calcium content is a measure of the demineralizing time and processing. If demineralization of at least 60 % of the material is not achieved, then a low inductivity will result because demineralization exposes the osteoinductive proteins. Therefore, if a high calcium content exists, then a highly mineralized graft makes the DBM less inductive [**20,21**]. The size of the particulate bone used in the formulation has been shown to be most inductive, within the range of 75 μm to 2 mm^2 [**22,23**].

Sterilization methods are probably one of the most widely diverse components of DBM processing. Most DBMs are produced under sterile conditions, and for those DBMs that are Class II products, they are also terminally sterilized. There has been

much published literature on all of the different methods, with the following serving as only an overview. Aseptic processing is the method of using sterile practices from recovery to packaging. It alleviates the need for end-term sterilization, but it is much more involved and expensive. Gamma irradiation has been shown to diminish or destroy the osteoinductive potential when used at levels of 2.0 mrad or higher [**24**,**25**]. Ethylene oxide has also been shown to reduce or eliminate osteoinductivity [**25–27**] and can cause an inflammatory response from residuals [**25**,**28**].

In summary, processing and sterilization techniques greatly affect the osteoinductivity of the product. The only accurate method to determine the osteoinductivity of the final product is to test it in an athymic rat (rnu/rnu) muscle pouch model [**14**,**29**]. Although in vitro methods are sometimes referenced as an acceptable methodology to determine osteoinductivity, these methods only show the potential to form new bone. However, the pouch model produces new bone in an ectopic site. Results using this model have been presented and show there is a wide range of osteoinductivity in current products [**30**]. It is also important to recognize the need to test final product and not intermediate steps because, as discussed, each processing and sterilization step may affect osteoinductivity.

Boden has established a ladder of hierarchy, sometimes referred to as the "Burden of Proof," which proposes that before making a decision on what material to use, consider what tests or studies have been done to show efficacy. The lowest proof would be using in vitro cell tests. These simply measure markers suggesting bone formation. Animal testing can range from rodents to larger mammals to nonhuman primates and finally to human clinical trials. Currently, some DBMs have gone through some testing; however, it varies considerably. Many processors test materials before or during processing; few test end product. However, the most alarming issue for the surgical community is that there are no standards for rejection. As long as the product is deemed "clean," it will go to market, but it may not be osteoinductive.

Biology and Incorporation of a DBM Bone Graft

Bone healing is known to be a symphony of events. Many factors and cell types are needed to achieve a strong union and repair of the graft site. Mohan [**31**] and Sampath [**32**] were able to show that demineralized bone powder possesses a considerable array of growth factors. Once implanted, DBM quickly revascularizes, followed by normal hematoma formation events including release of cytokines, growth factors, and leukocytes. Mesenchymal cells are recruited to the area and differentiate into chondrocytes in approximately 5 days after implantation [**33**]. DBM grafts are known to go through endochondral ossification, in which these chondrocytes form a cartilage matrix that is later mineralized. At approximately 10 days after implantation, osteoblastic cells appear and begin laying bone onto the mineralized cartilage matrix. Unlike the classical method of autogenous bone for grafting, there is not a large osteoclastic invasion because there is no/little mineralized component signaling for resorption. DBM promotes the cascade directly toward formation. Over time, the DBM is replaced by host

bone through remodeling. The sustaining presence of an array of growth factors in physiological amounts that are present in DBM allows for continued osteogenesis throughout the defect repair.

Preclinical DBM

Urist [**14**] first described the potential for DBM to induce new bone formation. He showed that DBM placed in a heterotopic muscle pouch could induce new bone formation in 28 days by endochondral ossification (cartilage mediary). Many studies since Urist's initial findings have proven the osteoinductive potential of DBM in various animal models [**34–37**].

Long bone critical-sized defect models such as the rabbit ulna [**35**] or rat femoral [**34**] models have shown the efficacy of DBM. Bolander et al. showed that DBM could heal the defect as well as ABG and exhibit similar torsional stiffness. He also demonstrated that if the DBM was augmented with bone proteins it had superior mechanical properties to ABG. Einhorn et al. assessed the healing profile by serial radiographs and the mechanical strength of the graft. They found a normal fracture repair sequence. The energy-absorption capacity and stiffness properties of the grafted femurs equaled that of intact femoral bone. The torsional stiffness was found to be consistent with normal fracture repair and achieved 35 % of the torsional strength of normal bone.

DBM has also been shown efficacious in several spine fusion models [**36–39**]. Martin et al. demonstrated the importance of formulation tailored to procedure. Their results reveal that fusion rates with fabric DBM sheets were superior to ABG and putty forms were equivalent to ABG in a posterolateral rabbit spine fusion model. Wang et al. studied the differences between three commercially available DBM putties (Osteofil, Grafton, and Dynagraft) with different processing using a posterolateral athymic rat model. Their results showed no statistically significant difference between the fusion rates of Osteofil and Grafton. None of the Dynagraft rats achieved fusion [**40**].

Clinical DBM

DBM has been used alone and to augment autogenous bone grafts in the repair of cysts, fractures, nonunions, and spine fusions. In several clinical situations, DBM has been used with considerable success. Glowacki and coworkers reported some clinical applications in craniofacial reconstruction [**41–44**]. Tiedeman et al. [**45**] evaluated the efficacy of DBM used alone and with autogenous bone marrow as graft material in the treatment of various osseous defects such as bone defects and comminuted and nonunited fractures in children. In their series, 30 of 39 (77 %) patients showed bony union in facial bones. They concluded that DBM and marrow composite grafts are comparable in efficacy to autogenous iliac crest bone grafts for use in certain clinical situations. Michelson et al. [**46**] compared hindfoot fusions augmented either by iliac crest bone graft or DBM alone. All 29 patients receiving DBM achieved complete fusion whereas 13 of 15 patients receiving autologous iliac crest bone graft went on to bony union. There was no difference in the time required for complete healing, with

both groups achieving union in 3–4 months. This study showed that DBM displayed comparable healing to iliac crest bone grafting in the hindfoot arthrodesis without the increase in blood loss, cost, and postoperative pain associated with iliac crest grafting. Killian et al. [**47**] used DBM to obliterate unicameral bone cysts in nine of eleven patients that were cyst-free at 2 years follow-up, clearly showing the effectiveness of using DBM in certain clinical situations. Recently, Leatherman et al. [**48**] showed considerable bone formation using DBM alone in the repair of mastoid obliterations.

In the one prospective evaluation, Geesink et al. [**49**] compared the osteogenic activity of OP-1® (a recombinant version of BMP-7), Stryker Spine, Annandale, NJ) to DBM (Grafton Gel®) in a fibula defect. Twenty-eight patients were enrolled in the study and received one of four treatments: OP-1® in collagen, collagen alone, DBM, or no treatment. Patients were evaluated at 1, 6, and 10 weeks and 4, 6, and 12 months by radiograph and dual-energy X-ray absorptiometry (DEXA; a means of measuring bone mineral density). Bone formed earlier with the OP-1®, but at 12 months there was no difference in bone mineral density scores between the DBM and OP-1®. In addition, at 12 months, the DBM had produced a radiographically more solid construct and homogenous fill in comparison to the OP-1® grafted fibula.

A recent retrospective review of patients who had undergone instrumented posterolateral lumbar spinal fusion with autogenous bone graft and Grafton Gel® was performed by Sassard et al. [**50**]. They compared Grafton Gel® implanted patients with an age-, gender-, and procedure-matched group of patients undergoing instrumented fusions with autograft harvested from the iliac crest. Using a bone mineralization rating scale, they did not find any radiographic differences between the groups based on films taken 3, 6, 12, and 24 months after surgery. The fusion rates in the Grafton Gel® with local bone group and the autograft group were 60 % and 56 %, respectively, statistically comparable. At 24 months, the fusion rates were less than had been reported in other studies of instrumented posterior fusion and were attributed to grading criteria. The choice of instrumentation was significantly related to fusion success and was the most important predictor of 24-month bone mineralization.

Although retrospective data and preclinical work are important and valuable, a prospective, clinical trial to study efficacy is still regarded as the most significant. In 2004, the first multicenter, prospective, clinical trial in a posterolateral spine fusion model studying the efficacy of DBM and autograft with pedicle screw fixation of 120 patients was published [**51**]. Autogenous bone graft from the iliac crest was implanted in one of the lateral gutters of the spine and a Grafton DBM/autograft composite was implanted on the contralateral side in the same patient. Fusion was achieved in 52 % of the lateral sides receiving the composite graft of DBM/autograft and in 54 % on the autograft side. Their conclusion was that they had shown Grafton DBM Gel to be a successful extender of autograft in spinal arthrodesis. However, it should be noted that gel formulations are the oldest forms of DBMs and newer fabric forms have improved osteoconductivity and are more suitable for this indication. Recently, in a prospective multicenter randomized clinical trial, Kang et al. [**52**]

compared the outcomes of Grafton DBM with local bone against iliac crest bone graft (ICBG) in a single-level instrumented posterior lumbar fusion. Forty-six patients were randomly assigned (2:1) to receive Grafton DBM Matrix with local bone (30 patients) or autologous ICBG (16 patients). An independent radiologist evaluated plain radiographs and computed tomographic scans, and 2-year time points reported that fusion rates were not statistically different with the Grafton Matrix group at 86 % versus the ICBG at 92 %.

In summary, DBM has been shown to be a successful extender to ABG in spine fusion. In some indications, it can perform equivalent to ABG. When making a decision on what to use, remember that DBMs differ by preparation and carrier and it is recommended to use a DBM that has been shown to be efficacious. As will be further highlighted in the next section, bone marrow aspirate enhances the activity of DBM and is always recommended to be used in conjunction with DBM when feasible.

Bone Marrow Aspirate

Autologous bone marrow is a potent osteogenic graft material. Harvested bone marrow by aspiration techniques contains a spectrum of cells ranging from undifferentiated stem cells to committed cells in the osteogenic lineage. Similar to autograft, it is commonly taken from the iliac crest, but it does not share the problems of morbidity.

The aspirate is taken at the time of surgery; therefore, it does not require any type of processing. However, there has been much literature discussing optimizing aspiration techniques. Muschler et al. [**53**] have described a fanning method in which only 2 cc are aspirated at each site. In this paper, they showed that aspiration technique had a significant effect in cell concentration of the harvested bone marrow. They found if more than 2 cc were aspirated at any one site that dilution from peripheral blood would occur. Their other important finding was that 70 % of the variation in cellularity could be accounted for by variation between subjects. In a later study, Muschler et al. [**54**] showed that there is a significant decrease in the number of osteoblastic progenitor cells in bone marrow with increasing age in humans. To improve cell volumes, systems have been introduced to concentrate the marrow before reintroduction.

Preclinical studies have examined the effectiveness of bone marrow when used in combination with and without grafting materials in spine fusion and long bone defects [**38,55–57**]. Paley et al. [**58**] demonstrated in a critical-sized defect model in the rabbit radii that bone marrow aspirate alone was able to successfully unite and form a solid bone construct. In a canine model, the combination of DBM and bone marrow proved synergistic in a tibial defect, resulting in more complete bridging and remodeling, greater density, and higher rigidity than the subjects with marrow or DBM alone [**56**].

Clinical studies confirm the osteogenic potential of bone marrow, particularly when used in healing defects [**59,60**]. Healey et al. reported being able to heal five of eight nonunions using injections of bone marrow obtained from iliac crest. Likewise, Connolly et al. reported the successful treatment of 18 of 20 tibial nonunions with

injected aspirated bone marrow combined with either fixation or cast and an intramedullary nail. In spinal fusion, the question of whether bone marrow aspiration from the vertebral body is a viable alternative to the iliac crest is often discussed. McLain et al. [**61**] demonstrated bone marrow from the vertebral body had comparable or greater concentrations of progenitor cells compared with matched controls from the iliac crest. Further, they showed no significant differences relative to vertebral body level, the side aspirated, the depth of aspiration, or gender and suggested vertebral body bone marrow aspiration was indeed a viable alternative.

There has been a significant upswing in commercially available systems to help concentrate the bone marrow aspiration. The actual number and concentration of these cells that are necessary to obtain bone repair is not well understood; however, several studies have shown that concentrating the bone marrow aspirate to concentrate the progenitor cells can be important and necessary in challenging applications [**62**].

Platelet-Rich Plasma

In the past few years, we have seen an emergence of platelet-rich plasma (PRP) collection systems come to market. PRP is collected by spinning down whole, autologous venous blood at the time of surgery. These systems aid the surgeon in collecting and separating the blood to concentrate the PRP. Unlike bone marrow, PRP has none of the original three signals essential for bone healing. It has been labeled as osteostimulatory. It is known in normal wound healing that platelets adhere quickly and release cytokines such as platelet-derived growth factor (PDGF) and TGF-β and others in the fibrin network. This led to the concept that PRP with a high concentration of platelets would stimulate the release of these cytokines. Because it is an autologous procedure, it is not regulated or reviewed by the FDA. The effect of the last statement is that little to no research had to be done before going to market to prove efficacy or safety. Being much easier to collect than marrow, it became attractive to the surgeon community. However, in the past 2 years we have seen conflicting literature regarding its efficacy and some suggestion that it may in fact not be helping and possibly hindering bone healing.

Weiner et al. [**63**], in a retrospective, consecutive series, evaluated patients who underwent a single-level intertransverse lumbar fusion for either degenerative disk disease (DDD) or degenerative spondylolisthesis. The control group (n = 27) was grafted with ICBG. The treatment group (n = 30) was grafted with ICBG augmented with autologous growth factors, or AGF (an ultra-concentration platelet system). Fusion was assessed radiographically at 1 and 2 years postoperatively. The authors reported the fusion rate for the control group was 91 % (24 of 27) and the treatment group was 62 % (18 of 32). They concluded that the use of AGF resulted in inferior rates of arthrodesis compared with autogenous bone graft alone. Carreon et al. [**64**] retrospectively compared ICBG versus ICBG + PRP in posterolateral lumbar fusion in one to three levels with 76 patients per group. At 2 years, there was not a statistically

significant difference in nonunion rates; however, the ICBG + PRP was higher, with 25 % versus 17 % nonunion in ICBG, and they recommend against the use of AGF. Newer versions of PRP-type systems that retain the fibrin component have recently been introduced and show great promise without the previous issues seen with AGF.

SYNTHETICALLY DERIVED BONE GRAFT OPTIONS

The innate issues of high variability and chance of viral transmission in naturally derived bone grafts pushed scientists to find synthetic alternatives. Synthetically derived bone grafts are rigorously tested for safety and efficacy to receive FDA approval before becoming available in the United States. Because they are manufactured materials that are classified as medical devices, they are produced in a consistent manner within small tolerance limits and have no viral transmission issues.

In this section, we will review the materials in the same manner as we did the natural bone grafts, focusing on their origin, processing, incorporation in the body, and the existing literature showing efficacy. Although BMPs are naturally present in the body, the commercially available products are genetically engineered; therefore, they are included in this section. Other options that will be reviewed are calcium sulfates, β-tricalcium phosphate, and collagen scaffold materials. Synthetic bone grafting options are a rapidly expanding field, but we concentrate on those options clinically available today.

BMP

Urist was the first to theorize that the osteoinductive activity of DBM was due to active protein molecules, and he named BMPs that were interrelated to bone healing [**65**]. Isolating these proteins from bone matrix proved difficult, and the first isolated extraction and recombinant form of BMP-2 was described almost 2 decades later in 1988 [**66**]. To date, although 15 BMPs have been identified and studied, BMP-2 and BMP-7 have been mainly shown to have the strongest bone-forming potential [**67**]. The recombinant protein versions available commercially today are synthetic, genetically engineered versions of the natural protein.

As members of the TGF-β superfamily, BMPs are known to be potent bone-forming agents. They can drive mesenchymal stem cells into the osteoblastic lineage. These proteins alone are considered osteoinductive and are usually added onto a collagen sponge or ceramic carrier. They initiate endochondral bone formation, presumably by stimulating local mesenchymal cells and enhancing bone collagen synthesis.

Preclinical Recombinant Human BMP-2

The first question obviously is how much of this potent protein should be administered to the site and if this dosage would be site or carrier specific. Sandhu et al. showed in a canine intertransverse spine fusion model that doses of recombinant human BMP-2 (rhBMP-2) on a polylactic acid polymer carrier from 58 to 920 μg were successful in

forming fusions at 3 months postoperatively [**68**]. Using this knowledge, they continued their work in lumbar interbody fusion in an ovine model. At 6 months postoperatively, they reported that all animals appeared radiographically fused; however, histological evaluation revealed something far more telling. Histologically, only 37 % of the animals treated with autograft-filled cages had achieved union compared with 100 % of the animals treated with rhBMP-2/collagen-filled cages [**69**]. This exemplifies the value of preclinical work.

Boden reviewed the existing knowledge base concerning the biology of spinal fusion and also conducted extensive research in the field [**70**]. Because the ovine model was successful, as demonstrated by Sandhu et al., Boden et al. [**71**] studied rhBMP-2 on a collagen carrier within a titanium interbody cage in rhesus monkeys. Because dosing was known to be vital, but the optimal dose for rhesus monkeys had not been previously established, two concentrations of rhBMP-2 (0.75 or 1.50 mg/mL) were tested. The results showed that both groups achieved fusion; however, the higher concentration resulted in faster and more dense bone formation. This study established the dose used in the Investigational Device Exemption (IDE) trial in the United States.

Although the collagen carrier is the optimal carrier for inside cages in interbody applications, posterolateral spine fusion is a different environment and requires a different carrier. In posterolateral spine fusion, the goal is to bridge bone between the transverse processes. The muscle layer surrounding the graft material is significant and will try to invade the space and cause mechanical compression of the graft material. This can lead to either nonfusion or an hourglass-type fusion that is not as dense in the mid-region. Therefore, a new carrier had to be identified and dosing concentration again needed to be established for rhBMP-2. Using a ceramic carrier (60 % hydroxyapatite [HA] and 40 % tricalcium phosphate), Boden et al. [**71**] studied in the nonhuman primate model three rhBMP-2 concentrations loaded with a solution containing 0, 6, 9, or 12 mg of rhBMP-2 per side in comparison to a control group, ABG. They reported solid fusions at all three concentrations of rhBMP-2 and even fusion in the ceramic carrier alone group. The ABG group did not achieve fusion in any of the animals. They concluded that the ceramic carrier was a suitable material for posterolateral applications. This study led to a clinical trial described below.

rhBMP-2 Clinical Trials

Because the preclinical testing showed efficacy and safety, the multicenter prospective randomized IDE trial was initiated. The study was designed for the treatment of DDD in the lumbar spine by interbody fusion. Patients were randomized to one of two groups: rhBMP-2 (1.50 mg/mL) on a collagen sponge with tapered titanium fusion cage (InFUSE/LT-Cage, Medtronic Sofamor-Danek, Memphis, TN) or autograft from the iliac crest with the same tapered titanium fusion cage. In the randomized arm of this trial, 143 patients received the InFUSE/LT-Cage and 136 patients received the LT-Cage with autograft (ABG/LT-Cage). In the continued access arm of the trial, an additional 134 patients received the InFUSE/LT-Cage. The study design was for 2-year

follow-up. The results showed radiographically no difference between the InFUSE/LT-Cage and ABG/LT-Cage groups, each receiving fusion rates above 90 % at 2-year follow-up (www.fda.gov). The FDA clearance was granted on July 2, 2002, to rhBMP-2 on a type I collagen sponge in conjunction with a tapered, threaded intervertebral fusion cage (LT-Cage; Medtronic Sofamor Danek, Minneapolis, MN) for the indication of DDD in the lumbar spine.

For the posterolateral spine fusion application, a prospective randomized multicenter clinical study to evaluate with rhBMP-2 on a ceramic carrier (60 % HA and 40 % tricalcium phosphate) was conducted in 25 patients whose spondylolisthesis did not exceed Grade 1 [**72**]. Patients were randomized to one of three groups: autograft with pedicle screw instrumentation (n = 5) (control), rhBMP-2 with pedicle screw instrumentation (n = 11), or rhBMP-2 without internal fixation (n = 9). In patients receiving rhBMP-2, the graft material consisted of 20 mg of rhBMP-2 on ceramic granules (10 cm/side). The radiographic fusion rate was 40 % (2 of 5) in the autograft with instrumentation group and 100 % (20 of 20) with rhBMP-2 group with or without internal fixation (P = 0.004). Patient questionnaires revealed a statistically significant improvement in Oswestry scores at 6 weeks in the rhBMP-2-only group and at 3 months in the rhBMP-2 with instrumentation group. However, the Oswestry scores did not significantly improve in the autograft with instrumentation group until 6 months. A systematic review on the biology of spine fusion healing and several fundamental principles required for the selection of a bone graft substitute is also conducted by Boden [**73**].

On April 30, 2004, InFUSE (rhBMP-2 and collagen sponge) was cleared with an intermedullary nail for the treatment of acute, open tibial fractures. In a prospective, multicenter clinical trial, the use of InFUSE with an intermedullary nail was evaluated for the treatment of tibial fractures. Patients all received an intermedullary nail and were randomized to one of three treatments (n = 150 patients): InFUSE at a 0.75-mg/mL concentration, InFUSE at a 1.5-mg/mL concentration, or control (standard of care defined as routine soft-tissue management). The primary endpoint in the study was at 1 year with the primary efficacy evaluated as the proportion of patients requiring secondary intervention because of delayed union or nonunion. At 12 months after operation, 421 patients were evaluated. The 1.50-mg/mL rhBMP-2 group had higher union rates, significantly lower occurrences of secondary interventions, a significantly higher healing rate at the postoperative visits from 10 weeks through 12 months, fewer hardware failures, fewer infections, and faster wound-healing than the control group. They concluded that InFUSE offered significantly superior care to the control [**74**].

On March 9, 2011, Medtronic received a nonapprovable letter from the FDA with regards to their AMPLIFY™ rhBMP-2 Matrix. This decision was a result of clinical and safety data from the IDE prospective, randomized, multicenter clinical trial in skeletally mature patients with DDD at one level from L1–S1 in 463 patients. Most of the controversy that resulted in the nonapproval stemmed from increased cancer risks in

the investigational group in comparison to the ICBG control, which was thought to be linked to the higher dosage than the previously cleared version.

In an unprecedented effort, the Yale University Open Data Access (YODA) project approached Medtronic for funding and access to all of their in-house safety and efficacy data on rhBMP-2. In 2013, two publications of the findings of the systematic reviews were published [**75,76**]. The major findings of the review by Simmonds et al. were rhBMP-2 increased spinal fusion rates at 24 months postoperatively and increased early postsurgical pain compared with ICBG. They also concluded that evidence of increased cancer incidence was inconclusive. Fu et al. reported with respect to lumbar spine fusion, rhBMP-2, and ICBG were similar in overall success and fusion. For anterior cervical spine fusion, rhBMP-2 was associated with increased risk for wound complications and dysphagia. Their findings differed from Simmonds et al. in regards to the cancer risk. Fu et al. found increased risk with rhBMP-2, but event rates were low and cancer was heterogeneous. The discrepancy between the two papers is derived from the differences in the studies they included to determine their analyses. In addition, there have been many recent publications describing retrospective cohort data from centers with regards to complications and incidences of cancer after rhBMP-2 usage with mixed findings [**77–80**].

Preclinical rhBMP-7/OP-1®

In animal models, large segmental defect studies were performed with OP-1® to understand the protein's ability to restore normal weight-bearing bone containing functional bone marrow [**81–85**]. The general finding of the works was similar to rhBMP-2: that bone formation was highly dependent on the dose of OP-1®. In the rabbit ulnar critical-size defect model, a 1.5-cm segmental defect was created and filled with a collagen carrier containing either one of nine concentrations of OP-1 or naturally occurring bovine osteogenic protein. The experimental dose levels of OP-1 were 3.13, 6.25, 12.5, 25, 50, 100, 200, 300, or 400 μg. The control groups were the collagen carrier alone and no graft. They found osseous unions in all of the OP-1 dose groups except the 3.13-μg dose and the bovine osteogenic protein at 8 weeks. The control groups did not heal [**81**]. The investigators continued their work in a canine model to further understand dose dependency [**82,84**].

Continuing the burden of proof, tests with larger mammals need to be completed before the material could be proved to be efficacious and be ready for an IDE trial. Cook et al. [**83**] proceeded with a segmental bone defect model in 28 African green monkeys. The two groups in this study consisted of either a 2.0-cm ulnar defect ($n = 14$) or tibial defect in the diaphysis ($n = 14$). Focusing on the tibial group, the tibial defects were subgrouped into defects filled with 250, 500, 1000, or 2000 μg of OP-1 in 400 mg of collagen carrier ($n = 5$), with collagen carrier alone ($n = 1$), with autogenous cancellous bone graft ($n = 6$), or unfilled control ($n = 2$). The tibial defects were stabilized with an intramedullary pin. Radiographically, at the 6- to 8-week window, four of five of the tibiae treated with OP-1 exhibited complete healing. Animals were

sacrificed at 20 weeks postoperatively. Although all of the defects treated with OP-1 exhibited new-bone formation, the degree of healing and remodeling differed. The autogenous group exhibited complete healing in five of six animals.

Similar to rhBMP-2 investigators, investigators studying OP-1 wanted to study expanding applications for the protein. Grauer et al. [**86**] evaluated OP-1 for intertransverse process lumbar fusion in the rabbit as a potential graft substitute. In their experiment, the three investigational groups were autograft, carrier alone, and carrier with OP-1. At sacrifice 5 weeks postoperatively, fusion was evaluated by manual palpation and resulted in 63 % in the autograft group, 0 % in the carrier-alone group, and 100 % in the OP-1 group achieving fusion. They concluded that OP-1 was found to reliably induce solid intertransverse process fusion in a rabbit model at 5 weeks. In a later investigation using this model by the same group [**87**], they found OP-1 was able to overcome the inhibitory effect of smoking on spine fusion. Combining this work with previous work, clinical trials began to evaluate OP-1 for posterolateral fusion and will be discussed in the next section.

Clinical rhBMP/OP-1

In a prospective, randomized, partially blinded, multicenter IDE clinical trial, OP-1 Implant® (Stryker, Allendale, NJ) was evaluated in the treatment of tibial nonunions to establish the safety and efficacy in comparison to the standard of care, ABG, in 122 patients for a total of 124 tibial nonunions, Each patient received an intramedullary rod and either OP-1 Implant® or ABG. The OP-1 Implant (Stryker, Hopkinton, MA) dose contained 3.5 mg of OP-1 mixed with 1 g of collagen carrier. Depending on the size of the fracture, up to two doses of OP-1 Implant® could be given. At 9 months, the primary endpoint of the study, the groups were not statistically different by radiograph, and it was shown that 75 % of the OP-1 Implant-treated and 84 % of the ABG-treated patients had healed fractures. The authors concluded that OP-1 on a collagen carrier was a safe and effective treatment for tibial nonunions [**88**]. In part on the basis of this study and the high rate of patient dropout, on October 17, 2001, OP-1 Implant received Humanitarian Device Exemption (HDE) clearance from the FDA for the treatment of long bone fractures. HDE clearance differs from rhBMP-2 device clearance in that institutions must receive Institutional Review Board approval for the patient.

There were two clinical trials involved in the evaluation of OP-1 Putty® for intertransverse process fusion of the lumbar spine. OP-1 Putty differs from the OP-1 Implant sold for tibial nonunions. Similar problems with mechanical compression issues with collagen sponge carriers mostly likely led to the decision to add carboxymethyl cellulose sodium as a bulking agent to the OP-1 Putty product. In the first multicenter clinical pilot study trial, OP-1 Putty was evaluated as a graft extender to autograft. The second trial evaluated OP-1 Putty as a substitute material.

In the first trial, twelve patients underwent intertransverse process fusion by placing iliac crest autograft and OP-1 Putty between the decorticated transverse processes.

No instrumentation was used. The investigators reported only 55 % of patients achieved solid fusion by the strict criteria used, but bridging bone was observed in 91 % of patients. In addition, nine of the twelve patients reported at least a 20 % improvement in their Oswestry scores. They concluded that in comparison to historical controls, autograft alone, with fusion rates at approximately 45 % that of OP-1 Putty, was an enhancer of autograft alone [**89**].

In the second trial, a prospective, randomized, controlled, multicenter clinical study was conducted to compare OP-1 Putty with ABG for one-level uninstrumented posterolateral fusion. Thirty-six patients were randomized (2:1) to either OP-1 Putty (3.5 mg of OP-1 per side) or ABG. At 1-year follow-up, 74 % of OP-1 Putty patients and 60 % of ABG patients achieved fusion. They concluded that OP-1 Putty had performed similar to ABG in fusion rates and patient questionnaires (Oswestry, SF-36), making it a valid alternative to ABG.

On April 7, 2004, OP-1 Putty was granted HDE clearance for posterolateral spine fusion in patients in which ABG and autologous bone marrow harvest are not feasible. The contraindications for OP-1 Putty are that it should not be used in patients under 18 years old, at or near the vicinity of a resected tumor, in patients with a history of malignancy, or in pregnant women. Women of childbearing potential should wait 1 year after implantation before becoming pregnant. Stryker has sold the rights to OP-1 to Olympus, pursuing it for nonorthopedic applications.

Ceramics

One of the essential elements of bone regeneration is osteoconduction, which provides a scaffold for the progenitor cells to proliferate and differentiate. In addition to providing support as a structural lattice, optimal osteoconductive materials must be biodegradable. Hence, the material must be remodeled into the newly formed bone. The role of ceramics in reconstructive orthopedics is primarily for osteoconduction. As discussed earlier in this chapter, one factor in bone regeneration does not constitute a stand-alone product. Osteoconductive matrices should never be used alone unless placed in a marrow-rich environment such as back-filling the iliac crest after ABG harvest. Osteoconductive matrices have been discussed throughout this chapter in conjunction with BMPs, bone marrow aspirate (BMA), and DBM. To further highlight their individual characteristics, this section will review them so that surgeons can make informed decisions when making their own combinations of bone grafting materials. The two major groups of synthetic ceramics that have clinical application are the calcium sulfate materials and the calcium phosphate materials. These compounds have been favored because they elicit very little immunologic reaction in adjacent tissues and have negligible systemic toxicity.

Calcium Sulfate

Calcium sulfate (plaster of paris) has been used as a synthetic graft material for well over 100 years, with one of the first reports observing its ability to completely heal

six of nine cavitary lesions [**90**]. Peltier [**91,92**] described his experiences of 26 patients with unicameral bone cysts with curettage and packing with calcium sulfate pellets. In this group, 24 patients went on to heal uneventfully, with 2 patients requiring subsequent surgery for recurrence. Coetzee reported on 110 patients treated for cranial osseous defect filled with calcium sulfate. He concluded that the material was an excellent bone graft substitute and allowed for bone formation and healing similar to ABG [**93**]. All of these earlier studies come with the caveat that they were not surgical-grade calcium sulfate and most likely varied in structure and properties. Calcium sulfate is known to have relatively rapid resorption in as little as 6 weeks after implantation and has no weight-bearing characteristics. It has shown promise as a carrier for antibiotics, DBM powder, BMPs, or any other small molecule that is difficult to deliver to a defect site.

The first surgical-grade calcium sulfate cleared by 510(k) clearance in the United States was OSTEOSET® (Wright Medical Technology, Arlington, TN). Today, there are a total of nine surgical-grade calcium sulfates available under FDA approval. Unfortunately for clinicians, when materials are 510(k) cleared, they do not go through the same types of rigorous IDE trials seen for Class III devices such as BMPs. Therefore, few data for efficacy in animal models or clinical trials are available. However, OSTEOSET has been part of two prospective trials. To our knowledge, none of the remaining eight materials have been prospectively evaluated. In the first trial, Kelly et al. reported on the treatment of 109 patients with up to 1 year of follow-up [**94**]. In a nonrandomized, prospective, multicenter study, calcium sulfate pellets were used in patients with various bone defects. The calcium sulfate was used alone or mixed with BMA, DBM, or ABG. At 6 months postoperatively, radiographic results showed 88 % of the patients exhibited new bone formation and almost all patients showed complete resorption of the calcium sulfate. They concluded that OSTEOSET was an efficacious bone graft material. However, it should be noted that 71 of 109 patients had OSTEOSET mixed with other products.

A different prospective randomized trial was conducted to investigate the effects of OSTEOSET pellets in critical-size defects created after standard anterior cruciate ligament reconstruction in 20 patients. Patients were block randomized either to no treatment ($n = 10$) or filled with the calcium sulfate pellets ($n = 10$). Patients were postoperatively evaluated by computed tomography to 6 months. The authors reported that they found the same amount of bone in the OSTEOSET as the untreated group, concluding that the OSTEOSET had no effect [**95**].

Calcium Phosphate

The general term calcium phosphate materials can be further broken down into three subgroups: tricalcium phosphates, calcium phosphate cements, and coralline-based HA. Each subgroup will be discussed separately because they have completely different chemistry and react differently in the body.

Tricalcium Phosphates

Tricalcium phosphates (TCPs) are less crystalline than HA and therefore more soluble. There are basically two forms of TCP: α-TCP and β-TCP. α-TCP is more soluble hence more degradable than β-TCP [**96**].

β-TCP

β-TCP is a widely available, FDA-cleared material. There are over six manufactures and various forms available in the United States. Unlike calcium sulfates that chemically dissolve within a few weeks of implantation, β-TCP is resorbed by osteoclastic activity [**97**]. This fundamental difference is very important. Calcium sulfates will leave regardless of bone formation. In comparison, β-TCP functions similar to ABG by causing osteoclastic resorption and hence signaling for osteoblastic activity. Therefore, the β-TCP will not go away until new bone is formed. Intuitively, the rate at which these materials can be resorbed is linked to the material properties. Because these materials are only osteoconductive, the necessity of osteoinduction by mesenchymal stem cells along the osteoblastic lineage is partially dependent on the microenvironment established by the scaffold chemistry and interactions with the host. β-TCP with higher porosity and larger pore size ranges will allow for greater cell infiltration and faster resorption. These two material characteristics are major differences in commercially available products.

Similar to calcium sulfates, β-TCP was cleared through the 510(k) pathway, and there is very little published support data for the cleared products. There are currently no prospective, randomized trials published. There are a few retrospective studies published that show efficacy. In posterolateral spine fusion, one β-TCP, Vitoss® (Orthovita, Malvern, PA) was evaluated in 50 patients as an adjunct to ABG. At 5–7 months postoperatively, 32 patients were available for follow-up. Of these patients, 100 % demonstrated good consolidation of their graft material. Their clinical impression was that Vitoss was facilitating bone formation and reducing the need for ABG harvest [**98**]. Linowitz and Peppers retrospectively reviewed seven patients with a 3- to 6-month follow-up who underwent anterior or posterior interbody fusion at twelve levels with an allograft spacer, Vitoss, and venous blood. At follow-up, the investigators reported that solid fusion was achieved in all patients [**99**]. There are numerous preclinical studies of laboratory-derived β-TCP, but these studies are difficult to interpret toward clinical relevance because, as discussed earlier, the material properties greatly dictate the results. There are very few preclinical studies on commercially available β-TCP.

Injectable calcium phosphates

Injectable calcium phosphate bone cements harden in situ but usually have no weight-bearing ability. When cured, they form an apatitic compound similar to bone mineral. They generally do not degrade during the patient's lifetime but are more bioactive than polymethylmethacrylate, a commonly used bone cement. After the powder and solvent have been mixed, the resulting ceramic is a paste-like material that

can be injected or molded into a non-weight–bearing defect with a setting time of approximately 10–30 min depending on formulation.

One of several of these calcium phosphate cements is Norian SRS (Synthes, USA, Paoli, PA), a powder composed of α-TCP, monocalcium phosphate monohydrate, and calcium carbonate combined with a solution of sodium phosphate. Norian SRS has an inherently low crystallinity with a low grain size as compared with HA blocks, which have a relatively high crystallinity and large grain size, making osteoclastic absorption easier. In a prospective, randomized multicenter study, Norian SRS was evaluated for closed reduction and immobilization in the management of distal radial fractures. The patients were either treated with or without Norian SRS for a total of 323 patients. Follow-up evaluations were conducted up to 1 year by radiograph and patient questionnaires (VAS, SF-36). The results showed early significant differences with patients treated with Norian SRS such as superior wrist range of motion, grip strength, digital motion, use of the hand, and social and emotional function as well as less swelling in the patients than in the control group ($P < 0.05$). However, at 1 year, there were no clinical differences. They concluded that fixation of a distal radial fracture with Norian SRS cement may allow for accelerated rehabilitation [**100**].

Another injectable calcium phosphate bone substitute material, called α-BSM (ETEX Corp; Cambridge, Mass), has been experimentally used as a delivery vehicle for antibiotics and growth factors because it solidifies at physiologic temperatures. In animal models from rabbits to nonhuman primates, α-BSM has been studied in combination with rhBMP-2 [**101–103**]. In male Cynomolgus monkeys at different time points up to 14 weeks, a fibular osteotomy model was used to evaluate injectable rhBMP-2 with different carrier formulations (buffer, calcium phosphate paste [α-BSM], hyaluronan gel, hyaluronan paste, and gelatin foam with and without TCP granules) in closed fractures. Of the evaluated combinations, the authors concluded that the α-BSM/rhBMP-2 was the best mixture with earlier healing and more complete restoration of mechanical competence.

Coralline-based HA

Over 20 years ago, the concept of converting coral to a viable bone graft was developed at Pennsylvania State University [**104**]. The process includes directly exchanging the calcium carbonate exoskeleton of the reef-building marine corals to calcium phosphate forming positive replicates [**105**]. To date, there is only one manufacturer of FDA-cleared coralline-based HA.

There are two genera derived from the scleractinian genus that are used for this process. The first, Goniopora, is similar to that of cancellous bone with large pores measuring 500–600 μm (Interpore ProOsteon500, Interpore Cross International; Irvine, CA) and the second, Porites, is similar to that of interstitial cortical bone with a pore diameter of 200-250 μm (Interpore ProOsteon200, Interpore Cross International). Newer, more resorbable versions are available in which only partial conversion of the

calcium carbonate to calcium phosphate occurs and mainly at the surface (Interpore ProOsteon500R and Interpore ProOsteon200R).

Preclinical research has been performed in long bone and spine models. Holmes [**106**] used ProOsteon 200 and ABG in a bilateral, distal radius diaphyseal model in 14 dogs. Implants were retrieved at 3, 6, 12, 24, and 48 months. They authors concluded by histological evaluation that implant specimens demonstrated good union and bone ingrowth at all time intervals. However, they observed slow ingrowth into the graft material and the absence of implant biodegradation. In an anterior cervical fusion model with and without plating in goats, Zdeblick et al. [**107**] studied the healing of multilevel anterior cervical fusion. Their histological findings at 12 weeks of the ProOsteon 500 implants without plating were that 48 % incorporated, 10 % possessed a fibrous gap, 29 % collapsed, and 14 % extruded; with plating, the results improved to 71 % incorporated, 24 % collapsed, and 5 % had a fibrous gap. The torsion testing showed those treated with the ProOsteon 500 implant were less stiff than autograft but equal to allograft. They concluded that ProOsteon 500 for anterior cervical fusion was biocompatible but had significant rates of implant collapse and extrusion issues could be solved by plating.

A clinical, retrospective study evaluated bone defects filled with Pro Osteon 500 after the removal of bone tumors in 71 patients with an average of follow-up time of 2.4 years. They concluded that Pro Osteon 500 showed good radiographic incorporation and was a viable option for this indication [**108**].

Collagen-Based Technologies

Collagen-based materials have been used successfully in many surgical applications. Usually animal derived, species-specific side chains are cleaved to produce a type I collagen bonded in a fibrillar structure. Nevertheless, patients should be tested before surgery to check for a positive antibody titer to bovine collagen. Initiated by its innate qualities of being a conducive material for mineral apposition and easily binding noncollagenous proteins, collagen became a popular platform carrier. However, collagen alone has been proven to be ineffective in healing diaphyseal defects [**109**]. All commercially available collagen-based materials are a mixture of collagen and another material. As discussed previously, both rhBMP products and at least one DBM material are available on collagen carriers. In this section, we will review collagen-based products mixed with ceramics such as TCP and HA.

Collagraft® (Zimmer; Warsaw, Ind/NeuColl; Palo Alto, CA) is a type I bovine-derived, fibrillar collagen and porous calcium phosphate ceramic (65 % HA and 25 % TCP). The composite is osteoconductive. It has been well studied in animal studies and clinical trials. In a multicenter, prospective trial for the treatment of long bone fractures, 267 patients were randomized to be treated with either cancellous iliac crest autografts or Collagraft with autogenous bone marrow. They reported at 6- and 12-month follow-ups that Collagraft appears to function as well as autogenous graft for this indication [**110**].

In a second clinical trial to study long bone fractures, a prospective, randomized, multicenter design was used to assess safety and efficacy. The two treatment groups were the same, and patients were followed for at least 2 years. Two hundred and thirteen patients (249 fractures) were treated. There were no significant differences found in daily living parameters, union rates, and complication rates. Again, the authors concluded that Collagraft was a viable substitute for ABG [**111**].

In spine fusion, Collagraft has had mixed results. No clinical trials have been performed, but there have been two preclinical studies. In a canine spinal fusion model, Muschler et al. [**112**] found the Collagraft material and the Collagraft mixed with ABG to be inferior to ABG in union scores and mechanical testing. In an ovine lumbar spine model with pedicle screw fixation, Collagraft was evaluated with and without marrow against ABG. At 6 months postoperatively, animals were killed and evaluated. Histologically, the Collagraft groups showed good incorporation and more solid fusion masses than ABG. Mechanical results were not statistically different between the groups. The authors supported the use of Collagraft in spinal fusion with pedicle screw fixation [**113**].

Healos® (DePuy Spine, Inc., Raynham, MA) are bovine-derived collagen fibers that are entirely coated with HA. There have been no published clinical studies of Healos. There are two preclinical studies both assessing its use in spinal fusion. The first study was posterolateral spine fusion model in a rabbit. The groups that were evaluated were Healos, Healos with ABM aspirate, Healos with ABM aspirate and heparin, and ABG. Animals were killed and evaluated at 8 weeks postoperatively. Healos alone was not a successful graft material, yielding an 18 % fusion rate. However, both Healos/marrow combinations had 100 % fusion rates in comparison to ABG with a 75 % fusion rate. Histological findings and mechanical testing showed ABG-treated animals to have the most mature fusion masses and highest stiffness followed by the Healos/marrow combinations [**114**]. In a sheep posterolateral spine fusion model with side-to-side comparison, Healos with MP52 (recombinant human growth/differentiation factor-5) was compared with ABG. Their results at 4 and 6 months postoperatively found no radiographic or histological differences between the two treatments [**115**].

CONCLUSION

The goal of this monograph was to evaluate bone graft substitutes in various indications. It is clear that there is not one material that applies to all applications. When making the decision of what to use, follow these simple guidelines. Begin with asking, "Has this material been proven for this indication in peer-reviewed publications?" If the material has been tested in the indication, then to what level in the hierarchy of mammals has it been successful? If it has been clinically evaluated, then remember that there are different classes of data. IDE trial data are the highest, most controlled data followed by prospective trials and lastly retrospective data.

If your choice is a DBM, one must ask additional questions. DBMs are regulated as a device but are only evaluated for safety. Efficacy falls upon the surgeon. It is imperative to understand how the material is screened, cleaned, and virally inactivated; what carriers are mixed; and finally what data exist regarding its end product osteoinductivity.

Lastly, know the indications and contraindications of your choice. Many materials are not stand-alone grafting options and must be combined to be successful. When using recombinant BMPs, know the approved indication and do not assume other applications will be successful because dosing is essential. Also, remember contraindications in some products may not make it a suitable for certain patient populations.

Bone grafting is an essential part of orthopedic surgery and an ever-evolving science. With each new advance, one needs to understand the characteristics of the material. The goal is to one day no longer view ABG as the standard of care but as a classical method of grafting that has become outdated.

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Chapter 3 | Xenograft Use in Orthopedic Surgery

Tamara Pylawka,[1] Ashim Gupta,[1,2] Taj Eubanks,[1]
Benjamin J. Main,[2,3] and Saadiq F. El-Amin III[1,2,4]

INTRODUCTION

In the field of orthopedic surgery, xenotransplantation is defined as any procedure that involves the infusion or implantation into a human of nonhuman animal tissue. This can include fluids, cells, tissues, or organs. Xenotransplants offer an answer to increasing demands for tissue implants. Allograft implants (tissue implants from same species: i.e., human to human) are available at limited quantity with demand currently superseding availability. The U.S. Public Health Service has further defined xenotransplantation as any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (1) cells, tissues, or organs from a nonhuman animal source or (2) human body fluids, cells, tissues, or organs that have had ex vivo contact with live nonhuman animal cells, tissues, or organs. Further clarification by the U.S. Public Health Service defines xenotransplantation products as tissues, cells, or organs [**1–3**].

One area of medicine that is particularly concerned with xenotransplantation is orthopedics. Tissues in the body can wear, break, or tear because of overuse, trauma, or degeneration. Damaged tissue must be repaired, removed, or replaced; however, recent orthopedic research has focused on replacement of injured tissue. The limited availability of allograft options and the uncertainty associated with integration and breakdown of synthetic composite options make xenografts an attractive option for application in orthopedic injuries. Xenografts are readily available, and the xenograft tissue often exhibits similar chemical and mechanical properties to that of human tissue [**4**]. Limited quantities of allograft (human) tissues and unlimited quantities of

[1] Division of Orthopedics and Rehabilitation, Department of Surgery, Southern Illinois University, School of Medicine, Springfield, IL
[2] Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University, School of Medicine, Springfield, IL
[3] University of Illinois at Springfield, Springfield, IL
[4] Department of Electrical and Computer Engineering, Biomedical Engineering Program, Southern Illinois University Carbondale, Carbondale, IL

xenograft (animal) tissues have made xenografts an interesting and potentially favorable alternative to human tissue implant sources [**5**]. Although its availability is plentiful, a concern of using xenograft tissues is the risk of cross-species disease transmission. This transmission can be viral, bacterial, or prion-mediated [**6**].

In this chapter, our goal is to discuss xenograft use in orthopedic surgery, explain the health risks involved in xenotransplantation, and explore current research in xenotransplantation.

XENOGRAFTS ROLE IN ORTHOPEDIC SURGERY

Xenografts can be fluid, cells, tissues, or organs transferred from one species to another. As a biologic scaffold it is composed of mammalian extracellular matrix (laminin, collagen, elastin, and fibronectin) [**7**]. Xenografts used as scaffolds allow for ingrowth and replacement by host tissue while concurrently providing structural support for the tissue deficit it is used to replace. Many products have been cleared for use as surgical mesh in tissue regeneration and have been labeled as devices by the U.S. Food and Drug Administration (FDA) [**8–15**].

Use of xenografts in orthopedics has increased because of need, and many products are commercially available and often stored in the operating room supply room.

Xenograft material that has been cleared by the FDA (**Table 3.1**) has undergone strict assessment for potential disease-causing viruses and microorganisms. Viral inactivation validation assessments of the manufacturing process are part of the FDA's strict review process. Postmarket surveillance consists of medical device adverse event reporting (MDR). This process is outlined by the Center for Devices and Radiological Health (CDRH) of the FDA [**16,17**]. For example, when collagen derived from another animal material is a device component, the FDA's device application for use identifies

TABLE 3.1 Xeno-Based Implants Currently Available for Orthopedic Surgery Applications.

Product (Manufacturer)	Product Composition				Indications
	Porcine	HAp	TCP	Bovine	
Collagraft (Neucoll, Campbell, CA)		•	•	•	Long bone fracture and bony void filler [**8**]
CuffPatch (Arthrotek, Warsaw, IN)	•				Soft-tissue repair reinforcement [**9**]
Bio-Gide (Geistlich Biomaterials, Wolhousen, Switzerland)	•				Autologous chondrocyte transplantation [**10,11**]
Bio-Oss (Geistlich Biomaterials)		•		•	Bone grafting [**10,11**]
Healos (DePuy Spine, Raynham, MA)		•		•	Bone grafting [**12**]
ZCR-Permacol (Zimmer, Warsaw, IN)	•	•			Soft-tissue repair [**13**]
Restore (DePuy, Warsaw, IN)	•				Soft-tissue repair [**14**]

HAp: hydroxyapatite; TCP: tricalcium phosphate.

several factors before component utilization. First, the species and tissue from which the animal material was derived is determined [**17**]. Second, the general health of each animal and how its health was monitored and maintained is investigated. Finally, the manner in which the health of the herd is maintained and monitored is evaluated. If the material is bovine, then further investigation regarding bovine spongiform encephalopathy (BSE) is obtained [**18**].

Products that are a combination of xenograft and synthetic material (polymeric, metallic) components will need to have additional information provided, such as the concentration of these products in the final device and the presence of any component that is potentially toxic, carcinogenic, or immunogenic (i.e., heavy metals, cross-linking reagents, or organic solvents). Furthermore, information must be provided regarding sterilization and methods for inactivating bacteria, yeast, and fungi [**19**].

SAFETY AND DISEASE TRANSMISSION RISK

In addition to the risk of infection that burdens all transplants, xenografts have the added risk of zoonoses. Zoonoses are infectious diseases that may be passed across species lines from animals to humans. These infections have been termed *xenozoonoses*. Xenozooneses are of particular concern because these pathogens may not cause disease in animals but have the potential to cause disease in humans. Potential xenozoonotic infections in orthopedic surgery have been divided into three categories: viral-, bacterial-, and prion-mediated.

Nonhuman to human viral disease transmission is a concern with xenotransplantation. Primates and pigs have retroviruses that have the potential to infect human cells. Porcine xenografts are more relevant to orthopedic surgery because we are using this tissue currently. Porcine endogenous retroviruses (PERV) are the most concerning because they can infect human cells. Retroviruses (i.e., PERV) are difficult to eradicate. Retrospective studies have not found any conclusive evidence of PERV infection. However, one study found that 23 of 160 human patients showed microchimerism [**20**]. Other viral pathogens of concern include swine influenza virus, swine fever virus, and parvovirus. Bacterial infection with xenografts (as in all transplants) is also a risk. For example, one possibility of cross-species transmission of bacterial infections is trichinosis (pig to human), although this has not been clinically documented.

A third xenozoonotic infection, and the most concerning, is prion-mediated infection. Prions occur naturally in nature and are glycoproteins found in the neuronal cell membranes. When normal tissue is exposed to abnormal prions (PrPsc), the conversion of normal prions (PrPc) to abnormal prions (PrPsc) can result. Exposure to prions can occur multiple ways, from ingestion and mutation to iatrogenic induction [**21,22**]. A notable and publicized prion-related disease is BSE [**21**].

A systematic review of prion disease transmission through bone xenografts (bone substitutes) was completed by Dr. Kim in 2011 [**23**]. In this review, the author used electronic databases to search over 1700 titles to better assess the risk of BSE

transmission through bovine bone substitutes. The author determined that no studies were identified regarding effective BSE prion inactivation using current treatment of bone manufacturing. Thus, the risk of BSE prion transmission when using bovine bone graft substitutes is present, but the risk as of 2011 cannot be quantified [**23**].

Furthermore, inconsistent results when looking at different BSE diagnostic tests were not uncommon. Balkema-Buschmann et al. [**24**] further illustrated a discrepancy between BSE infectivity and actual detection of the disease. Detection of BSE prion disease infectivity is currently completed through the use of a marker, PrP [**23–27**]. Balkema-Buschmann et al. ascertained their inconsistency and discrepancy between BSE infectivity and detection of the marker [**24**].

Most xenografts in orthopedic surgery are of porcine or bovine origin. As previously mentioned, the main concerns with these tissues are PERV and BSE. The relative risk of transmission of each is very low. Xenograft tissue is unique in that infectious or pathogenic agents (which may be undetectable or considered non-pathogenic in the animal source) can still be pathogenic in the human host. This can occur via recombination or reassortment of the infectious agents with nonpathogenic host infectious agents, resulting in the formation of new pathogenic agents.

There is a dichotomy of opinion among clinicians regarding the use of xenografts. Some feel that the process of allowing donor animals to be used, obtaining consent, and the requirement of lifelong monitoring of patients presents a significant degree of burden that may outweigh the benefits of xenograft use [**18**]. Furthermore, concerns exist about the lack of international regulation of xenotransplantation. For example, a questionable practice has emerged and been coined "Xenotourism," which has been defined as patient travel to another country for a xenotransplantation, primarily because of its lack of availability in the domestic country. There is currently a lack of international collaboration and coordination of surveillance of potential infections that can, in theory, present a significant public health risk [**28**].

GUIDELINES FOR CLINICAL USE AND INDUSTRY PRODUCTION OF XENOGRAFTS

Few guidelines have been published regarding xenotransplantation, its long-term effects, or its proper use. However, the Center for Biologics Evaluation and Research (CBER, a subdivision of the FDA) publishes the Guidance for Industry, which addresses the use of animal transplant tissue in humans [**29**]. In general, the guidance protocol suggests that animals should not be imported from any country or region where transmissible spongiform encephalopathy exists. Further, microbiologic testing of xenotransplantation products should also be performed. If the tested tissue is found to have positive results, then the FDA should be notified immediately.

Patient informed consent should be obtained by the clinician and should cover the risks of using xenotransplantation devices, including the risk of pathogenic infection.

The clinician is responsible for educating and updating patients on any relevant information that may develop after transplantation and throughout the clinical course.

XENOTRANSPLANTATION RESEARCH

Xenotransplantation research focuses on transplantation of organs, tissues, and cells from an animal source to a human source. Although a significant challenge, researchers continue to investigate the viability of xenografts in various areas of science and medicine.

Recent xenotransplantation research has focused on cell (e.g., pancreatic islets, neurons, or red blood cells), tissue (e.g., epithelial or connective tissues), and organ transplants. A 2012 review by Ekser and colleagues [**30**] suggests that cellular and tissue xenotransplantation present the most promise, whereas organ xenotransplantation does not appear to be a reality in the imminent future.

As for the field of orthopedics, recent clinical porcine/bovine-to-human xenotransplantation research is relatively limited. The current literature exhibits successful and unsuccessful clinical results. An emphasis appears to be placed on tendon augmentation using xenografts to reinforce native soft tissues.

The repair of torn tendons is a challenge to the field of orthopedics because of a high tendency for the repaired tendon to fail. Several xenotransplantation methods have recently attempted to circumvent these issues by reinforcing the repaired tendon with animal tissue. In a 2009 study, Phipatanakul and Petersen [**31**] augmented massive rotator cuff repair using porcine small intestinal submucosa. Eleven patients received tendon repair augmented with a porcine small intestinal submucosa patch, Patients were reevaluated at a mean of 26 months, with results exhibiting 91 % patient satisfaction and partial or total repair in 44 % of patients. Despite high patient satisfaction, the authors do not recommend small intestinal submucosa augmentation of rotator cuff repair because of inflammatory concerns. In a 2013 study, Gupta and colleagues showed that torn tendon(s) of the rotator cuff were effectively repaired using a porcine dermal tissue matrix xenograft [**32**]. This xenograft improved joint flexibility and strength while decreasing pain. At a mean follow-up of 36 months, results of the repairs exhibited no inflammation or tissue rejection and partial or total repair in 21 of 22 shoulders (95 %) [**32**].

In vitro, cadaveric and animal model xenografts also show promise in repairing, replacing, or augmenting the repair of injured tendons, ligaments, cartilage, and bone [**25–27,33**]. Although the results of these types of experiments are encouraging, a comprehensive, long-term, clinical in vivo assessment of a particular xenograft implant is required before an implant can be deemed effective.

CONCLUSION

Because of scarcity and cost, xenograft options appear to be a much more sustainable option for orthopedic issues than allograft options. However, orthopedic-related xenotransplantation has recently been delayed from clinical implementation because

of uncertainties about host immune rejection and cross-species disease transmission. The field of xenotransplantation has progressed in recent years. As tissue sterilization methods improve, xenotransplantation could soon become a reality.

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Chapter 4 | Bone Grafts Based on Demineralized Bone Matrix

Mark Borden[1]

INTRODUCTION

The discovery of the proteins capable of inducing bone formation can be traced back to the work by Marshall Urist in the mid-1960s [**1**]. Urist found that after removing the mineral component of bone and creating demineralized bone matrix (DBM), the residual collagen particles contained entrapped noncollagenous proteins in a cross-linked collagen matrix. Urist discovered that the implantation of DBM particles into a rat muscle pouch resulted in the formation of ectopic bone. To further investigate this response, he subjected DBM to various protein extraction solutions and was able to isolate a group of proteins that were responsible for inducing bone formation in the muscle. These proteins were later named bone morphogenetic proteins (BMPs), and this initial work is often referred to as the "discovery of BMP." In addition to identifying BMP, this work introduced the scientific community to the osteoinductive properties of DBM. Osteoinductivity is the ability of a material to cause stem cells to differentiate into osteoblasts. After Urist's initial work, several studies confirmed the osteoinductive nature of DBM [**2–8**]. The success of DBM in the laboratory eventually translated into its use as a clinical bone graft material. Particulate DBM saw its first use in patients as a bone void filler in dental and periodontal surgery [**9**]. In this application, the DBM powder worked well because of the contained nature of the defect. However, the use of DBM in more open bone graft applications (e.g., fracture repair and spine fusion) was limited because of issues with the delivery and containment of the particulate material.

In the early 1990s, Osteotech, Inc. (currently part of Medtronic) solved this problem with the introduction of Grafton Gel®. This was the first commercially available product that greatly improved DBM handling and opened the door for its widespread use in orthopedic and spine surgery [**10–13**] as well as maxillofacial surgery [**14–17**]. Using glycerol as a carrier, the DBM in Grafton Gel® was effectively delivered to the graft site, thereby improving intraoperative handling and placement. After Grafton

[1] Synergy Biomedical, Collegeville, PA

FIG. 4.1 Unique DBM fibers found in Grafton putty.

FIG. 4.2 Grafton family of DBM products includes Grafton Gel® (upper left), Grafton putty (upper right), Grafton flex (lower left), and Grafton crunch (lower right).

Gel®, Osteotech introduced Grafton putty, which utilized DBM in a unique fiber form (**Fig. 4.1**). This was then followed by a sheet form of the putty (Grafton flex) and a putty form that included demineralized cortical cubes, Grafton crunch (**Fig. 4.2**). Since the introduction of the Grafton product line, various DBM products have been introduced to the market by different manufacturers. Each of these products enlists

a different approach to delivering and containing DBM at the graft site. In addition, there have been many improvements in how DBM is manufactured and processed into various bone graft products. Although the concept of DBM mixed with a carrier is relatively straightforward, there are several factors that can affect the product's osteoinductivity and resulting bone-forming abilities. The focus of this chapter is to discuss the methodology for developing a DBM product while maintaining osteoinductivity, and to review some of the key characteristics of currently available DBM products.

DEVELOPMENT OF A DBM BONE GRAFT

After the introduction of Grafton in the 1990s, additional DBM putties and gels were also introduced. The manufacturers of these types of DBM implants originally considered the products as human tissue that fell under U.S. Food and Drug Administration (FDA) regulation 21, CFR Part 1271, Human Cells, Tissues, and Cellular and Tissue Based Products (HCT/P). Because of this classification, products were not required to be cleared as medical devices through the traditional 510(k) review process. During this time, tissue banks and DBM companies viewed their products as a simple combination of human DBM with an inert carrier. However, as more DBM putties and gels entered the market, FDA began to closely look at the combination of DBM with carriers that had various chemical compositions. In the early 2000s, FDA contacted manufacturers and informed them that DBM products containing nonbiologic carriers were considered combination products (human tissue and medical device). FDA stated that these products would be considered class II medical devices, which required manufacturers to clear their products through the 510(k) process. After the notifications, the manufacturers were allowed to keep their products on the market while 510(k) clearance was obtained. All DBM products on the market were eventually cleared as bone void fillers through the 510(k) process. All new DBM products must currently be cleared for use by the FDA before entering the market.

The original position of the DBM product manufacturers was that the carrier was an inert material that had the sole function of aiding in delivery and containment of the DBM at the implant site. However, the development of a DBM product is more involved than just simply mixing DBM particles with a biocompatible gel. The osteoinductive proteins in DBM are sensitive to various factors and processing conditions that need to be considered during the development of a DBM putty or gel. The list of available biomaterials that can effectively function as a carrier for DBM is reduced by adding a few bone-specific requirements. The development of a DBM bone graft must incorporate specific design criteria related to bone regeneration to maximize the osteoinductive potential of DBM. First and foremost, the carrier must be biocompatible with surrounding soft tissue and bone. However, this does not immediately qualify a material for use as a DBM carrier. Development of a fibrous capsule around a biocompatible carrier in a bone defect can interfere with proper bone healing and must be avoided. For bone grafting applications, the carrier must also be osteocompatible. Osteocompatibility is defined as the ability of a material to provide a suitable

environment for bone regeneration without interfering with the bone healing mechanism. An osteocompatible carrier will allow the DBM to interact with the surrounding tissue and allow for bone formation to occur.

In addition to being compatible with the defect site, the carrier should also be compatible with the DBM. The main purpose of the DBM carrier is to effectively deliver the particles to the graft site and to maintain graft placement. However, this must be accomplished without compromising the biological activity of the DBM powder. The ability of DBM to induce bone formation has been associated with the diffusion of BMPs out of the cross-linked collagen matrix [**18**]. Once released, the BMPs interact with local cells and stimulate bone healing. Work by Landesman and Reddi shows that the presence of host enzymes during the initial inflammatory phase of healing may be responsible for the release of osteoinductive proteins by breaking down the highly cross-linked collagen matrix of DBM [**19**]. Therefore, the exposure of DBM to the local host environment is crucial to the success of a DBM product. To accomplish this task, the carrier must be resorbable within a relatively short period of time (optimally <7 days) or be porous enough to allow the host enzymes to interact with the DBM particles. The use of rigid cements such as calcium sulfate or calcium phosphate is not recommended because the DBM particles would be isolated from the surrounding cells while the cement slowly resorbs over time. Carriers that isolate the DBM during the initial healing response because of slow resorption may prevent the proteins from the DBM particles from interacting with the local cells and may reduce the ability of the graft to regenerate bone.

With the exception of collagen and gelatin sponges embedded with DBM, most carriers are solid gels and pastes. These types of carriers are designed to resorb within the first few days of implantation. Because of their viscosity and fast resorption, these types of carriers do not confer any mechanical strength to the DBM product. This results in DBM putties and gels that have a soft, moldable consistency and do not harden. Although the DBM products lack any significant compressive strength, the most common use is to fill gaps and voids around stabilizing hardware such as fracture plates, spinal rods, and spinal screws. Because the hardware provides the main mechanical support of the implant site, the DBM bone graft does not need to possess significant mechanical properties.

In addition to the in vivo properties of the carrier, the long-term interaction between the DBM and its carrier is important to the success of the graft. From the time of manufacture, DBM products may sit on the shelf for extended periods of time before implantation. Manufacturing shelf-life validations typically assess only the sterility of the packaging over time, not the osteoinductivity of the DBM. It has been shown that freeze-dried DBM is only stable for up to 9 months [**20**]. However, many manufacturers have expiration dates beyond 1 year. In addition, the 9-month time frame applies to dry DBM and not DBM in prolonged contact with a carrier. Therefore, it is important to evaluate the long-term effects of the carrier on the osteoinductivity of the DBM. Potential problems that may occur with extended contact

include (1) extraction of the BMPs from the DBM, (2) a reduction or complete loss of activity due to denaturation of the osteoinductive proteins by the carrier, and (3) a change to the handling properties of the DBM/carrier formulation. In his original work, Urist found that the osteoinductive proteins in DBM could be extracted from the residual collagen matrix using various ionic solutions [**21,22**]. In DBM putties and gels in which the carrier is capable of partially or fully extracting BMPs, prolonged exposure of the DBM to the carrier could potentially lead to inactivation and a reduction in the osteoinductive potential of the DBM. The denaturation of the BMPs could also potentially occur through the absorption or swelling of the DBM by the carrier. As long as the carrier is in direct contact with the DBM particles, there could be a chance of inactivation.

In addition to the loss of osteoinductivity, a long shelf life may also change the physical properties of the putty or gel. Hydrogels and natural carriers such as gelatin, hyaluronic acid, alginate, or collagen can degrade over time. This can change the handling of the DBM product, resulting in a sticky or crumbly bone graft that is difficult to use. Shelf life is also an issue for synthetic carriers, which can be slowly absorbed by the DBM, thereby changing the consistency and handling of the product over time. Although the osteoinductivity may be maintained, if the graft is difficult to handle or does not maintain its placement, then its effectiveness is reduced.

As seen by these issues, the carrier plays an important role in the DBM product. However, there are several additional characteristics that also affect the product's performance and function. A list of optimal DBM product properties is shown in **Table 4.1.** The optimal product should consist of an easy-to-handle, osteoinductive putty or gel that effectively delivers DBM to the graft site. It should be stable on the shelf for extended periods of time, provide excellent graft handling and containment, and be resistant to irrigation at the implant site. The carrier should also be resorbable in a short period of time and be compatible with the DBM so that long-term exposure would not reduce its osteoinductive potential. In addition, the product should be compatible with minimally invasive delivery systems and be capable of being mixed with other graft materials (e.g., autograft, growth factors, or marrow cells).

TABLE 4.1 Characteristics of an Optimal DBM Product.

Maintains osteoinductivity over time	Excellent handling/moldability
Stable handling properties	Excellent graft containment
Quick resorption of the carrier	Resistant to irrigation
Biocompatible and osteocompatible	Mixable with bone marrow/autograft
No mixing/heating required	Compatible with minimally invasive surgery delivery systems
No special storage	History of clinical use (carrier)

CHARACTERISTICS OF COMMERCIALLY AVAILABLE DBM PRODUCTS

After the initial introduction of Grafton Gel® by Osteotech in 1991, various DBM products were introduced. **Table 4.2** shows an example of various DBM products currently on the market and provides details on their composition, method of sterilization, and method for testing osteoinductivity. Although the core formulation for all products is similar (DBM mixed with a carrier), the individual bone-forming properties and characteristics of the products vary. This variability can originate from the donor age, the demineralization process, the sterilization process, and the composition of the DBM product [**18,23–29**]. These factors may influence bone formation alone or in combination, and they are tied to the osteoinductive potential of the DBM and the clinical performance of the product. To develop an optimal DBM product, it is important to identify the key processing parameters that can affect osteoinductivity.

Bone Demineralization

DBM is a useful and effective bone graft material because of the presence of osteoinductive proteins within human bone. However, in the normal bone structure, the proteins are trapped within a mineralized collagen matrix and remain inactive. For the proteins to actively affect bone formation, the mineral component of the bone must be removed through demineralization. This process produces a residual cross-linked collagen particle with exposed osteoinductive proteins. The goal of demineralization is to take recovered bone and gently process the tissue to create DBM without denaturing the proteins and reducing the osteoinductivity. Although each tissue bank may use its own technique for creating DBM, the process is generally the same. DBM is typically produced from ground cortical bone, which is harvested from the long bones of the tissue donor. This includes the tibia, fibula, femur, humerus, ulna, and radius. On the other hand, cancellous bone can also be demineralized to create a porous, spongy bone graft. Once the bone has been harvested, the soft tissue is manually removed, and the bones are processed using various solutions intended to decellularize, defat, disinfect, and clean the bone. The processed bone is then dried, ground to a specific particle size range (typically 125–800 μm), and demineralized in a 0.5-N hydrochloric acid solution. After demineralization, the tissue is neutralized, rinsed in sterile water, and then freeze-dried. In this form, the DBM can either be used as a particulate bone graft material or further processed into various bone graft forms (putties, gels, sheets, etc.).

Although the key processing steps are common amongst tissue banks, the finer details of demineralization can vary and can have a substantial effect on the resultant osteoinductivity of the DBM. The osteoinductive proteins in DBM are susceptible to thermal, physical, and chemical denaturation. During processing, care must be taken to avoid overexposure of the DBM to any potentially denaturing conditions. Processing conditions that can negatively affect DBM include long-term storage of the recently

TABLE 4.2 Examples of Various Commercially Available DBM Products.

Company	Product	Particulate	Carrier	Sterilization	Osteoinductivity Assay
Biomet (formerly distributed by Interpore Cross)	InterGro	DBM particles	Lecithin	Aseptic production	In vitro alkaline phosphatase
	InterGro Plus	DBM particle and Pro Osteon 500R	Lecithin	Aseptic production	In vitro alkaline phosphatase
Exactech	Optecure	DBM particles	Hydrogel polymer	Aseptic production	In vivo athymic mouse
	Optecure and CCC	DBM particles and cancellous chips	Hydrogel polymer	Aseptic production	In vivo athymic mouse
Integra LifeSciences (formerly distributed by Isotis/GenSci)	Accell Evo 3	DBM particles	DBM-based gelatin and poloxamer 407	Electron beam radiation	In vitro alkaline phosphatase
	Connexus	DBM particles	DBM-based gelatin and poloxamer 407	Electron beam radiation	In vitro alkaline phosphatase
	TBM	DBM particles	Freeze-dried DBM-based gelatin	Electron beam radiation	In vitro alkaline phosphatase
	DynaGraft II	DBM particles	Poloxamer 407	Electron beam radiation	In vitro alkaline phosphatase
	Orthoblast II	DBM particles and cancellous chips	Poloxamer 407	Electron beam radiation	In vitro alkaline phosphatase
Medtronic (formerly distributed by Osteotech)	Graton Gel® and putty	DBM particles or fibers	Glycerol	Aseptic production	In vivo athymic rat
	Grafton crunch	DBM fibers and cortical cubes	Glycerol	Aseptic production	In vivo athymic rat
	Progenix putty	DBM particles	Bovine collagen and alginate	Aseptic production	In vivo athymic rat
	Progenix Plus	DBM and cortical chips	Bovine collagen and alginate	Aseptic production	In vivo athymic rat

(Continued)

TABLE 4.2 (Continued)

Company	Product	Particulate	Carrier	Sterilization	Osteoinductivity Assay
RTI Surgical	BioReady DBM putty	DBM	DBM-based gelatin	Gamma radiation	In vivo athymic rat
	BioSet paste	DBM	Porcine gelatin	Gamma radiation	In vivo athymic rat
	BioSet IC paste	DBM and cancellous chips	Porcine gelatin	Gamma radiation	In vivo athymic rat
	BioSet RT	DBM	Freeze-dried porcine gelatin	Gamma radiation	In vivo athymic rat
Synthes	DBX paste and putty	DBM particles	Hyaluronic acid	Aseptic production	In vivo athymic rat assay or in vitro alkaline phosphatase or both
	DBX mix	DBM particles and cortico-cancellous chips	Hyaluronic acid	Aseptic production	In vivo athymic rat assay or in vitro alkaline phosphatase or both
	DBX strip	DBM particles	Hyaluronic acid/gelatin	Aseptic production	In vivo or in vitro alkaline phosphatase or both
Wright Medical	AlloMatrix Custom	DBM particles and cancellous chips	Calcium sulfate and carboxy-methylcellulose	Electron beam radiation	In vitro proliferation

harvested tissue at room temperature, grinding to particles sizes less than 250 μm, excessive heat generation during grinding, and excessive exposure to damaging solvents and solutions [**26,30,31**]. In addition, the hydrochloric acid demineralization step can also directly affect the osteoinductivity of the DBM. During demineralization, exposure of the bone mineral to acid causes it to solubilize, allowing the mineral to diffuse out of the bone particle. This process occurs on the surface of the particle and works toward the center until the particle is fully demineralized. However, if the particle is exposed to the acidic environment for long periods of time, the proteins can denature. On the contrary, if the demineralization cycle is shortened, then the mineral is not effectively removed and the osteoinductive proteins remain trapped in the mineral matrix.

The goal of DBM processing is to fully demineralize the tissue without overexposing the DBM to the acid. This can be done by monitoring the residual calcium levels of the DBM as a marker for the degree of demineralization. Zhang, Powers, and Wolfinbarger showed the effect of various residual calcium levels on the osteoinductivity of the DBM [**26**]. It was found that a 2 % residual calcium level provided the best osteoinductivity. Wolfinbarger et al. concluded that the presence of residual calcium served as a nucleus for new bone mineralization. In addition, demineralizing bone to the 2 % residual calcium level resulted in a processing time that minimized damage to the osteoinductive proteins. On the basis of this work, tissue banks modified their processes to monitor demineralization so that a 2 % residual calcium level could be achieved. This was done by linking residual calcium to the pH of the demineralization solution. As the hydrochloric acid reacts with the bone mineral, it is partially neutralized and the pH increases. This continues until the bone is fully demineralized, at which point the pH stabilizes. To link pH to residual calcium, the pH of the demineralization solution is monitored and bone samples are removed at specific time points. The samples are then tested for residual calcium and matched to their respective pH measurements. A plot of pH versus residual calcium is then created, and the pH equivalent to 2 % residual calcium is determined. Because the rise in pH is dictated by the ratio of bone to acid, the density and particle size of the bone, and other factors, a standard curve of pH and residual calcium must be specifically generated for each tissue bank's process. This method gives tissue banks the ability to precisely control the residual calcium level in their DBM and eliminate acid overexposure.

Although pH monitoring does provide better control over the demineralization process, it does not affect the time the DBM spends in the acidic demineralization solution. Once the bone mineral is removed from a DBM particle, the acid begins attacking the newly exposed osteoinductive proteins. This is particularly an issue for smaller DBM particles that demineralize at a faster rate than larger particles. Because tissues banks want to maximize the yield of DBM from a single donor, a relatively large particle size range is used (typically 125–850 μm). During demineralization, the proteins in the smaller particles are more susceptible to acid denaturation. However, one tissue bank, LifeNet, has developed a unique demineralization process

that significantly reduces the exposure time of the tissue to the acid solution [**32**]. In LifeNet's pulsatile acidification wave demineralization (PAD) process, the bone/acid mixture is stirred at a high speed (1350 r/min) throughout the demineralization process. This agitation increases acid flow around the particles and increases the demineralization rate. In addition, the acid is completely drained and replaced with fresh acid at select intervals. These exchanges counter the increase in pH, which typically diminishes the ability of the acid to demineralize the bone. The combination of rigorous stirring and acid exchanges allows the bone to be demineralized up to ten times faster than normal demineralization techniques. In addition, the process also incorporates Wolfinbarger's optimal 2 % residual calcium level by measuring pH throughout the process. This combination of processing and residual calcium control results in DBM with a high osteoinductivity.

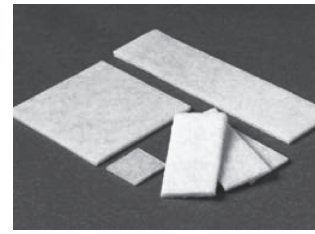
Carriers

The role of a DBM carrier is to provide particulate DBM in a form that improves intraoperative handling, graft delivery, and graft placement. The common forms of DBM products are shown in **Fig. 4.3.** DBM putties are the most commonly used form and typically consist of DBM particles (25–40 % wet weight) mixed with an aqueous carrier (60–75 % wet weight). Putties can also include compositions with additional cancellous or cortical bone chips mixed in ("crunch" and "plus" forms). DBM sheets are formed by altering the DBM/carrier ratio, adding a flexible component to the carrier, or freeze drying a DBM putty with an aqueous carrier. DBM is also provided in a gel or paste form that allows the DBM product to be extruded from a syringe. DBM gels and pastes typically have the same components of a putty product but with an increased carrier content to allow extrusion.

The various DBM product forms are achieved by combining particulate DBM with a suitable carrier. The carrier allows the DBM particles to be easily delivered to the surgical site by manual or syringe placement. The carrier also aids in containing the DBM particles at the graft site and allowing the products to be combined with autograft or other graft materials. In orthopedic and spine surgery, putty handling is a key feature of the product and is linked to the choice of carrier. Surgeons want a doughy, moldable putty that easily conforms to the irregular defects they are trying to fill. In addition, because most putties are manually placed into the body, the putty must not be sticky, crumbly, or fall apart during placement. The putties must also resist dissolution during exposure to irrigation fluids or blood from the exposed bone. Examples of irrigation and blood-resistant putties are shown in **Fig. 4.4.** In addition, the carrier must also have a relatively short resorption time (optimally within 5–7 days) and must not interfere with the bone healing process.

In the early phase of the DBM market, the initial putty products used synthetic-based carriers such as glycerol and an aqueous gel that is based on the copolymer Poloxamer 407. These carriers were inert materials that provided an effective means for delivering DBM to the surgical site. Over time, device companies started

FIG. 4.3 Common forms of DBM products.

DBM Product Form Examples	Photograph
DBM putty (InterGro® putty, Biomet/Interpore Cross)	
DBM putty with cancellous chips (AlloMatrix® Custom, Wright Medical)	
DBM sheet (Grafton® flex, Medtronic/Osteotech)	
DBM gel (Grafton Gel®, Medtronic/Osteotech)	

looking at carriers that could perform these same functions, but they could also contribute to the bone healing process. Han and Nimni showed that phosphotidylcholine (lecithin) was not only an effective DBM carrier, but it also enhanced osteoinductivity by restoring lipids to the DBM [**33**]. Gertzman et al. combined DBM with hyaluronic

FIG. 4.4 Examples of a DBM putties with carriers that are resistant to irrigation fluid and blood. Top: AlloMatrix Putty, Wright Medical. Bottom: InterGro putty, Biomet/Interpore Cross.

acid to take advantage of hyaluronic acid's positive effect on vascularization and bone healing [**34–36**].

In addition to using carriers that can aid in the bone healing process, new techniques were also developed to create DBM carriers directly from human bone tissue. Initially developed by Borden et al., these carriers are based on processing DBM or cortical bone into human gelatin [**37,38**]. A completely bone-derived DBM product can be formed by mixing the gelatin carrier with DBM from the same donor. Because the carrier and DBM are derived from the same tissue source, there is no concern that the carrier will have a negative effect on the DBM. It is important to note that the same donor must be used for the carrier and the DBM component. Federal regulations prevent mixing donors in one product. In one method, the carrier is derived from the DBM itself. The lot of DBM powder is initially split into a carrier portion and a DBM

portion. After separation, the carrier DBM is treated with a weak acid (e.g., citric acid) to gently break down the collagen within the DBM particles and to extract the osteoinductive proteins. This is done in such a manner that the osteoinductive proteins are not denatured while the gelatin is being generated. Once the process is complete, the solution is neutralized and then freeze-dried to isolate the DBM gelatin/osteoinductive protein mixture. The result is a DBM-derived gelatin that retains its osteoinductivity. This gelatin is then recombined with water to form a gel carrier (typically 8 % concentration) and then mixed with DBM to form a putty or gel product. This process creates a DBM product entirely derived from bone with a carrier that has osteoinductive properties.

Tissue Cleaning and Sterilization

During the processing of DBM, it is vitally important to ensure that the end product is free from viral and biological contamination while still maintaining the product's osteoinductivity. To minimize the potential transmission of disease, all tissue banks use a rigid donor screening processes to preselect donors. This involves questionnaires and interviews with the donor's family, a review of autopsy findings, a physical exam of the donor, and other methods to assess the donor's tissue. Although these procedures are designed to weed out any donors with adverse medical backgrounds, the process is not 100 % accurate, and secondary levels of decontamination and sterilization are required. This includes tissue cleaning and disinfection, acid demineralization, and terminal sterilization.

In the cleaning and disinfection process, the recently harvested bone is manually cleaned of all soft tissue. The bone is then subjected to various solutions to completely remove all lipids, blood, and cellular material. The solution cleaning also aids in reducing any viral or microbiological contamination. In general, detergents and surfactants are used in the process to aid in removing lipids and to break down residual cellular material. Alcohol and antibiotics are used to aid in tissue disinfection whereas peroxides are used to oxidize colored elements of the bone and give it a white appearance. In addition, solvents may be used to aid in the dehydration of the tissue. In general, the type of solutions used at various tissue banks is similar. However, the main challenge of a solution-based cleaning process is getting the solutions to fully penetrate through the entire tissue. Because of this issue, several tissue banks have developed patented methods to improve the cleaning of bone used for DBM. A summary of the common cleaning and disinfecting methods is shown in **Table 4.3**. These techniques are based on improving cleaning by increasing fluid flow through the tissue. This is done through the use of sonication (ultrasound) to vibrate the tissue and the cleaning solutions and the use of vacuum/pressurization cycles to pull the solutions into the tissue. These techniques are designed to ensure complete cleaning throughout the bone.

Once the bone is fully cleaned, it is then subjected to a hydrochloric acid demineralization process. Because of the caustic nature of hydrochloric acid, the solution

TABLE 4.3 Common Cortical Bone Cleaning Processes.

Process Name	Developed by	Solutions Used	Tissue Penetration Techniques	U.S. Patents
AlloTrue™	AlloSource	Antibiotics, alcohol, peroxide	Sonication, tissue and cleaning solution stirring	7,658,888 7,776,291 7,794,653 7,919,043 8,303,898 8,486,344
AlloWash®	LifeNet	Detergents, surfactants, antibiotics, alcohol, peroxide	Sonication, vacuum penetration, dynamic fluid flow of cleaning solutions	5,556,376 5,797,875 5,820,581 5,976,101 5,977,034 6,024,735
BioCleanse®	RTI Biologics	Detergents, surfactants, antibiotics, peroxide, water rinses	Sonication, cyclic pressurization/vacuum	6,482,584 6,652,818

not only solubilizes the bone mineral to create DBM, but it also inactivates any viral and microbial contamination. Studies have shown that the 0.5-N hydrochloric acid solution used to demineralize bone is capable of a 10^6 viral load reduction [**39,40**]. Because of the variability in demineralization processes, most tissue banks conduct viral inactivation validations on their own process. Based on the method of Scarborough, White, Hughes, Manrique, and Poser, these validations involve spiking the cortical bone powder with a known amount of virus, subjecting the tissue to the full demineralization process, and then measuring residual viral levels [**39**]. In Scarborough and colleagues' study, the data showed that demineralization was effective in a greater than 10^6 reduction for various viruses, including HIV and hepatitis A, B, and C.

In addition to tissue cleaning, disinfection, and demineralization, termination sterilization is often used to add an additional level of sterility assurance to DBM and DBM products. Of the available sterilization methods, radiation sterilization is primarily used because of its ability to penetrate through the entire material. Radiation sterilization is conducted by either using a gamma or electron beam (e-beam) radiation source. Although gamma sterilization has a better ability to penetrate a material, it requires longer radiation exposure times (4 kGy/h) and can generate heat within the material [**41**]. An electron beam provides a significantly faster processing time (20 kGy/s), but it can also generate heat and requires product units to be placed in a low-density arrangement because of penetration limits of the e-beam radiation [**42**]. Applied to DBM product manufacturing, radiation sterilization can be used to presterilize the DBM or to sterilize the finished DBM product. For DBM products

produced by aseptic processing, all components (including the DBM powder) used to create the finished product must be sterile. Therefore, the DBM raw material is sterilized in a freeze-dried form before aseptic compounding into a putty or gel. If aseptic manufacturing is not used, then the DBM putty or gel is sterilized at the end of the manufacturing process in its finished packaged form. In this process, the DBM powder is not presterilized before mixing with the carrier and does not receive a double radiation dose.

Although the use of radiation sterilization can provide additional sterility assurance, the sterilization method and radiation dose can have a negative effect of the osteoinductivity of DBM. Gamma radiation has particularly been shown in several studies to have a deleterious effect on DBM powders and DBM putties [**27,43,44**]. Buring and Urist initially showed that the use of gamma radiation caused a reduction the osteoinductivity of DBM [**27**]. Han, Yang, and Nimni showed a similar result with DBM powder and DBM putties [**43**]. They found that higher gamma doses resulted in larger decreases in osteoinductivity. In addition, they found that the gamma sterilization of a DBM putty with an aqueous carrier significantly increased the drop in osteoinductivity. This was confirmed by Connor and colleagues, who showed that the presence of water in gamma and e-beam sterilized samples amplified the initial decrease in osteoinductivity and led to a faster loss of osteoinductivity during long-term storage [**44,45**]. In these studies, the negative effect of sterilizing DBM in the presence of water was attributed to the formation of free radicals from the water component. The free radicals increased the initial denaturation of the osteoinductive proteins and continued to negatively affect the proteins over time.

Although radiation can reduce the osteoinductivity of DBM, there are methods to counter these effects. One approach involves using low temperatures during radiation sterilization to avoid heat generation within the DBM. Dziedzic-Goclawska, Ostrowski, Stachowicz, Michalik, and Grzesik showed that maintaining the DBM at a low temperature (–72°C) during gamma sterilization preserved the osteoinductivity of the DBM [**46**]. Wientroub and Reddi showed a similar result with e-beam radiation [**47**]. However, Gertzman, Sunwoo, Raushi, and Dun found that if the low-temperature gamma sterilization method was applied to a DBM product with an aqueous carrier, the presence of water negated the benefit of the low-temperature process. Their results showed that the osteoinductivity of the DBM still decreased (53 %) [**48**]. This result emphasizes the importance of not using gamma radiation on DBM products that contain water.

Another approach to minimizing the decrease in osteoinductivity has been the use of low-dose radiation (<25 kGy). This is based on the data showing that higher doses result in greater decreases to osteoinductivity. To use low-dose radiation, the biological contamination of the DBM or DBM product that is generated during manufacturing must be minimized. In DBM products and other medical devices, radiation doses are set and validated according to International Standardization Organization (ISO) 11137 ("Sterilization of Healthcare Products—Radiation—Part 2: Establishing

the Sterilization Dose") [**49**]. This procedure provides various methods for validating the sterilization dose on the basis of the bioburden level and targeted radiation dose. For DBM products, the goal is to achieve a radiation dose in the range of 15–25 kGy. According to ISO 11137, this can be done by following a Method 1 sterilization validation. In this validation, three production lots are sampled for bioburden and sterility testing. For the bioburden testing, eleven nonsterile product samples are obtained from each lot. This provides three samples for recovery efficiency testing (which determines if a correction factor needs to be applied to the bioburden results). The remaining 30 samples are used for determining the average bioburden level (based on bacterial and fungal counts). Once the average bioburden level is calculated, this is used to determine a verification dose. This is a radiation dose that will reduce the bioburden to a sterility assurance level (SAL) of 10^{-2} (contamination in 1 of 100 units). One hundred product samples are then radiated at the verification dose and tested for sterility. Three additional samples are also tested for bacteriostasis and fungistasis (B&F testing) to ensure that the product does not interfere with the sterility test. If the product passes the sterility test, then the full SAL 10^{-6} sterilization dose is determined by ISO 11137. This dose is typically two to four times higher than the verification dose and results in a chance of contamination of 1 in 1,000,000. Using this method, DBM product manufacturers can establish a low radiation dose that will terminally sterilize the DBM product while minimizing the damage to the osteoinductivity.

Osteoinductivity Testing

As seen throughout this chapter, DBM is sensitive to many factors that can significantly affect the osteoinductivity of the end product. Variables such as donor age [**24,25,50**], method of demineralization [**26**], and method of sterilization [**27,43–45**] all have been shown to affect the osteoinductivity of DBM. In addition, short- and long-term carrier interactions and DBM product composition also contribute to this issue. Because the osteoinductivity of DBM is the main characteristic that makes DBM an effective bone graft, it is important to have an accurate and reproducible assay that can measure the lot-to-lot osteoinductivity of the final DBM product.

Historically, a few methods have been established to measure the osteoinductivity of commercially available DBM products. Initially, the standard model used to assess osteoinductivity was the athymic rodent assay developed by Edwards et al. [**51**]. The use of an athymic animal allows for the implantation of human DBM into a rodent without causing a xenogenic immune response. This model has been shown to be reproducible and sensitive to varying levels of inductivity [**52–55**]. In this assay, small samples of DBM powder or a DBM product are placed in a muscle pouch in athymic rats. The muscle pouch is typically created in the biceps femoris muscle above the posterior femur (limited to two sites per animal) or in the abdominal muscle (up to eight sites per animal, four sites per side). The implant is then excised at 28 or 35 days and assessed for bone formation using a semiquantitative histological analysis developed by

Edwards, Diegmann, and Scarborough [**51**]. In this scoring system, the implant area is scored from 0 to 4 on the basis of the amount of explant area involved in new bone formation (0 = no bone, 1 = 1–25 %, 2 = 26–50 %, 3 = 51–75 %, and 4 = 75–100 %). Additional analytical methods can also be used in the analysis, including a qualitative assessment of mineralization by radiography and a quantitative measurement of in vivo alkaline phosphatase. However, these methods produce complementary data and should not be solely used to establish osteoinductivity. Only histological evaluation can accurately determine if normal ectopic bone formation has occurred. In particular, abnormal healing processes such as dystrophic calcification can give false positives in a radiographic assessment. Overall, the athymic rat assay has been the gold standard of determining DBM osteoinductivity. It is able to directly visualize the presence of DBM-induced bone formation at an ectopic muscular site. It is also robust enough to allow for the testing of the DBM putties and gels that contain a carrier. However, it is a time-consuming assay, with a 28- to 35-day implantation time that requires additional time for histology processing and analysis.

As a faster alternative to the athymic rat assay, in vitro cell culture assays were developed to determine the osteoinductivity of DBM [**56–62**]. In one method, cells from a readily available mouse muscle cell-line (C2C12 cells) were cultured with DBM [**63**]. The osteoinductivity of the DBM was measured by the conversion of the muscle cells (myoblasts) into osteoblasts. This was done by quantifying the expression of the bone-specific marker alkaline phosphatase. Because myoblasts have a low basal level of alkaline phosphatase, increases in alkaline phosphatase expression can indicate conversion into osteoblasts and can be used to predict the osteoinductivity of the DBM. Work by Han, Tang, and Nimni compared the in vitro results with data from the in vivo implantation of the same DBM in the athymic rat assay [**63**]. The results showed that the in vitro alkaline phosphatase levels correlated with the in vivo alkaline phosphatase levels and the semiquantitative histological data. In a separate method, the osteoinductivity of DBM was linked to the proliferation of osteoblast cells. This was based on the concept that the osteoinductive proteins in DBM cause osteoblasts to proliferate during endochondral bone formation. In a study by Adkisson et al., DBM powder was cultured with a human osteosarcoma cell line (SAOS-2) [**64**]. Cell proliferation was measured using a radiolabeled DNA assay (tritiated thymidine). Similar to the Han study, the in vitro results were compared with in vivo athymic rat data from the same lots of DBM. The results indicated a correlation between the in vitro proliferation data (converted to an osteoinductive index) and the percentage of new bone seen in the in vivo histomorphometry analysis.

Although both in vitro assays were correlated with in vivo bone formation, they are primarily used for testing DBM powder. The use of these assays to measure the osteoinductivity of DBM putties and gels is complicated by the presence of the carrier. Depending on the carrier, the dissolution of the carrier in the cell culture system can add interference to the assay and reduce or eliminate the ability of the assay to accurately and repeatedly detect osteoinductivity. For DBM putty and gel testing,

the athymic assay appears to be the preferred choice. However, there has been some controversy on whether the specific assay methods reported in the literature can be universally used for the wide variety of DBM products on the market. It appears that the experimental conditions of the assay must be specifically tailored to the material being tested. For example, studies using the athymic rat assay have shown a large variability in the inductive response of several commercially available DBM putties [**23,65,66**]. The data indicated that some DBM materials were highly osteoinductive whereas others did not show any osteoinductive properties at all. Although the results have shown prominent differences in the osteoinductivity of the different products, variations in the experimental parameters of the athymic model (e.g., implantation time, implant amount, and implant site) can have significant effects on the resulting data. For example, one DBM putty was shown to be highly osteoinductive in one study [**66**] whereas separate studies showed that it was highly toxic with 100 % mortality [**67,68**]. The main difference between these two studies was an increased implant volume. Other unpublished work by Borden has shown that putties implanted for 28 days in the abdominal region of the athymic rat showed little to no induction whereas the same putty implanted for 35 days in the biceps femoris muscle showed excellent induction. This demonstrated that by modifying the experimental parameters of the athymic rat assay, completely different results could be obtained.

To address these issues, ASTM recently released a new standard for DBM osteoinductivity testing in the athymic assay—**F2529-13** "Standard Guide for In Vivo Evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone (DBM)" [**69**]. This standard provides detailed guidance on the implantation of DBM in athymic rodents. This includes recommended implant mass/volumes, implant numbers, and implant locations. The standard also details sample preparation, surgical technique, and techniques for assessing bone formation. More importantly, it comments on establishing and maintaining a validated assay. Although the standard recommends ideal assay parameters, it does provide ranges that allow for the assay to be specifically tailored for each DBM product.

In choosing the appropriate assay for lot-to-lot osteoinductivity testing, it is important for manufacturers to closely evaluate the effect of their DBM product on the assay system. This may require optimization of the assay's experimental conditions to account for product composition. Overall, in vitro assays work well with testing DBM powder. However, this may not be representative of the final DBM product. The athymic assay works well for finished DBM products, but the DBM must be fully manufactured before a determination of osteoinductivity can be made. For failing lots of DBM, this results in unnecessary manufacturing time and cost. The solution may be to perform an incoming in vitro test on the DBM powder to preselect passing DBM lots. After production, the finished DBM product could then be tested in the athymic assay.

CONCLUSION

Since the introduction of the first DBM products in the 1990s, many DBM putties, gels, and sheets have entered the bone graft market. Despite a similar formulation of DBM particles mixed with a resorbable carrier, these products vary widely in their composition, processing, and method of assessing osteoinductivity. As seen in this chapter, development of a DBM product is not a simple exercise of combining DBM with a resorbable carrier. Since Urist's initial discovery of the inductive properties of demineralized bone, there has been continued research that has identified several key factors that can affect the osteoinductive potential of DBM. This includes the effects of tissue processing, demineralization, terminal sterilization, and DBM product composition. The development of a DBM product and its manufacturing process must carefully consider the effect of these factors on the osteoinductivity of the end product. Combined with donor variability, this emphasizes the importance of using a validated osteoinductivity assay as a lot release criterion. Because of the wide variety of DBM product compositions and forms, it is difficult to adopt a specific test method that can be universally used for all DBM products. However, following detailed standards such as ASTM F-2529 can ensure that an assay is appropriately developed and validated. The assay experimental parameters should be optimized based on each product being tested. Assays should be conducted on the finished product and not in-process DBM. In addition, assays should be validated to show sensitivity to varying levels of osteoinductivity and should be linked to a histological determination of bone formation. With the implementation of these guidelines, manufacturers can ensure that they are providing surgeons with a DBM bone graft with a high osteoinductive potential.

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Chapter 5 | Clinical Perspectives on the Use of Allogenic Tissue Substitutes

Steven Gitelis,[1] Brian J. Cole,[1] Joshua D. Harris,[1] Kristen Hussey,[1]
Yale Fillingham,[1] Rachel M. Frank,[1] and Ross Wilkins[2]

INTRODUCTION

Bone and soft tissue human allografts are used extensively to replace or repair damaged tissue. Their use extends beyond bone reconstruction. Cartilage restoration and ligament substitution are common indications. Bone allograft is also processed into bioactive proteins to aid bone repair. Allograft mesenchymal stem cells (MSCs) are now available and used as a bone graft substitute. The following is a review of the clinical perspectives on the use of allogeneic tissue substitutes. Published clinical outcomes studies will be discussed.

Allograft in Tumor Reconstruction

Bone allograft is an attractive alternative for the reconstruction of the skeleton after tumor surgery. There is no donor site morbidity or pain, and they are readily available and cost-effective. There still are some unanswered questions, including graft incorporation, disease transmission, strength, and the most effective means of processing of the allograft. The first use of bone allograft in tumor reconstruction dates back to the late 1800s. Lexer reported on the substitution of a whole or half joint from freshly amputated extremities by free plastic operation in 1908 [**1,2**]. In 1912, Carrel described the preservation of tissues and bone allograft application in surgery [**3**]. During the 1940s and 1950s, the U.S. Navy Bank was established, and it popularized tissue banking. Three surgeons around the world championed the use of bone allografts for tumor reconstruction. They published their experience and include Ottolenghi [**4**] from Argentina in 1972, Parrish [**5**] from M.D. Anderson in Houston, TX, in 1973, and Volkov [**6**] from the Soviet Union in 1976. In general, they reported that one third of their patients had excellent results, one third had good results, and one third failed. This high failure rate was unacceptable and was largely related to technical complications. It was not until the late 1970s that Henry Mankin at Massachusetts General Hospital reported on his extensive use of bone allografts in tumor reconstruction [**7**].

[1] Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL
[2] Colorado Limb Consultants, Denver, CO

He noted that frozen allografts had diminished immunogenicity, they needed to be rigidly fixed, that sizing is critical, and that there is a need to be prepared for complications. Despite the diminished immunity associated with freezing of bone allografts, an immune response is easily detectable. It is a cell-mediated response to surface antigens on the allograft tissue. The most active immune response is CD4 and CD8 cytotoxic T cells. It is known that the more robust the immune response, the poorer the outcome with large bone allografts. It is also known that residual bone marrow is highly immunogenic and for that reason it should be removed. If cartilage is transplanted along with bone, then it is minimally immunogenic because of the antigen isolation. The active antigen is embedded in a proteoglycan matrix, which protects it from the immune response. Most bone allografts for tumor reconstruction are fresh-frozen. Although freezing is advantageous to the bone, it has a negative effect on the articular cartilage. Articular cartilage is largely water, and freezing creates crystals that tend to damage the chondrocytes. Several techniques have been tried to minimize cell death, including immersion in glycerol or dimethylsulfoxide (DMSO) for a period of time before freezing. The cryoprotection achieved with these techniques is quite minimal; thus, one of the major complications of a frozen osteoarticular allograft is cartilage degradation. William Tomford published his research on approaches to articular cartilage preservation, and his work represents a major source of our knowledge [**8–11**].

Bone allografts are currently used clinically in three reconstructive techniques for tumors [**7–31**], including osteoarticular allograft arthroplasty, intercalary reconstruction of long bones, and in allograft prosthetic composite arthroplasty. Although there are still some enthusiasts around the world promoting the use of osteoarticular allografts, many have abandoned this technique for allograft prosthetic composite arthroplasty. The reason is that the articular cartilage degrades over time. One other problem with this technique is joint instability. Even if meticulous ligament reconstruction is performed, the joint remains unstable; thus, there is significant risk of cartilage and joint degradation along with fracture of the graft. Muscolo et al. [**23**] published their outcomes with osteoarticular allografts of the distal femur in 2005. They reported on 75 distal femoral osteoarticular allografts with a minimum follow-up of 7 years. The graft survival at 5 and 10 years was approximately 78 %. The Musculoskeletal Tumor Society score was good at 26 of 30 points. In the series of patients, joint deterioration secondary to anatomical mismatch and joint instability were cited as the primary cause for failure of the osteoarticular allografts of the distal femur. The same group published their outcomes with proximal tibial osteoarticular allografts, which did not perform as well [**25**]. The allograft survival was approximately 65 % at 5 and 10 years, but still with good functional outcomes. Unlike distal femoral osteoarticular allografts, proximal tibial osteoarticular allografts most commonly failed from infection secondary to prolonged wound exposure, dead space created by tumor resection, and insufficient soft tissue coverage. They also reported on hemi-joint osteoarticular allografts for tumor reconstruction in 2007. They reported

on 40 unicondylar osteoarticular allografts with a survival of 85 % at 5 and 10 years, thus making this application for tumor reconstruction the most successful [**22**]. Similar to osteoarticular allografts of the distal femur, unicondylar osteoarticular allografts would fail because of anatomical mismatch and joint instability.

Intercalary allografts represent another application in tumor reconstruction [**10,11,17,21,24,28,31**]. Here, the center of a long bone is transplanted without involvement of the proximal or distal joint. These grafts need to be rigidly fixed, either with a rod, or better yet a plate, to achieve osteosynthesis at both allograft host bone junctions. Locking plates are now frequently used to fix an intercalary allograft. An intercalary allograft can be combined with an onlay vascularized autograft to improve healing and minimize complications. Frisoni from the Rizzoli Institute reviewed 101 patients treated with an intercalary allograft of the femur [**32**]. The mean age was 20 years with a mean follow-up of 9.3 years. The rate of allograft failure was 24 %. They observed several adverse variables, including the use of a rod instead of a plate, chemotherapy, and grafts greater than 17 cm. The Italian group recommended combining a vascularized fibular autograft to optimize outcome. Muscolo et al. [**21**] published their experience with 124 femoral and tibial intercalary allografts. Their patients had a mean follow-up of 6 years. The allograft survival was 82 % at 5 years and 78 % at 10 years. The functional score using the Musculoskeletal Tumor Society system was 27 of 30 points. Farfalli et al. [**33**] from Buenos Aires reported on 26 intercalary allografts after tumor reconstruction of the tibia. Their mean follow-up was 6 years. Their survivorship was 84 % at 5 years and 79 % at 10 years. The most common complications included infection (three patients), fracture (three patients), and nonunion (two patients). Intercalary reconstruction can also be used in children after a transphyseal resection. This is when the sarcoma involves the metaphysis of the long bone near the growth plate. The sarcoma can be resected through the physis, preserving the joint, and an intercalary allograft transplanted. Fixation is somewhat difficult with this type of reconstruction. Only a small wafer of epiphysis remains for the upper fixation. Locking plates are a good choice in fixing an intercalary allograft after a transphyseal resection.

Allograft prosthetic composite arthroplasty is a technique that combines a long bone allograft with metallic implant arthroplasty. The metallic implant is either in the form of a total hip or total knee replacement. It is attached to the allograft to not only restore the bone stock but also to replace the joint. Allograft prosthetic composite arthroplasty does not require maintenance of cartilage viability. The joint is replaced with a metallic and plastic implant. Joint stability is also improved because of the mechanics of the metallic arthroplasty. Donati from the Rizzoli Institute reported on 62 patients treated with allograft prosthetic composite arthroplasty of the upper tibia [**34**]. Their reconstructive survivorship was 74 %. They did have a significant infection rate of 24 % and recommended the common use of a gastrocnemius flap to cover the allograft prosthetic composite arthroplasty.

When allografts are used in tumor reconstruction, complications should be anticipated [12]. This is particularly true when an allograft is used to reconstruct the pelvis. Campanacci from Florence, Italy, reported on 33 pelvic allografts with 33 months of follow-up [35]. There was a 24 % incidence of sciatic nerve palsy, an 18 % incidence of hip dislocation, and a 15 % incidence of infection. Mankin and Hornicek [36] reported on a 30-year experience with allografts for giant cell tumor. They had 144 patients in their series, and their complication rates included allograft fracture, 21 %; nonunion, 8 %; and infection, 8 %. Gebhardt also saw a significant complication rate in his review of 53 patients for high-grade extremity osteosarcoma [17]. His mean follow-up was 25 months. There were 16 infections, 12 nonunions, 6 fractures, and 6 cases of instability. Eighteen of 53 grafts failed. However, most of his complications were salvageable with preservation of the limb.

Overall, allografts are a reasonable alternative for limb reconstruction after tumor surgery. The most common applications currently include osteoarticular allograft, intercalary allograft, and allograft prosthetic composite arthroplasty. Survivorship of the bone transplant remains reasonably good out to 10 years. Complications should be anticipated, such as infection, fracture, and nonunion. Osteoarticular allografts are associated with a significant incidence of joint degradation. To minimize that risk, the allograft can be combined with an implant in which the cartilage is not necessary to restore the joint.

Fresh Osteochondral Allograft for Joint Restoration

Observation of focal chondral pathology in the knee is common during knee arthroscopy [37]. A wide spectrum of chondral disease exists and ranges from superficial articular cartilage injury to large, full-thickness osteochondral lesions. Defects may progress to osteoarthritis on the basis of several patient-, limb-, knee-, and defect-specific factors [38]. The ideal candidate for cartilage restoration surgery is the symptomatic, young or middle-aged, motivated individual with either normal or correctable comorbidities (alignment, meniscal, or ligament deficiency). However, patients that meet these criteria only make up 5 % of those with articular cartilage injury in the knee [39]. The challenge in the identification of symptomatic chondral pathology warrants caution in proceeding with the surgical techniques used to treat them; thus, "treat the patient and not the MRI." The exact mechanism of symptom initiation and progression with isolated chondral lesions is not completely known. Nonetheless, it is recognized that chondral lesions may cause significant pain and limitation of function [40]. In symptomatic patients who have failed conservative treatment, there are several viable surgical treatment options. Although many procedures are simple and inexpensive arthroscopic procedures (e.g., debridement, drilling, microfracture), others require considerable financial and time investments (e.g., cell-based therapies or allograft transplants [osteochondral, meniscal]). Furthermore, comorbidities are addressed simultaneously or sequentially: (1) meniscal repair or transplantation, (2) high tibial valgus-producing osteotomy (for varus) or distal femoral varus-producing

osteotomy (for valgus), (3) tibial tubercle osteotomy (for patellofemoral compartment), and (4) ligament reconstruction as indicated. Therefore, it is the responsibility of the surgeon to understand the advantages and disadvantages of all potential options and educate the patient for the best treatment option for "the here and now." Prophylactic surgery for the incidental lesion is not recommended.

In the setting of symptomatic, large lesions with subchondral bone involvement, treatments such as microfracture, osteochondral autograft, autologous chondrocyte implantation (ACI), and other cell-based therapies are insufficient to address underlying osseous deficiency. Thus, fresh osteochondral graft is advantageous with viable hyaline cartilage and structural subchondral bone transplanted as a single-stage procedure. Grafts traditionally were frozen or cryopreserved (inferior chondrocyte viability, matrix preservation, and clinical outcomes vs. fresh grafts) whereas now they are aseptically processed and stored at 4°C [**41**]. Although chondrocyte viability is decreased beyond 14 days after allograft harvest, this is a necessary step to allow for disease testing [**41**]. Modern tissue banks have created guidelines to ensure the safety of implanted grafts. Most banks recommend transplantation by 28, to a maximum of 35, days postharvest.

The indications for osteochondral allograft transplantation include symptomatic chondral or osteochondral defects of the knee that have failed prior cartilage repair techniques and previously untreated primary chondral or osteochondral lesions greater than 1–2 cm^2 on the femoral condyles, trochlea, or patella. The surgical technique varies based on lesion location. Exposure typically involves medial or lateral parapatellar mini-arthrotomy. Defect preparation involves recipient site sizing, ensuring sufficient surrounding osteochondral walls to support the donor plug. Preoperative sizing images match the recipient and donor sites. Once the recipient site is reamed to a healthy subchondral bone bed (typically between 6 and 9 mm), a surface area- and depth-matched donor plug is press-fit with gentle manual pressure. It is imperative to ensure flush placement of the donor plug because proud or recessed graft placement significantly increases the contact pressure and subsequent degeneration [**42**]. If graft fixation security is in doubt, then a recessed bioabsorbable compression screw (Arthrex, Inc., Naples, FL) may be placed in the center of the graft. High-quality evidence using reliable and validated patient-reported outcomes is currently lacking for cartilage repair in the knee [**43**]. However, new meta-analyses have indicated significant recent improvements in quality [**43**]. For focal and diffuse single compartment chondral or osteochondral lesions, osteochondral allograft predictably and significantly improves patient-reported outcomes and results in high patient satisfaction (**Table 5.1**) [**44**]. At short-, mid-, and long-term follow-up, nearly half (46 %) of patients undergo concomitant or staged osteotomy or meniscal surgery [**44**]. At 5 years follow-up, overall satisfaction approaches 90 %, and 65 % of patients have little or no radiographic osteoarthritis [**44**]. Short-term complications are infrequent (<3 %). Although failures are variably defined (repeat surgery, revision cartilage surgery, osteotomy, or conversion to arthroplasty), they are uncommon (<18 %). Survival rates

TABLE 5.1 Selected Clinical Outcomes after Fresh Osteochondral Allograft Transplantation.

Authors	Year	(*N*)	Age (years)	Defect Size (cm^2)	Defect Location	Follow-Up (years)	Method Preservation (days to implant)	Primary Outcomes
Lyon et al. [**137**]	2013	13	15	5.1	11 FC, 2 PF	2.0	Fresh (14–21)	• Merle d'Aubigne-Postel 12.7 to 16.3 at final follow-up • All patients returned to unrestricted sports at 9-12 months
Giorgini et al. [**138**]	2013	11	34	10.3	7 FC, 4 TP	2.2	Fresh (14–21)	• Subjective IKDC 27 to 59 ($P < 0.001$) • Defect size < 8 cm^2 IKDC improved 38 vs. >8 cm^2 IKDC improved 23 ($P = 0.01$) • One failure (conversion to unicompartmental arthroplasty)
Haudenschild et al. [**139**]	2012	1	48	10.2	Trochlea, FC	3.0	Fresh (12)	• Gene expression, proliferation rate, chondrogenic potential graft/host • No chondrocyte chimerism, shorter doubling times in host • Retained XX host and XY donor (FISH)
Krych et al. [**140**]	2012	43	33	7.3	80 % FC	2.5	Fresh (7–30)	• Preinjury level return to sport in 34 of 43 (79 %)—9.6 months • Age > 25 years ($P = 0.04$) and preoperative duration symptoms ($P = 0.003$) decreased RTS • Improved ($P \leq 0.01$) in IKDC subjective, KOOS ADL, and Marx activity score
Scully et al. [**141**]	2011	18	27	2.2	100 % FC	3.4	Fresh	• One soldier returned to previous military position • Ten soldiers to Medical Evaluation Board for discharge (23 months) • Seven soldiers still active duty but with permanent running/athletic restrictions

(Continued)

TABLE 5.1 (Continued)

Authors	Year	(N)	Age (years)	Defect Size (cm^2)	Defect Location	Follow-Up (years)	Method Preservation (days to implant)	Primary Outcomes
Gortz et al. [**142**]	2010	22	24	10.8	100 % FC	5.5	Fresh (5–21)	• 89 % graft survival rate (avoided reoperation) • Knee Society Score improved ($P = 0.0005$) 60–86 • Merle d'Aubigne-Postel ($P < 0.001$) 11.3–15.8 • IKDC pain 7.1 to 2.0 ($P < 0.001$) and IKDC function 3.5 to 8.3 ($P = 0.002$)
LaPrade et al. [**143**]	2009	23	31	4.8	100 % FC	3	Fresh (15–28)	• IKDC subjective 52 to 68.5 ($P < 0.03$) • Modified Cincinnati score increased 27.3 to 36.5 ($P < 0.01$) • Zero failures
Pascual-Garrido et al. [**144**]	2009	16	34	4.5	100 % FC	4	Fresh	• Tegner 0 to 6 ($P < 0.001$); Lysholm 25 to 37 ($P = 0.015$); IKDC subjective 31 to 45 ($P = 0.004$) • KOOS: Pain 52–74 ($P = 0.002$); Sport 32–46 ($P = 0.037$) • Lower ($P < 0.03$) increase in KOOS Sport and Quality of Life scores vs. ARIF and LBR
Gross et al. [**145**]	2008	35	53	n/a	n/a	21	Fresh	• At retrieval study, long-term graft survival shows viable chondrocytes, functional matrix, complete replacement of graft bone with host bone at 1–25 years after operation

ADL: activities of daily living; ARIF: arthroscopic reduction and internal fixation; FC: femoral condyle; FISH: fluorescent in-situ hybridization; IKDC: International Knee Documentation Committee; KOOS: knee injury and ostoearthritis outcome score; LBR: loose body removal; n/a: not applicable; PF: patellofemoral; RTS: return to sport; TP: tibial plateau.

decline with time: 91–95 % at 5 years [**45,46**], 76–85 % at 10 years [**45,46**], and 74–76 % at 15 years [**45,46**]. Prognostic factors that may negatively influence clinical outcomes include diagnosis of spontaneous osteonecrosis of the knee (SONK), bipolar lesions, age greater than 50 years, patellofemoral lesions, Workers' Compensation status, preoperative duration of symptoms greater than 12 months, and failure to address malalignment or meniscal deficiency [**46–51**].

Patients with osteochondral lesions can frequently present with meniscal pathology. In the past, full-thickness chondral defects were considered to be a contraindication for a meniscal allograft transplant [**52**]. As a result of advancement in the treatment of osteochondral lesions, it is no longer a considerable risk factor for failure of a meniscal allograft transplant [**52**]. In fact, clinical outcomes have demonstrated excellent results in concurrent procedures with osteochondral allograft and mensical allograft transplant [**53**]. The options for mensical allografts include free soft tissue allografts, separate anterior and posterior bone plugs, and bone bridges. In the presence of concomitant procedures, the bone bridge-in-slot has been cited as offering secure bony fixation along with the flexibility for concomitant procedures [**54**]. The most important factor of successful meniscal allograft transplantation when using bone plugs or bridge-type allograft is accurate size matching of the allograft to the native meniscus [**53,55,56**]. Overall, mensical allograft transplantation has offered encouraging results, with good to excellent results in almost 85 % of patients [**54**].

Allograft for Cruciate Ligament Reconstruction

Despite autograft being considered the gold standard in anterior cruciate ligament (ACL) reconstruction, the use of allograft tissue has recently become more widely used in cruciate ligament reconstruction [**57–59**]. Allograft tissue had become unpopular in the 1990s because of concern over the increased risk of viral disease transmission [**57**]. However, one institution between 1986 and 2006 demonstrated a significant increase from 2 % to almost 50 % of the patients using allograft tissue for ACL reconstruction [**59,60**]. Other recent estimates of allograft utilization in ACL reconstruction have been reported between 20 % and 30 % [**61–64**]. When allograft is used for ACL reconstruction, several options exist that include grafts with or without a bone block(s). Allograft options with bone block(s) are the patellar tendon, Achilles tendon, and quadriceps tendon. The available options for soft-tissue-only allograft include the quadriceps tendon and the semitendinosus, tibialis anterior, tibialis posterior, peroneus longus, and iliotibial band. The choice of graft is often tailored to the patient because no study has been able to identify a single allograft option as superior to another in ACL reconstruction [**65**].

The use of allograft tissue for ACL reconstruction offers advantages over autograft tissue that have caused a greater demand for allografts. Commonly cited advantages include decreased donor site morbidity, shorter operative time, decreased rehabilitation period, improved cosmesis, decreased postoperative pain, the ability to easily customize the bone blocks, lower overall cost for primary ACL reconstruction, use in

patients with insufficient or poor quality donor tissue for autograft, and readily available grafts for complex ligamentous injuries [**57–59,61,62,65–71**]. Advantages to the patient for allograft versus autograft were noted in a survey in which 63 % of the patients would have chosen allograft instead of their bone-patella tendon-bone (BTB) autograft despite being satisfied with the overall results [**72,73**].

Most orthopedic surgeons consider allograft tissues safe for use; a survey of American Orthopedic Society for Sports Medicine members cited that 86 % of the respondents stated that they use allograft tissue [**58**]. Despite a strong belief in the safety and efficacy of allograft tissue, the commonly cited disadvantages include disease transmission, immunogenic response of the host toward the graft, slower incorporation, and the possibility for higher failure rates [**57,65,74**]. Allograft incorporation after ACL reconstruction was previously believed to have been completed after 18 months; however, a histological study of allografts retrieved during autopsies at 2 years demonstrated poor central vascularization of the allografts [**57,75,76**]. On the basis of the more recent findings, allograft tissue incorporation is likely further delayed from prior estimates. Regardless of the limited number of documented cases of disease transmission from allograft tissue, the risk of human immunodeficiency virus (HIV), hepatitis C virus (HCV), bacteremia, and septic arthritis must still be considered when using allograft tissue. The transmission of viral disease from properly screened allograft tissue has been cited as approximately 1 in 1.5 million [**57**]. Documented cases of viral transmission have primarily been isolated to case reports [**77,78**]. Bacterial transmission from allograft tissue has been documented in a series of 14 patients with an allograft-associated Clostridium infection during the period of 1998–2002, which resulted in one patient death [**79**]. An investigation of the series of Clostridium infections showed that the same tissue bank processed all 14 allografts [**79**]. Overall, the risk of septic arthritis after ACL reconstruction has been reported to range between 0.2 % and 4.0 % [**80**]. Despite the theoretically higher risk of bacterial transmission with allograft tissue, Greenberg et al. demonstrated no statistical significance in the rate of septic arthritis in ACL reconstruction with allograft and autograft tissue [**81**].

The advent of improved sterilization techniques, which retain the biomechanical properties of the graft, are credited with the repopularization of allograft tissue in ACL reconstruction [**57,82**]. The most commonly used method of allograft harvesting involves an aseptic technique. Processing of allograft tissue for orthopedic procedures has not been standardized, which results in varying processes between tissue banks. The protocol of allograft tissue processing typically involves terminal sterilization with gamma-irradiation, freeze-drying, or chemical disinfection, or combinations thereof. Most tissue banks use a low-dose irradiation with 1–3.5 Mrd, which is only effective in killing bacteria [**83,84**]. The high-dose irradiation required to kill viral contamination is no longer used because of its deleterious effects on the allograft tissue's biomechanical properties [**85,86**]. Chemical disinfection is used as an adjuvant to the process with the attempt to limit the effects on graft integrity and minimizing the risk of disease transmission. Significant differences exist regarding the chemical disinfectant used by

tissue banks because of the proprietary techniques such as Allowash (Lifenet, Virginia Beach, VA), Biocleanse (Regeneration Technologies, Alachua, FL), and Clearant Process (Clearant, Inc., Los Angeles, CA) [**58**].

Because gamma-irradiation has proven to exhibit dose-dependent deleterious effects on allograft tissue, it is important to understand the key variables of gamma-irradiation, including target dose, dose range, and temperature of irradiation. Gamma-irradiation doses are typically reported as a single target dose or dose range. When a single target dose has been reported, it represents the intended minimal irradiation exposure of the tissue. Because the method of irradiation does not allow for all tissue in a given batch to receive the same dose, some tissue will have received a much higher dose of irradiation. On the other hand, the dose range provides a more accurate representation of the irradiation exposure of the allograft tissue in the batch. The temperature during exposure to irradiation affects free radical generation, with lower temperatures working to minimize free radical generation and successive tissue damage [**87–89**]. As a result, low-dose irradiated allograft tissue with a narrow dose range and performed at low temperatures will provide an ideal condition for minimizing the deleterious effects of irradiation on allograft tissue. The dose-dependent effects on allograft tissue integrity have been extensively researched in controlled laboratory studies with the conclusion that irradiation doses below 2.0–2.5 Mrd at low temperatures minimizes the biomechanical effects compared with matched nonirradiated grafts [**89**]. Performing clinical outcome research to compare irradiated and nonirradiated grafts is difficult because of the variable processing between tissue banks and multiple forms of allograft available. The current literature has not provided a consensus on whether or not a clinically measurable difference exists between irradiated and nonirradiated allograft tissue [**89**].

Regardless of any potential measurable difference between allograft and autograft tissue in a controlled laboratory setting, the primary concern is how allograft tissue performs clinically compared with autograft tissue. Providing an accurate evaluation of allograft and autograft tissue in ACL reconstruction is difficult because of the multiple types of graft, the variable processing of allograft tissue, and differences in graft fixation and surgical procedures (e.g., single vs. double bundle). The orthopedic literature has multiple level II–IV evidence studies investigating the clinical outcomes between autograft and allograft tissue in ACL reconstruction, with results ranging from no statistical difference to more favorable outcomes for autograft tissue. However, many of the studies lack randomization because the patients are provided the option to choose the graft. In contrast to most studies, Sun et al. published a prospective randomized study comparing 86 BTB autograft knees and 86 BTB allograft knees with an average follow-up of 5.6 years for both groups [**71**]. The results demonstrated no statistical difference between the allograft and autograft groups with the Lachman test, pivot-shift test, mean laxity with KT-2000 arthrometer testing, and percentage of knees with laxity greater than 3 mm [**71**]. In addition, three meta-analysis studies have been performed to investigate the clinical outcomes

between allograft and autograft tissue [**70,90,91**]. Two earlier meta-analysis studies showed no statistical difference between the groups in regards to the Lachman test, pivot-shift test, and laxity on arthrometer testing [**70,90**]. The most recent meta-analysis demonstrated with statistical significance a mean laxity with arthrometer testing of 1.4 ± 0.2 mm for allograft and 1.8 ± 0.1 mm for autograft ($P < 0.02$) [**91**]. Despite the small difference in measured knee laxity, no statistical difference existed in the percentage of knees with less than 3 mm of laxity between the two groups [**91**]. The literature has not provided a consensus regarding the clinical outcomes between allograft and autograft tissue, but the belief is currently that allografts and autografts are clinically equivalent in ACL reconstruction.

DEMINERALIZED BONE MATRIX

Demineralized bone matrix (DBM) is a material derived from donor bone that is essentially the pure protein of bone. The cellular, fatty, and calcium components of the bone are removed during processing. DBM is used as a conductive and inductive biomaterial to produce bone healing in humans. Within this material are multiple bone growth factors (bone morphogenic proteins [BMPs]). These proteins have been shown to be active and important in bone formation. Therefore, DBM is a biological biodegradable substance that promotes bone formation in the proper environment. DBM can be provided by itself, but it is most often combined with a carrier for improved handling properties. DBM has a long clinical and scientific history, and it is the most commonly used bone-promoting agent in the allograft market, being involved in approximately 20 % of all procedures done per year [**92**].

It was in the 1930s that it was discovered that acid digestion of bone resulted in a material that would induce ectopic osteogenesis when injected into skeletal muscle [**93,94**]. Marshall Urist subsequently published his landmark paper in *Science* [**95**] that demonstrated that demineralized bone would induce osteogenesis when implanted into a nonbony site. It was Dr. Urist who coined the terms BMP and osteoinduction. Subsequent to Dr. Urist's work, Hari Reddi [**96**] characterized the various BMPs that were present in DBM. This work eventually led to the production and commercialization of individual BMPs—BMP-2 and BMP-7. It has been shown through extensive laboratory and clinical research that DBM is osteoconductive and osteoinductive and is effective in bone healing situations in humans [**97–98**].

DBM is acquired through the procurement of human bone tissue through the tissue donation system. This process and the subsequent manufacturing of this Class I medical device is regulated by the U.S. Food and Drug Administration (FDA) and is further overseen by the American Association of Tissue Banks [**99**]. Once the bone is initially cleaned, it is further processed into very small particles of various diameters and then demineralized, freeze-dried, and prepared for application. The various tissue processing facilities have developed detailed and proprietary techniques for preparing these materials. Although DBM can be used by itself, it comes in a dry powder form and is somewhat difficult to handle and introduce into a surgical site. Therefore, most

DBM is combined with a carrier material to produce a product that can be injected or packed into and around a surgical site where bone healing is necessary. There are a myriad of types of carriers, which may or may not affect the activity of the DBM. Examples of these carriers are calcium sulfate, hyaluronic acid, glycerol, and various polymers.

Because DBM is acquired from tissue donors, each individual donor lot may have varying characteristics in regards to its initial biologic activity, processing methods, sterilization technique, and its eventual combination with a carrier substance. Multiple studies have been performed that show varying quantities of BMPs within various lots of DBM, although processing within each facility may be equivalent [**100–102**]. As expected, these biologic differences are difficult to predict and measure [**103–105**]. There have been many efforts to standardize a bioassay for the activity of DBM, but because of these variables, specific protein assays and in vitro tests have been unreliable. The in vivo tests using an athymic rat implant model seem to be the most reliable method of assessing the overall osteoinductive potential of DBM products [**106**]. The commercial providers of DBM products have the option of testing the biologic activities of their materials before release. Some manufacturers test the DBM before sterilization and the addition of carrier materials; other manufacturers test the end product. It seems logical that the second method would give the surgeon the best indication of biologic activity.

DBM has been used in almost all bone healing instances, including dental, craniofacial, neurosurgical, and orthopedic applications [**92,100,101**]. There have been many papers using preclinical animal models that illustrate the bone healing capabilities of DBM [**92**]. There have also been numerous studies exhibiting its effectiveness in general orthopedic and spine grafting situations [**97,100,101,107,108**].

Over 50 % of allograft procedures in the United States involve spine grafts [**109**]. Of these, a high percentage involves the use of DBM product. Although most of the studies reported are case series, there have been several comparative studies. A study by Kang et al. [**107**] compared fusion rates in patients who underwent single-level instrumented posterolateral lumbar fusion with either local autogenous bone and DBM or iliac crest autograft. At 2 years follow-up, the groups demonstrated statistically equivalent computed-tomography-verified fusion rates. In the general orthopedic area, there have been multiple papers published on the effectiveness of DBM for the treatment of unicameral bone cysts [**108**], fractures, and nonunions. Tiedman et al. demonstrated that demineralized bone, with or without autogenous bone marrow aspirate, was effective in bone healing application [**97,107**] comparing BMP-7 to demineralized bone protein in fibular defects. At 1 year, there was no difference in bone mineral density scores between those two products.

Controlled studies and anecdotal reports suggest that DBM is a product that may induce local bone healing and improve outcomes. DBM is osteoconductive; osteoinductive; and, with a carrier compound, is easily used clinically. It can be used to expand

the volume of autograft procedures, such as spine surgery, and it can be used effectively in any area where bone growth is necessary.

HUMAN ADULT STEM CELLS

Over recent years there has been a tremendous amount of interest in using stem cells for the regeneration and repair of injured and missing tissues. Embryonic and adult stem cells have been investigated for their regenerative properties. These studies have exhibited a dramatic potential for tissue repair [**110,111**]. Because of many factors, embryonic stem cell technology has been difficult to access and commercialize because of limited availability and cellular mechanism complexity [**112**].

Adult stem cells are multipotential, undifferentiated cells with proliferative and self-renewal capacity. With the appropriate environment and local growth factor signals, adult stem cells can be directed toward specific cell lineages, including musculoskeletal tissues [**113**]. These adult MSCs have been shown in laboratory and clinical situations to assist in the regeneration of connective tissues [**114–119**].

There are two basic sources for adult MSCs: autologous and allogeneic. Autologous MSCs are generally derived from bone marrow and have been shown to have an effect in particular on bone healing. Multiple studies have demonstrated that MSCs will differentiate into an osteoblast line in vitro with the appropriate growth factors and nutrients [**120,121**]. As these cells mature in the appropriate environment, bone formation occurs.

Various clinical studies have supported these properties. Connolly et al. [**122**] show the effects of autologous marrow-derived MSCs as well as Hernigou et al. [**123**] in healing nonunions. There appeared in the Hernigou study to be a dose response related to the number of MSCs present in the marrow aspirate. Clinical and preclinical studies alike have demonstrated that a higher number of bone marrow cells may enhance fracture repair [**123,124**]. The optimal number or biologic activity of MSCs necessary for bone regeneration has not been elucidated. One of the difficulties in dealing with autologous bone marrow stem cells is their relative paucity within the aspirate or the bone graft material [**125**].

The other source of adult MSCs is from allograft donor tissue. Allograft MSCs have been shown to be nonimmunogenic when applied to local areas [**112,126**]. These cells are isolated from tissue from donors that have been designated for tissue and organ donation. Strict adherence to FDA and American Association of Tissue Bank criteria is mandatory for these donors [**99**]. These are naturally occurring MSCs and have not been cultured and expanded. There are two common sources of allograft MSCs. One source involves the actual in situ cells found in cancellous bone where the non-stem-cell components of the bone marrow have been removed [**127,128**]. The actual number of stem cells present in these materials is not well understood. At least one study suggested that bone marrow contains less than 1000 MSCs per cubic centimetre [**129**].

The other source of adult MSCs is from allograft adipose tissue. It has been shown in vitro and in vivo that adipose-derived MSCs have at least as much potential or perhaps more potential to form along an osteoblastic line than marrow-derived cells [**112,130–133**]. The presumed advantage of using adipose-derived MSCs is that they preferentially bind to demineralized bone and in numbers much higher than that found in naturally occurring cancellous bone [**112,130–132,134,135**].

The ideal materials necessary for bone generation involve an appropriate substrate or scaffold, MSCs that are able to respond and proliferate, and the appropriate growth factor signals to stimulate the differentiation and proliferation of those cells. Currently available adult MSC products are available in two varieties. The first and most prevalent is a cancellous bone material that has been procured from a donor and processed in an attempt to save the MSCs but remove the myeloproliferative cells and bone inhibitor cells. These products generally come in a particulate form and are commonly used in spine fusions, arthrodesis, and problem bone healing situations [**119,128,136**]. The second material comprises DBM upon which adipose-derived MSCs have been added, which biologically bind to the scaffold. Although both of these materials are in common use, there has been no consensus on a method to measure their overall osteogenic activity. It seems important that methods be developed that can accurately measure the numbers of active stem cells and quantitate the growth factors necessary to provide adequate bone formation.

Human-derived stem cells are already being used in clinical medicine to promote bone healing in various situations. Further work is necessary to define and quantitate their actual biologic potential and regenerative properties.

SUMMARY

A wide range of soft tissue and osseous allografts are currently available for clinical use in orthopedic surgery. Allograft tissue has more recently gained popularity because of the abundant supply and lack of donor site morbidity. However, the primary concern regarding allograft tissue has been related to disease transmission and a perception that allograft tissue is not an equivalent substitute for autograft tissue. When using allograft tissue, the goal is to provide a comparable or superior outcome to the use of a synthetic implant or autograft tissue. In the process of deciding on an allograft tissue, the surgeon must take into account many considerations, including the type of operation, patient demographics, patient expectations, and the patient's willingness to use allograft tissue.

Human allograft remains a viable alternative for bone and joint reconstruction. We have been able to demonstrate the successful use of allograft tissue in a broad range of orthopedic applications; however, continued research and development is needed to improve allograft tissue. The focus of future research must include studies with high levels of evidence to confirm the equivalence of allograft and autograft tissue. The primary barrier toward achieving equivalent biomechanical properties of allograft tissue with autograft tissue is centered on the processing of the allograft.

We must work to develop improved methods of tissue processing that limit disease transmission without altering the biomechanical properties of the tissue. In addition, new tissues are being developed that have a significant potential for skeletal repair. Although much is known about human allograft, questions still remain. What are the clinical outcomes and how do they compare to autogenous tissue or manufactured product? How do they work? What is the risk of disease transmission? Does processing affect performance? What are the long-term effects of implantation of these bioactive materials? These are other questions that need to be answered before we have a thorough understanding of human allografts and their use in clinical practice.

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Chapter 6 | Cell-Based Approaches for Bone Regeneration

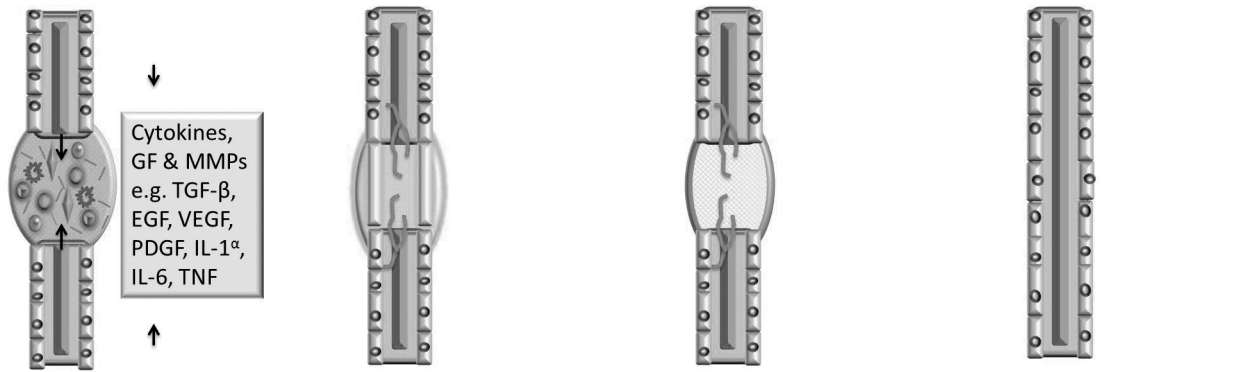
Paiyz E. Mikael[1,2] and Syam P. Nukavarapu[1,2,3,4]

STAGES OF NEW BONE FORMATION

Bone is a dynamic tissue that provides many functions; therefore, it has evolved into a highly specialized natural material [**1**]. Because of its complex structure, the process of bone fracture healing consists of a cascade of intricate events that requires the interplay of many elements, including cells, growth factors, and extracellular matrix. As illustrated in **Fig. 6.1**, these events can be described in four stages: inflammation, soft callus formation, hard callus formation (primary bone), and finally the remodeling into cortical or trabecular bone or both [**2**]. Inflammation is naturally the first step and is triggered by the damaged vasculature because of the distortion of the marrow architecture caused by a fracture. This results in the activation of nonspecific wound healing pathways and the release of many factors that, in turn, attract macrophages, mesenchymal stem cells (MSCs), and other progenitor cells (PCs) to the wound site. The macrophages not only migrate to the affected site to remove any damaged tissue or debris, but they also release factors that invite more cells. The second stage begins with the soft callus formation, which consists of a mesh of clotted blood, fibroblasts, and fractured bone fragments. These components come together to form a temporary scaffold that bridges the gap and is mechanically sufficient to allow for the new vasculature and bone formation. In the meantime, the migrated MSCs and other PCs proliferate and subsequently differentiate into their prospective lineages. Depending on the site of injury, the MSCs in particular differentiate into either osteoblasts or chondrocytes to begin the transmembranous or endochondral ossification processes, respectively; this stage marks the hard callus or primary bone formation [**3**]. The final stage is the bone remodeling process, in which the hard callus transitions into cortical or trabecular bone or both [**2**].

[1] Institute for Regenerative Engineering, University of Connecticut Health Center, Farmington, CT
[2] Materials Science & Engineering, University of Connecticut, Storrs, CT
[3] Biomedical Engineering, University of Connecticut, Storrs, CT
[4] Orthopedic Surgery, University of Connecticut Health Center, Farmington, CT

FIG. 6.1 Stages of fracture repair or bone regeneration.



In the case of large or segmental bone defects, the healing process is impaired; thus, the use of tissue engineering techniques becomes a necessity. To best mimic the natural bone healing process, three major components are required: a mechanically stable graft, a suitable cell source, and chemical and biological factors. Extensive research has been done in all three components mentioned above; however, we are still at the bench stage, in which biomaterials, growth factors, and cell sources are being examined and optimized for the regeneration of bone. In this chapter, the focus will be on the different cell sources available and their potential clinical use.

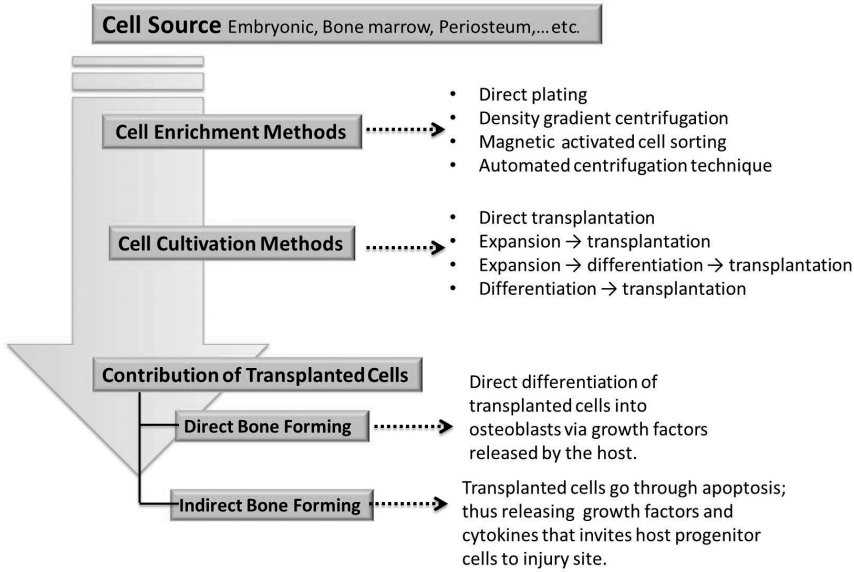
CELL APPROACHES FOR BONE REGENERATION

The body has the natural ability to recruit PCs to affected areas; this mechanism helps in the repair and maintenance of physiological balance. Bone in particular is continuously remodeling through absorption and deposition, which are controlled by two cell types, osteoclasts and osteoblasts, respectively. However, this strategy proves to be insufficient when the damage occurring is greater/faster than the repair mechanism. In such scenarios, cell-based therapy becomes a valuable regenerative approach and is proven to be very effective and extremely critical in the early stages of the bone healing process. In addition to traumatic injuries, cell-based therapy may be used in many skeletal developmental diseases such as osteogenesis imperfecta and osteoporosis.

Cell Sources for Bone Regeneration

Osteoblasts, which are highly specialized bone-forming cells, seem to be the more appropriate choice for bone repair. However, the limited proliferation capability of osteoblasts makes their use extremely difficult for segmental bone regeneration because large quantities are required. Therefore, the need to identify other osteoprogenitor cell sources is very essential. Few sources have been identified and well characterized for the cell-based approach for bone defect repair. Some resources can directly be implanted into the bone defect area, such as MSCs and the periosteum, in this case predifferentiation is not necessary. Others, such as embryonic stem cells (ESCs), cord-blood cells, and induced pluripotent stem cells (iPSCs) must be differentiated prior to implantation into the bone defect area. Each of the mentioned cell sources holds great promises and challenges. The selection of the more appropriate sources will depend on many parameters, such as isolation, expansion, immunogenicity, stability, and bone-forming capability. Regardless of the cell source chosen, there are still questions regarding the mechanisms by which these cells contribute to bone formation. As illustrated in **Fig. 6.2**, transplanted cells can either directly participate in the regeneration process, or participate indirectly through the release of growth factors, which facilitate recruitment of the host cells required for the bone healing process. The following section will briefly describe each cell source and its advantages and disadvantages.

FIG. 6.2 Stem cell or osteoprogenitor cell participation/contribution to the process of bone regeneration.

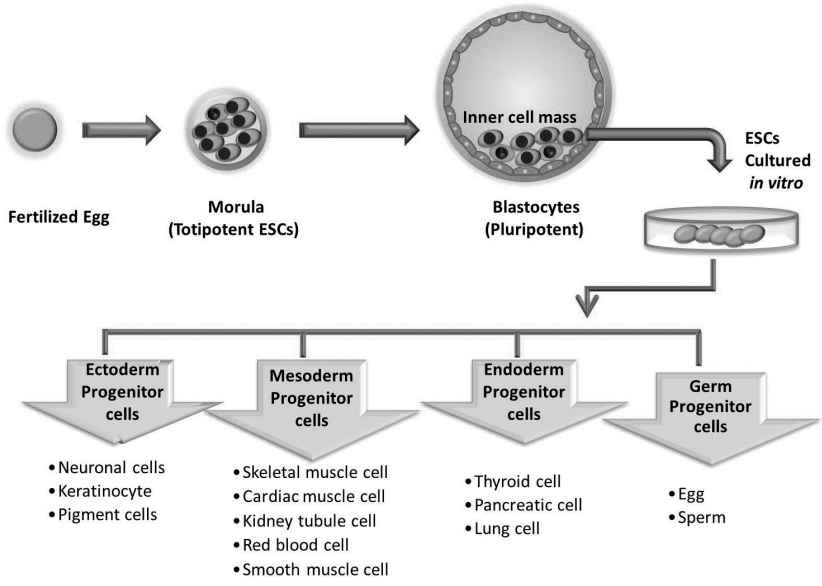


ESCs

ESC cultures were first successfully established in 1981 by researchers from the University of Cambridge, and these cells were obtained from a mouse embryo [**4**]. Later, in 1998, Thompson and colleagues isolated and established five ESC lines (H_1, H_7, H_9, H_{13}, and H_{14}) derived from the human blastocyst [**5**]. By definition, ESCs refer to cells that are derived from the inner mass of blastocyst (an early-stage embryo), as illustrated in **Fig. 6.3.** Several cell surface markers specific to undifferentiated human ESCs (hESCs) have been identified, such as stage specific embryonic antigens 3 and 4 (SSEA3 and SSEA4), which are glycolipids; thymocyte antigen 1 (Thy1) and human leukocyte antigens (HLA), which are glycoproteins; and transcription-associated proteins-1-60 and -81 (TRA-1-60, TRA-1-81), and germ cell tumor marker 2 (GCTM2), which are keratan sulfate proteoglycans [**6–8**].

ESCs are characterized by their unlimited proliferation and ability to differentiate to any somatic cell type, which makes them a great cell source for tissue regeneration. However, there has been a great deal of ethical debate regarding the use of ESCs. For this reason, hESCs are obtained from extra embryos formed by in vitro fertilization methods [**7**]. The capability of these stem cells to rapidly proliferate is due to their unique abbreviated cell cycle, in which the G_1 phase is reduced. The typical doubling time for a somatic cell is 30–36 h whereas that for a stem cell is only 15–16 h. In fact, the self-renewal potential of ESCs is intertwined with their pluripotency: as the ESCs progress in their commitment lineages, the proliferation ability decreases [**9**].

FIG. 6.3 Diagram representing human embryonic stem cell isolation and differentiation.



ESCs have gained much attention in the field of organ regeneration because of their ability to differentiate into many cell types, including osteoblasts, chondrocytes, cardiomyocytes, adipocytes, hepatocytes, endothelial cells, neurons, and hemopoietic cells. The osteogenic differentiation of ESCs can be achieved by directly culturing in media containing ascorbate 2-phosphate, β-glycerophosphate, and dexamethasone [**10**]. ESCs can also be differentiated by forming embryoid bodies, followed by their growth and differentiation in the osteogenic media [**11**].

Most studies have shown the feasibility of using hESCs in bone regeneration by predifferentiating them into osteoblasts or directly implantating undifferentiated hESCs co-cultured with an osteogenic-like cell population. Ahn et al. differentiated hESCs into osteoblasts by allowing them to adhere onto culture plates containing primary bone-derived cells (PBDs). Results show that bone nodule formation was possible 14 days after co-culturing [**12**]. Kuhn et al. studied the potential of using mesenchymal-like cells derived from hESCs (hESC-MCs). These cells were cultured on a collagen/hydroxyapatite (HA) scaffold and implanted into calvarial defects in mice. After only 6 weeks, cells had formed a vascularized new bone that bridged the defect site [**13**].

There are many challenges concerning ESCs that can impair their potential use in regenerative therapy. Because ESCs are isolated from an early-stage embryo, the cell population is very small (typically 100–150 cells/embryo), which requires extended cell

culturing to obtain a sufficient cell population. Although hESCs maintain their normal karyotype through the activation of the G_1/S checkpoint, mutations can still occur. This is largely due to a lack of understanding of the molecular mechanisms underlying the culture adaptation of hESCs [**14,15**]. These conditions include different growth factors, the combination of media, or coated culture plates. The differentiation mechanism of hESCs requires delicate and extremely intricate cascades of events. In fact, it is well established that hESCs must be differentiated to the desired cell type before implantation. However, it is unclear if differentiated ESCs are detected by the immune system postimplantation [**16**]. On the other hand, the direct implantation of undifferentiated hESCs into defects resulted in uncontrollable differentiation and teratoma formation.

MSCs

MSCs offer a great alternative to ESCs in the treatment of skeletal injuries. One of the main differences between ESCs and MSCs is that MSCs are much less committed or more primitive than the ESCs. Therefore, it takes more steps to differentiate an ESC to a specific somatic cell type than it would for MSCs. One other great advantage in using MSCs is their ability to inhibit the immune function of T and B lymphocytes and natural killer cells. Although abundant in the bone marrow, MSCs can be found in amniotic fluid, umbilical cord blood, fat tissue, and many other tissues. MSCs are characterized by their ability to differentiate into multiple lineages when properly stimulated, and they are culture adherent [**17**]. However, MSCs are a heterogeneous population in terms of morphology and surface markers; therefore, a combination of surface markers must be recognized for their identification. STRO-1, named after Simmons and Torok-Storb, is one of the markers recognized in MSCs, but only in a small subpopulation. Other markers include cluster of differentiation (CD): CD29, CD44, CD73, CD90, CD105, CD106, CD146, and CD166. MSCs do not express hemopoietic and endothelial cell surface markers such as CD11, CD14, CD31, CD33, and CD45 [**18–20**]. MSCs have two main inherent functions: (1) their ability to secrete various factors such as cytokines with autocrine and paracrine activities, vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), leukemia inhibitory factor (LIF), granulocyte colony stimulatory factor (G-CSF), interleukins (ILs), and others and (2) the ability to orchestrate the differentiation process with differentiated or undifferentiated residing cells [**21**]. One of the main functions of MSCs is to maintain a constant balance of an activated and quiescent population. MSCs are typically quiescent until activated by the need to repair and maintain tissue; this ability is referred to as "homing" [**22**]. In addition to the aforementioned MSC sources, research has shown the possibility of MSCs residing on blood vessels; these are referred to as "pericytes" [**23**]. When examined in culture, these vascular-associated cells display MSC-like characteristics; likewise, bone MSCs have pericyte characteristics [**24**].

Bone marrow stromal cells (BMSCs) or bone marrow MSCs are a subpopulation of the stromal cells within the bone marrow, as illustrated in **Fig. 6.4.** The complex microenvironment of bone marrow consists of fibroblasts, endothelial cells, adipocytes,

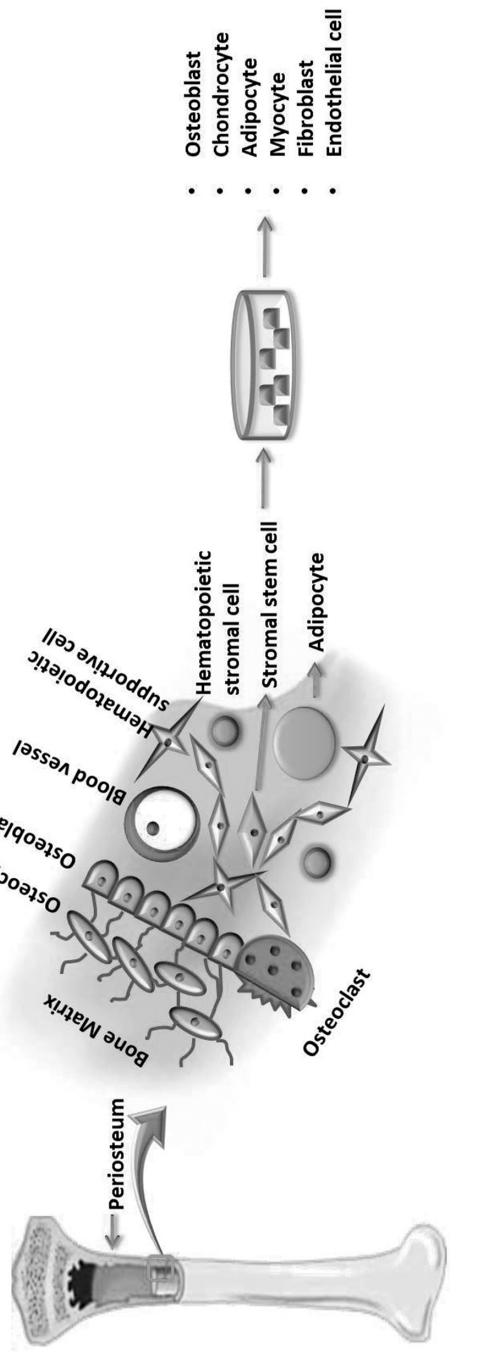


FIG. 6.4 Human bone marrow mesenchymal stem niche, MSCs isolation and differentiation.

osteoclasts, plasma cells, hemopoietic cells, and monocytes. MSC-like cells were first identified in 1960 by Alexander Freidanstien, and these cells were capable of differentiating into osteoblasts [**25**]. However, BMSC isolation and multilineage potential came much later in 1994. Their ease of isolation from either the iliac crest or from long bone makes them an ideal source for bone regeneration. Whether alone or in combination with three-dimensional scaffolds, BMSCs are extensively studied for their potential in bone tissue regeneration. However, unlike ESCs, MSCs have a limited proliferation capability and their population decreases dramatically with age [**24**]. In addition, isolation of autologous MSCs for clinical treatments of large segmental bone defect are very invasive and can cause complications because of donor site morbidity. Researchers have demonstrated bone formation when BMSCs are predifferentiated; however, this limits their clinical potential [**26**].

Periosteum-derived PCs have greater potential in bone and cartilage regeneration. The periosteum of endochondral and transmembranous bone consists of two layers. The outer fibrous layer, which attaches muscles, tendons, and ligaments to bone, consists of fibroblasts and is rich in collagen fibers. The inner layer consists of PCs that are involved in bone regeneration. Periosteum membrane with PCs has been used in many surgical procedures for bone repair; however, the periosteum-derived cells are less studied and understood [**27,28**]. PCs are regarded as MSCs because of their potential to differentiate into bone and cartilage, but their adipogenic potential has not been established. A recent study populated PC cells onto a collagen scaffold to replicate similar morphologies as the natural periosteum layer. The construct was then populated with stem cells, and the results showed enhanced osteogensis in comparison with controls (pseudoperiosteum-free scaffold) [**29**]. The superior capacity of PCs to form bone was examined in vitro and in vivo using BMSCs and PCs isolated from the same patient. Both cell types were cultured onto porous β-tricalcium phosphate (β-TCP) scaffolds and implanted subcutaneously. The PC samples showed enhanced mineralization in vitro but a higher degree of neovascularization and mature bone formation in vivo [**30**]. The disadvantage of using a periosteum cell population is the difficulty in harvesting these cells and the limited numbers available. This requires long periods of ex vivo expansion; therefore, they may possibly lose their multipotency. PCs are among the least studied MSCs; thus, in vitro expansion and maintenance are not well established.

On the other hand, adipose-tissue-derived MSCs (ADSCs) are a much more abundant source of autologous stem cells. In general, ADSCs have higher frequency (~1–5 % of isolated cells vs. <0.1 % for MSCs). ADSCs are easily isolated and expanded compared with BMSCs or PCs. ADSCs express the same surface markers as BMSCs with the exception of Stro-1, and, similar to BMSCs, these expressions can vary with passage number.

One study showed that the isolation of ADSCs from a highly vascularized fat produced a several-fold increase in the number of colony forming units compared with the poorly vascularized fat specimen [**24,31**]. Many researchers have studied the plasticity of ADSCs, and these cells have the potential to differentiate into osteogenic,

chondrogenic, and endothelial linages [**32**]. In vivo studies of ADSCs cultured on poly(lactide-*co*-glycolide) (PLGA) scaffolds have shown that preculturing ADSCs in osteogenic media for at least 14 day before implantation resulted in robust bone formation [**33**]. Another study conducted by Jeon and colleagues demonstrated the feasibility of bone regeneration by direct implantation of ADSCs seeded onto poly(lactide-co-glycolide)/hydroxyapatite (PLGA/HA) composite scaffolds loaded with bone morphogenic protein-2 (BMP-2) [**34**]. The ease of isolation and abundance in numbers of ADSCs is counteracted by their inferior osteogenic potential compared with that of BMSCs and PCs. The osteoblastic priming of ADSCs poses a great obstacle in clinical applications because culture duration and conditions can potentially lead to phenotypic changes.

Umbilical-cord-blood-derived MSCs (UCMSCs) are considered the youngest and most primitive source of MSCs. They are isolated from the connective tissue (Wharton's jelly) of the umbilical cord. During fetal development, umbilical cord cells are derived from the extraembryonic mesoderm layer and grow to form a 30- to 50-cm-long helical organ at birth [**35**]. UCMSCs express similar cell surface markers to BMSCs, such as STRO-1, CD44, and THY-1. Because of their origin, UCMSCs have a much higher self-renewal rate than other MSCs and differentiate to osteogenic, chondrogenic, and adipogenic lineages. It is interesting to note that studies comparing the osteogenic potential of UCMSCs to BMSCs found that the former proceeds more rapidly than the latter; these results were attributed to the higher number of osteoprogenitor cells in UCMSCs in comparison with BMSCs [**36**]. In this same study, it was also found that in vitro culture of human UCMSCs (hUCMSCs) did not demonstrate any contact-inhibited cell growth even after 20 days of continuous culture; rather, the proliferation of hUCMSCs continued after 100 % confluency by forming aggregates layered over the confluent cells. Another study by Chen and colleagues demonstrated the feasibility of seeding hUCMSCs onto macroporous calcium phosphate cement (CPC). The constructs generated new bone and blood vessels [**37**]. Although UCMSCs hold great promise, these cells have not been extensively studied, and their differentiation mechanisms and interaction with other cells have yet to be determined.

Combination of Bone-Forming and Vessel-Forming Cells

Treating large bone defects remains a grand challenge because bone is a highly vascularized tissue; thus, its repair must include new vascular network formation. This is vital for the viability and functionality of the newly forming bone. Blood vessels are the body's cargo shuttles that transport nutrients, oxygen, growth factors, and cells and remove waste. In fact, during the first stage in the fracture healing process, endothelial PCs (EPCs) and MSCs migrate to the injury site [**38–40**]. As previously mentioned, MSCs orchestrate the differentiation process with neighboring cell populations. One particular cell-cell crosstalk is found between MSC and EPCs (i.e., vessel-forming cells). Studies have found that the greatest amount of bone formation occurs where sufficient vascularization is present [**41–43**]. Recent tissue engineering techniques attempt to promote vasculature within the constructs. These attempts include the use

of angiogenic growth factors and in vitro prevascularization [20]. Our own studies examined the potential of a clinically relevant EPC population on the enhancement of bone formation [44]. In this study, circulating blood-derived EPCs were isolated and characterized for endothelial cell morphology (Fig. 6.5A) and vascular function in vitro (Fig. 6.5B). Also, EPCs and MSCs co-cultured at different ratios resulted in enhanced expression of key osteogenic and angiogenic markers. In addition, the 4:1 MSC:EPC ratio had the highest alkaline phosphatase activity in comparison with other combinations, MSCs or EPCs alone culture (Fig. 6.5C). This study not only showed the synergy in using EPCs and MSCs together, but it also demonstrated the optimum ratio of the two cell populations for enhanced bone regeneration and neovascularization [44].

Genetically Modified Cells

iPSCs

To overcome the shortcoming of the limited proliferation ability of somatic and MSCs and to bypass all moral and legal issues associated with the use of human embryos, iPSCs were developed by genetically reprogramming adult cells into an embryonic-like state. iPSCs were first established in 2006 by Shinya Yamanaka from the Institute for Frontier Medical Sciences in Japan. Dr. Yamanaka was able to induce pluripotency from mouse embryonic and adult fibroblasts using four factors: transcription factors 3 and 4 (Oct3, Oct4), Proto-oncogene c-Myc (the transforming gene of the avian myelocytomatosis virus), and Kruppel-like factor 4 (Klf4) (Fig. 6.6) [45]. Later in 2007, Dr. Yamanaka and group successfully generated iPSCs from adult human fibroblasts using the same method [46]. iPSCs express similar markers to ESCs, are able to proliferate indefinitely, and can differentiate to all three germ layers. iPSCs have become a great tool in drug development and the study of diseases. These cells are also used to understand the developmental process of embryos and the mechanisms of cellular repair and differentiation. Because of the lack of understanding of iPSCs, a more thorough evaluation is required to characterize and establish iPSC lines. Specific gene and protein expression is evaluated using reverse-transcription polymerase chain reaction and immunocytochemistry, respectively. A global expression of genes using a microarray analysis is also used. This in combination with in vivo and in vitro differentiation assessment is used to confirm their pluripotency [47]. Autologous iPSCs are a great source for regenerative engineering because of the lack of immune rejection. However, iPSCs programmed using viral vectors carry the risk of virus-induced toxicity and immunogenicity. Although researchers are attempting to chemically induce somatic cells, the nonviral vector-based techniques are still in their infancy and are far from optimized. In addition, the efficiency of these processes remains extremely low (<1 %) [48].

Engineered Cells to Release Growth Factor

Genetically engineering cells consists of transferring the genetic materials of a cell to induce the production of desired growth factors or proteins. Genetic engineering or gene therapy was first established for the treatment of diseases; however, it is currently

FIG. 6.5 Effect of MSC and EPC co-culture on vascularization and bone formation. (A) Cobblestone morphology of EPCs; (B) two-dimensional assay showing vascular network formation; and (C) synergy of EPC with BMCs for enhanced osteogenic ability.

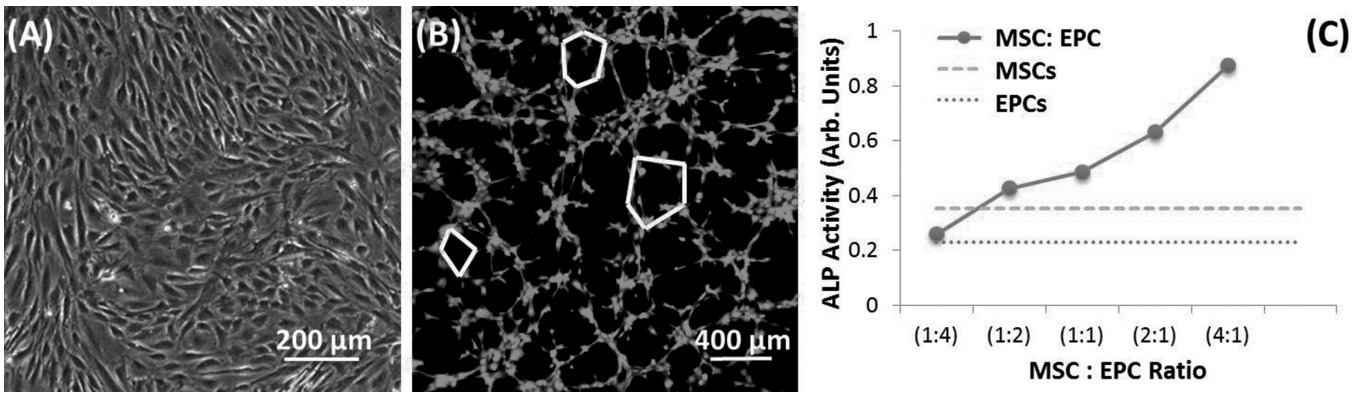
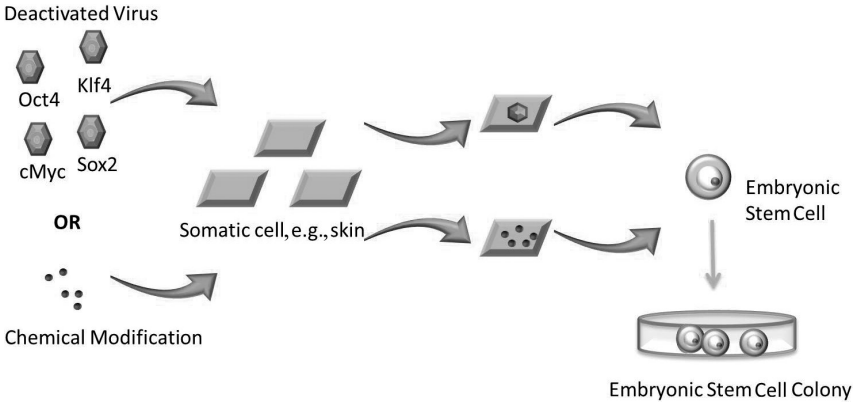


FIG. 6.6 Diagram representing reprograming of adult cells into induced pluripotent stem cells.



being used for tissue regeneration purposes. The purpose of gene therapy techniques applied to bone regeneration is to increase osteoconductive and osteoinductive growth factors and cytokines. The current efforts using exogenous growth factors in the treatment of segmental bone defects are limited and often insufficient to simulate and sustain adequate bone growth. Bone morphogenic proteins (BMPs), in particular BMP-2 and BMP-7, are the most common factors used in bone repair. Laurencin and colleagues investigated the development of an osteoinductive bone replacement system by culturing BMP-2-producing cells onto a PLGA-HA matrix. When implanted subcutaneously, the construct induced heterotopic bone formation [**49**]. Park and colleagues studied the effect of using BMP-2-producing BMSCs on bone formation in a critical size defect in rat mandible. This study compared the use of adenoviral and liposome-mediated therapy, and complete bone healing was observed within 4 and 6 weeks, respectively [**50**]. Lieberman and colleagues studied bone healing in a rat femoral critical size defect using five experimental groups: 20 μg of recombinant human BMP-2, BMP-2-producing bone marrow cells, β-galactosidase-producing bone marrow cells, uninfected bone marrow cells, and DBM alone. The results revealed that significantly greater bone formation occurred using BMP-2-producing bone marrow cells in comparison with other groups. The study also concluded that BMP-2-producing cells supplied adequate amounts of necessary proteins for effective bone healing [**51**]. There are many factors involved in bone regeneration, such as VEGF for the induction of neovasculature, and receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage colony-stimulating factor (M-CSF), IL-1, and IL-6, which are involved in secondary bone formation and remodeling. Similar to the effect of co-culturing on bone healing, the combinations of these growth factors also enhance bone formation. Peng and colleagues investigated the synergistic effects of

stem cells expressing VEGF and BMP-4 on bone formation. Although cells that expressed VEGF alone did not improve bone formation, genetically modified cells that expressed VEGF and BMP-4 in combination showed significantly enhanced bone formation [**52**]. Gene therapy proves to be a very effective strategy in bone healing. However, the methodologies used to induce cells are mostly based on viral transfection, which can cause immunogenicity reactions and uncontrollable mutagenic insertion, leading to malignant transformation.

Clinically Relevant Cell Sources

As with any cell-based engineering techniques, the interplay of the autologous cell population and growth factors is essential; the combination provides enhanced stimulus for bone regeneration. As previously described, there are several viable cells sources in addition to several growth factors that play a pivotal role in bone regeneration. However, there are a few clinically viable sources that include BMA, concentrated BMA (cBMA), platelet-rich plasma (PRP), and PRP containing BMA (PRP-BMA).

BMA and cBMA

Orthopedic surgeons currently treat a fractured area by directly infusing BMA harvested from the iliac crest or any of the long bones. The bone marrow niche is rich in several PCs and growth factors, such as BMPs, VEGF, and platelet-derived growth factor (PDGF), which makes it a great candidate for segmental bone defect repair. However, the volumetric amount of BMA required for a certain size defect might not contain a sufficient PC population, thus leading to limited new bone formation, and a second surgical procedure is often required. These numbers significantly decrease with age and health status. Researchers have investigated the possibilities of enriching a larger volumetric sample of BMA such that PC populations are concentrated in a small sample; these are referred to cBMA. Most BMA enrichment methods use centrifugation techniques to eliminate unwanted extra volume while maintaining the desired BMA component, including MSCs, EPCs, and growth factors. Jäger and group studied the potency of cBMA to regenerate bone. BMA was isolated from 39 patients; cBMA was obtained by a density centrifugation technique using a SmartPReP® centrifuge. Collagen sponges and bovine HA were used as scaffolding materials. Patients showed bone healing in both types of scaffolds, and complete bone healing was achieved after 17 weeks in the case of the bovine HA scaffold and 22 weeks in the case of the collagen sponge [**53**]. Although the centrifuge-based cell enrichment technique is currently used in clinics, this method has the potential of contaminating samples. Ridgway and colleagues developed a compacted, single-use, acoustically assisted filtration device that can be used to obtain cBMA at the point of care [**54**]. Others use a magnetic cell sorting (MACS) technique to separate large numbers of cells according to their surface markers. Our own studies are to use completely automated and U.S. Food and Drug Administration (FDA)-approved cell enrichment devices (MAGELLAN® from Arteriocyte Medical Systems, and CliniMACS® from Miltenyl Biotech) [55,56]. These efforts are to develop completely intraoperative tissue engineering strategies for bone defect repair and regeneration.

PRP

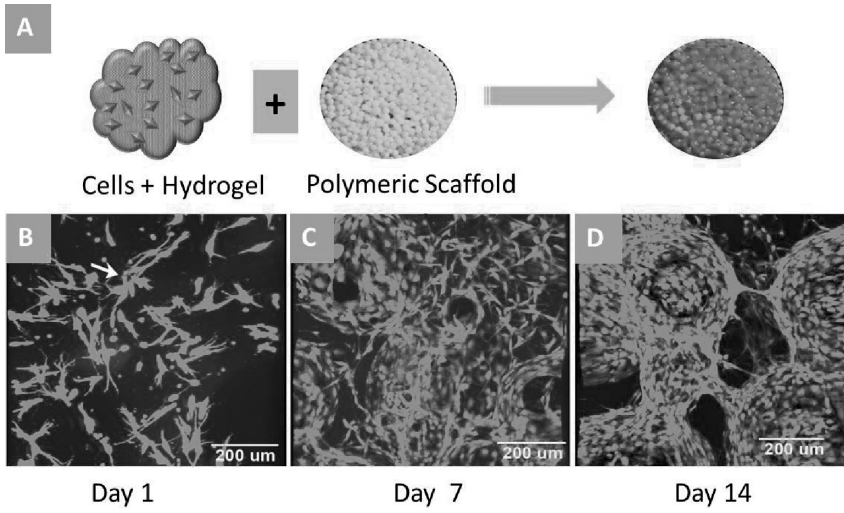
Platelets are rich in growth factors, such as transforming growth factor-β (TGF-β), PDGF, and VEGF. These factors are involved in chemotaxis, cell proliferation, differentiation, and extracellular matrix synthesis. Therefore, by concentrating platelets, growth factors are also enriched. Autologous PRP is easily isolated from freshly drawn whole blood and activated by thrombin and calcium. Although PRP does not include cells, it can enhance bone formation through the abundance of multiple growth factors and is proven to be more potent than the individual use of each growth factor. Despite the progress shown by using PRP in vitro, the in vivo models exhibit various outcomes. This can be attributed to the variance of growth factor levels in different samples, which can change with age and health status [**57**].

PRP alone is not useful in the regeneration of bone; however, PRP readily forms into a fibrin mesh that can be used as a scaffolding material for bone healing applications. Marx and colleagues showed a significantly higher bone healing rate and bone density when using PRP in combination with grafts [**58**]. Yamada and colleagues used a combination of PRP as an autologous scaffold with in vitro expanded MSCs to increase osteogensis. Compared with scaffold alone, PRP-MSC grafts showed significantly higher mature bone and neovascular formation by 8 weeks of implantation [**59**]. In a recent study describing the treatment of osteonecrosis of the femoral head using a combination of PRP and MSCs, significant pain relief was found in 86 % of patients [**60**].

Efficient Cell Loading

After identifying a cell source, the next important and most challenging step is efficiently delivering these cells to the area of interest. Tissue engineering techniques combine cells with graft materials to design different geometries, chemistry, and biocompatibility. In non-load-bearing settings, there are several biodegradable hydrogel options that can be used to support tissue regeneration. The challenge is to design matrices that are load-bearing and yet support rapid and efficient cell loading. Our laboratory has developed an advanced matrix system (polymer-hydrogel matrix) composed of a load-bearing polymeric scaffold and a hydrogel phase in which cells are encapsulated [**61**]. In this design, the gel phase is chosen such that it is transient in nature. By choosing a transient gel, one could design matrices that support rapid and efficient cell transplantation without affecting the polymer matrix transport properties. This study demonstrated the feasibility of encapsulating high cell seeding density within the polymer-hydrogel system with a significant cell proliferation and survival (**Fig. 6.7A**). Twenty-four hours postencapsulation, cells were located in the pores and void spaces of the polymeric scaffold where the hydrogel resides, as shown in **Fig. 6.7B**. After 7 days of culture, cells began migrating toward the surface of the polymeric microsphere scaffold (**Fig. 6.7C**). As shown in **Fig. 6.7D**, by day 14, the hybrid scaffolds were fully cellularized.

FIG. 6.7 Hybrid scaffold loaded with cells. (A) diagram showing cell encapsulation and loading onto 3D scaffold (hydrogel was stained with Ponceau S red staining for visualization purposes). (B), (C), and (D) live/dead assay to show growth and survival of cells in hybrid scaffold at days 1, 7, and 14 respectively. Adopted from Igwe et al. [61].



SUMMARY

The field of bone regeneration has undergone tremendous advancement in terms of understanding the cellular mechanisms and developmental stages of bone formation. Cell-based therapy approaches hold great promises in the field of bone tissue engineering. However, bone repair/regeneration is not only based on the selection of an appropriate and clinically viable cell source but it is also guided by the chemical, biological, and physical microenvironment. Many cell sources have been identified, such as ESCs, adult MSCs, and iPSCs. Cell-based approaches such as cBMA, PRP, and PRP-BMA have shown to be clinically valuable tools for bone regeneration. However, the most challenging problem facing bone regeneration therapy is the treatment of critical size defects. In this case, a mechanically strong scaffold is required to physically support the regeneration process. In addition, an abundant osteoprogenitor cell population and their isolation via FDA-approved methods as well the use of osteoinductive factors are necessary to ensure a compete healing of bone.

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Chapter 7 | Review of State of the Art: Growth Factor-Based Systems for Use as Bone Graft Substitutes

Aditya Arora,[1] Arijit Bhattacharjee,[1] and Dhirendra S. Katti[1]

INTRODUCTION

Decades of research have been directed toward the development of synthetic bone graft substitutes that can efficiently replace the current gold standard autograft procedures. However, success in the area of synthetic bone graft substitutes has been limited because of unpredictability of the biological responses to these substitute materials, especially in the case of large-sized defects [**1,2**]. One of the critical factors that has contributed to the low success rates of synthetic grafts is their inability to provide adequate osteogenic and osteoinductive cues [**2**]. Therefore, a successful bone graft substitute should possess an appropriate three-dimensional substrate for cell adhesion and proliferation, appropriate cells (or an ability to recruit such cells), and appropriate growth factors to initiate regeneration [**3,4**]. Growth factors at the site of interest can be released from a carrier matrix in a controlled manner. The matrix often doubles as a scaffold that provides surface area for cell adhesion and proliferation and as a carrier matrix for growth factor delivery. Growth factors, which are essentially soluble signaling molecules, can facilitate all cell fate processes necessary for bone regeneration. The matrix sequesters growth factors using various physical cues, thus giving rise to the possibility of delivering the soluble signals of interest in an appropriate sequence (by controlling the proximity to scaffold-fluid interface), at an adequate rate (by controlling strength of interaction), and in suitable doses (by controlling total encapsulation). The factors can then act to produce an orchestrated sequence of events that recapitulates the natural bone healing process reminiscent of those occurring during embryonic development.

Classically, only molecules such as bone morphogenetic proteins (BMPs) were thought to be ideal candidates for providing osteoinductive cues. However, extensive work on the molecular mechanisms of osteoinduction has demonstrated that

[1] Department of Biological Sciences and Bioengineering, Indian Institute of Technology Kanpur, Kanpur, India

the osteoinductive capacity of proteins such as BMP may be enhanced by other cytokines and growth factors that influence cellular responses and are involved in native bone healing. These factors include those that influence inflammation, cellular proliferation, migration, angiogenesis, and osteogenic differentiation. Recent approaches have focused on using a combination of factors so as to evoke a synergistic response in the healing of nonunion fractures [**5–9**]. These combinations are delivered in a manner such that they artificially recreate the native microenvironment of healing bone. However, to recreate this microenvironment it is necessary to understand the process of bone regeneration and the role of various growth factors in this process.

BONE REPAIR AND REGENERATION

Fracture healing is a complex process that consists of a cascade of events involving multiple players such as cells, mechanical cues, and spatiotemporally controlled presentation of soluble factors. The healing response is classified into primary and secondary fracture healing. Primary healing involves active participation of the bone cortex to re-establish its continuity. This response is observed only in cases in which there is rigid internal fixation and minimal interfragmentary strain, making it relevant only to the cases involving rigid compression fixation and small bone cracks [**10**].

Thus, secondary fracture healing has gathered more interest in the healing of large nonunion fractures. This response is characterized by active involvement of periosteum and external tissues, in which regeneration proceeds with the help of committed cells and mesenchymal stem cells (MSCs). Secondary healing response recapitulates the sequence of events that occur during embryonic development of bone involving intramembranous and endochondral ossification. The process can be divided into multiple stages:

- Hematoma formation and inflammation due to damaged vasculature
- Intramembranous ossification leading to outer hard callus
- Cartilage formation leading to inner soft callus
- Induction of hypertrophy followed by vascularization
- Endochondral ossification and finally remodeling [**10–12**].

The process starts with rupturing of the blood vessels at the site of injury, leading to formation of platelet-rich clot and influx of inflammatory cells. The inflammatory cells in and around the clot secrete various inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukins (IL), which in turn recruit progenitor cells to initiate regenerative processes. The intramembranous ossification involves formation of woven bone without an intermediate cartilage-like stage whereas endochondral ossification involves induction of hypertrophy in the transient cartilage, which is finally replaced by cancellous bone, after vascularization. Herein, vascularization plays a crucial role as blood vessels deliver the chondroclasts (cells that resorb cartilage) and mesenchymal progenitors to the transient cartilage [**10,13**].

ROLE OF SOLUBLE FACTORS IN BONE REPAIR AND REGENERATION

Each stage of the healing process is characterized by a set of soluble factors and cell types acting in a coordinated manner. A repertoire of soluble factors is presented to the cells in a spatiotemporally controlled manner. These factors most often are proteins secreted by a cell that bind to specific cell surface receptors and can influence inflammation, cellular proliferation, migration, angiogenesis, and differentiation (**Fig. 7.1**). They can have two types of effects, a *paracrine effect*, meaning that they stimulate neighboring cells to proliferate and increase matrix protein synthesis, or an *autocrine effect*, meaning that they can stimulate themselves for additional metabolic activity, or both.

All musculoskeletal tissues produce and respond to growth factors because they initiate the cascades of cellular events that lead up to bone healing (**Fig. 7.2**). Some of the most popular growth factors that have been associated with fracture healing include platelet-derived growth factors (PDGFs), IL-1, IL-3, IL-6, colony-stimulating factors (macrophage colony-stimulating factor, granulocyte macrophage colony-stimulating factor), the transforming growth factor-β family (TGF-β), BMPs, insulin-like growth factors (IGFs), fibroblastic growth factors (FGFs), parathyroid hormone (PTH), wingless type proteins (Wnt), hedgehog proteins (Hh), and vascular endothelial growth factor (VEGF). On the basis of the response that the factors elicit,

FIG. 7.1 Schematic of early and late events occurring during bone healing. After bone injury, growth factors are released from the fracture callus and local fracture site to stimulate precursor cells on the endosteal and periosteal surfaces to proliferate and differentiate, initiating a healing response.

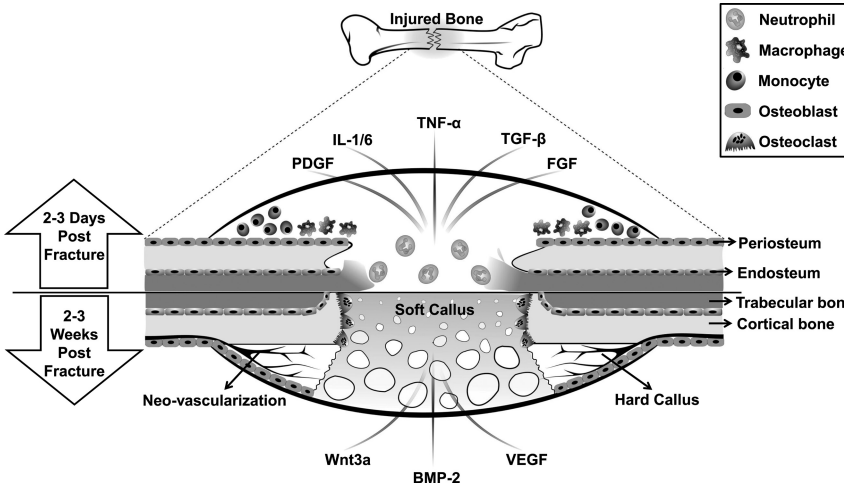
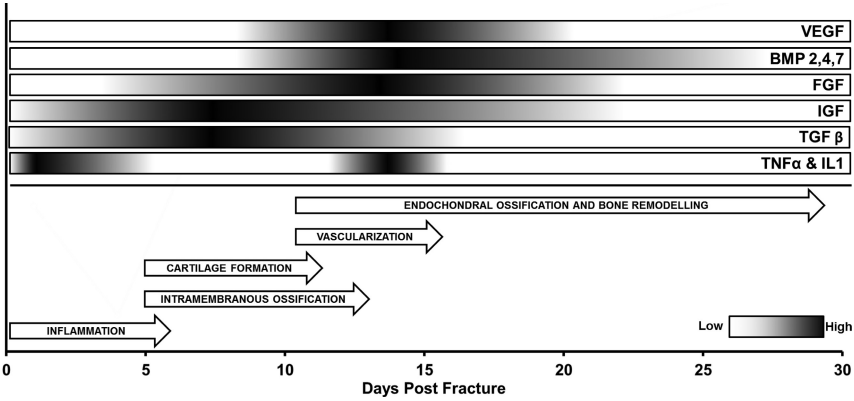


FIG. 7.2 Schematic of sequence of events that occur during natural bone fracture healing and temporal sequence of expression of growth factors involved in the process.



they can broadly be classified into three categories: (1) inflammatory factors, (2) proliferative and osteoinductive factors, and (3) angiogenic factors [**14,15**]. In addition, platelets secrete a mixture of several growth factors that have also been used clinically in the form of "platelet-rich plasma" for enabling musculoskeletal regeneration.

Inflammatory Factors

Inflammation is well known for its catabolic effects in skeletal diseases such as arthritis. However, the same inflammatory factors have been shown to be crucial in the speedy regeneration of fractured bones. This contradictory behavior of inflammatory factors is context dependent and varies greatly depending on the receptor type that is expressed on the target cells. The key inflammatory factors that play a role in fracture healing are TNF-α, IL-1, and IL-6. They serve functions such as chemotactic effects on inflammatory cells, recruitment of fibrogenic cells, and enhancing extracellular matrix (ECM) synthesis and angiogenesis [**16–19**]. During healing, their expression is precisely controlled and is biphasic in nature with the first peak being at 24 h postinjury and the second peak being initiated at approximately 14 days postinjury (initial stage of endochondral ossification) [**12**].

TNF-α

In the early phase of healing, TNF-α is responsible for eliciting a chemotactic effect, which in turn helps in the recruitment of various cell types for regeneration [**20**]. However, in the later stage (at ~14 days), TNF-α induces apoptosis in hypertrophic chondrocytes and matrix metalloproteinase (MMP) expression for turnover of

mineralized cartilage, thus enabling progression of endochondral ossification [**21,22**]. The applicability of TNF-α in regenerative strategies was recently demonstrated by the work of Glass et al., in which they showed that injecting recombinant human TNF-α (rhTNF-α) during the first 2 days postinjury improved mineralization of fracture callus at later stages of fracture healing [**23**].

IL-1

Effects of IL-1 are very similar to those of TNF-α. IL-1 is also responsible for release of other proinflammatory molecules such as IL-6 and prostaglandins [**24,25**]. A recent study demonstrated that injecting IL-1β for 3 days postinjury accelerated in vivo bone regeneration [**26**]. In another study, which used prostaglandin agonists, the authors demonstrated that incorporation of prostaglandin agonists specific for prostaglandin E2 receptors in poly(lactic acid-co-glycolic acid) (PLGA) matrix significantly enhanced healing of critical-sized radial and tibial bone defects in dogs [**27**].

Because of the well-established role of inflammatory factors in catabolic pathways, very few studies have explored their potential in bone healing. However, initial studies have shown exciting results and more in vivo studies need to be performed. Furthermore, to harness their full potential in influencing bone regeneration, it has to be kept in mind that these factors must be presented under precise temporal control because prolonging or early cessation of these factors can impede bone regeneration [**28**].

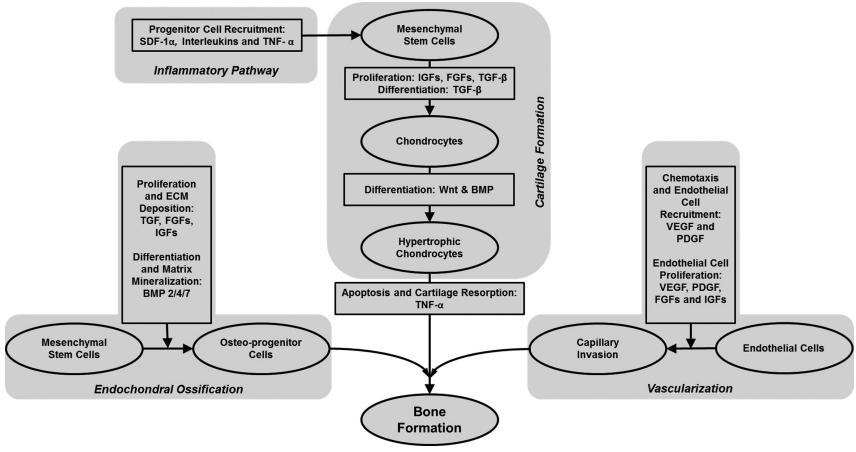
Proliferative and Osteoinductive Factors

Proliferation and differentiation are two crucial events that are prerequisites for successful bone formation. There are several growth factors that play either one or both of the roles in native fracture healing; thus, they have been used for augmenting fracture healing (**Fig. 7.3**). These include TGF-β, BMP, FGF, PDGF, and IGF; in addition to proliferation and osteoinduction, they also play a role in cell recruitment and ECM synthesis. Because of their crucial role in bone fracture healing and regeneration, they have been extensively explored in the area of bone repair. Each of these factors is discussed in further detail in the following subsections.

TGF-β

The discovery of large amounts of TGF-β in bone matrix, its effect on matrix synthesis in chondrocytes and osteoblasts in vitro, and its release into fracture hematoma by platelets has led to the belief that TGF-β is a major cytokine involved in regulating cartilage and bone formation during normal growth and remodeling and after injury [**29,30**]. Endogenous TGF-β is most often secreted as a latent complex consisting of latency-associated peptide, which renders secreted TGF-β inactive; thus, dissociation of the two is crucial for its activation. However, this complex is further stabilized by a latent TGF-β binding protein—1/3/4 (LTBP)—that leads to the formation of a stable ternary complex. LTBPs are extracellular fibrillin-like proteins that orchestrate the function of TGF-β at multiple levels, including folding, secretion, spatial

FIG. 7.3 Schematic depicting involvement of various growth factors in different cell fate processes that govern natural bone fracture healing.



distribution, and activation. The role of LTBP in bone was demonstrated in a study in which it was shown that LTBP-3-null mice have significant alterations in the skeletal system with osteosclerosis, premature obliteration of synchondroses, and osteoarthritis [**31**]. Furthermore, it has been demonstrated that LTBP-bound TGF-β is released at the site of bone resorption via proteolytic cleavage by enzymes such as MMPs secreted by the osteoclasts, thereby helping in bone remodeling in vivo [**32**]. In addition, TGF-β is released from the latent complex in a plasmin-dependent manner in many situations. Herein, plasmin can be secreted by various cells such as interferon-γ-activated macrophages and basic FGF (bFGF)-activated endothelial cells [**33,34**].

However, most studies involving the use of TGF-β for supporting bone regeneration do not rely on this cell-mediated activation and use an active recombinant form of the protein that is not bound to the LTBPs. In an in vitro study involving treatment with recombinant human TGF-β (rhTGF-β) in murine parietal bone organ culture, it was shown that rhTGF-β suppressed the formation of mineral in osteoid, probably by downregulating the expression of mediators of mineralization such as osteocalcin despite its stimulation of osteoid formation [**35**]. Therefore, although TGF-β initiates new bone formation, when provided exogenously, endochondral ossification started only after cessation of TGF-β injections in rat femur [**36**].

Nevertheless, several in vitro and in vivo studies show that TGF-β is a potentially osteoinductive substance and has been studied in various animal models using subperiosteal injections in the femur, tibia, calvaria, critical-sized defects, and bone in-growth into prosthetic devices [**36–39**]. Hock et al. showed that when calvarial

osteoblasts were incubated in vitro with TGF-β, there was an increase in osteoblast proliferation and bone matrix formation [**37**]. Likewise, Joyce et al. demonstrated that injections of rhTGF-β in the rat femur could stimulate periosteal cells to undergo endochondral ossification. In addition, TGF-β2 was found to be autoregulatory, increasing the production of TGF-β1 in osteoblasts and chondrocytes [**36**]. These actions suggest the potential of TGF-β in therapeutic applications for osseous defects.

Lind et al. delivered continuous infusion of human platelet-derived TGF-β (with either 1 or 10 μg per day for 6 weeks) in rabbit tibial diaphyseal fractures fixed with a plate and found that there was a significant increase in callus formation, and a significant increase in bending strength, in comparison to nontreated control specimens. The group receiving 1 μg per day demonstrated superior mechanical strength in three-point bending as compared with the untreated and the higher-dosed groups [**39**].

Nielsen et al. also reported mechanical strength of bone in a rat fracture model that received a local treatment of human platelet-derived TGF-β (either 4 or 40 μg every other day for 40 days) and demonstrated that the fractures that received TGF-β showed a significant increase in callus formation and strength. The group that received the 40-μg dose demonstrated a significant increase in ultimate load to failure than the lower-dosed and untreated groups [**40**].

Critchlow et al. investigated the effect of purified porcine TGF-β2 injection around the developing callus of rabbit tibial fractures healing under stable (fixed with a dynamic compression plate) or unstable (without plate fixation) mechanical conditions 4 days after fracture. Their results demonstrated that TGF-β2 cannot stimulate fracture healing under unstable mechanical conditions, but it led to a small increase in bony callus under stable mechanical conditions [**41**].

In addition to these, several studies have been performed in recent years that demonstrated that TGF-β can induce enhanced chondrogenic differentiation, osteoblast proliferation, and increased bone formation [**42–44**].

Although these studies [**39–44**] confirm that TGF-β enhances cellular proliferation, its potential as an osteoinductive substance and the concentration at which it is an effective osteoinducer remain equivocal [**45,46**]. Different isoforms and doses of TGF-β have been used in various studies that made use of different animal models. The positive effects of TGF-β in the studies by Lind et al., Nielsen et al., and Critchlow et al. suggest that relatively large dosages (supraphysiological) are required to enhance bone repair [**39–41**]. However, large dosages may not be possible in the clinical setting because TGF-β enhances proliferation in various cell types, which may cause undesired side effects. On the basis of studies thus far, it can be concluded that TGF-β has potential for being developed as an agent for clinical use, but further preclinical studies need to elucidate dosing parameters, safety, and appropriate methods of application/administration.

BMP

BMP was discovered in demineralized bone material to have the unique ability to ectopically induce bone formation [**47,48**]. The BMPs are a subfamily of the TGF-β superfamily, consisting of 20 known members [**49**], and they have the highest osteoinductive effect amongst all known growth factors [**50**]. These factors play a key role in bone formation by stimulating migration and proliferation of osteoblasts and MSCs, after which they also promote the osteogenic differentiation of MSCs [**5,50–52**].

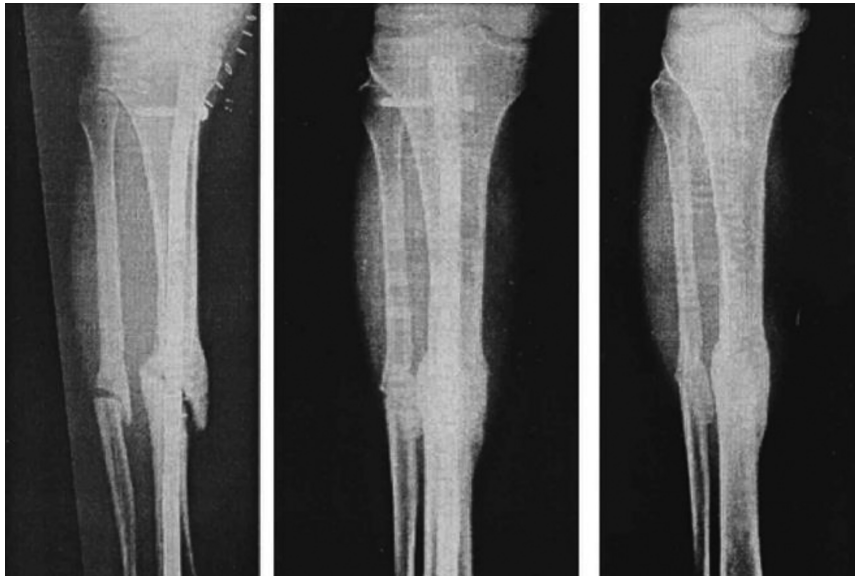
Amongst the various BMPs, BMP-2, BMP-4, BMP-7, and growth differentiation factor-5 (GDF-5/BMP-14) are well-known osteoinductive factors that stimulate chondrogenic differentiation of MSCs, followed by endochondral bone formation [**53**]. BMP-3 is a bone-inductive protein also known as osteogenin. Osteogenin has shown potent ability to induce the rapid differentiation of extraskeletal mesenchymal tissue into bone [**54**].

The use of BMP in humans is currently restricted to spinal arthrodesis, treatment of spinal nonunions, fractured bone, and periodontal defects [**55–57**]. BMP-2 and BMP-7 (also known as osteogenic protein-1 [OP-1]) have been studied extensively for their ability to induce bone regeneration. The administration of recombinant human BMP (rhBMP) has been simplified by incorporating it into a collagen sponge/ gel that acts as a reservoir for the growth factor and can be implanted at the site where bone induction is desired. Sheehan et al. demonstrated the effectiveness of type I collagen gel combined with rhBMP-2 and autogenic bone from iliac crest by comparing bone formation at the sites implanted with and without rhBMP-2. Biomechanical testing of the harvested specimens showed superior strength of the rhBMP-2-treated sites in comparison to the control group [**58**].

Geesink et al. reported the use of rhBMP-7/OP-1 in humans for the first time in 1999. OP-1 was combined with a type I collagen carrier and placed at the defect site [**59**]. At 6 weeks, the OP-1-treated group showed radiographic evidence of bone formation, whereas the group receiving only collagen without OP-1 did not show significant bone formation. This study validated the osteogenic activity of OP-1 in a critically sized human bony defect [**59**].

In a prospective, randomized, multicenter study of tibial nonunions treated with intramedullary nailing, Friedlaender et al. showed that recombinant human OP-1 (rhOP-1) implanted with a type I collagen carrier was comparable to autografts. Parameters measured were radiographic assessment, immunologic assessment, complications, and clinical assessment by physician satisfaction. In 124 tibial nonunions at the 9-month follow-up time point, 75 % of those in the OP-1-treated group and 84 % of those in the autograft-treated group had radiographic union. By clinical criteria, 81 % of the OP-1-treated patients and 85 % of the autograft-treated patients had achieved union. At the 2-year follow-up, these results continued at similar levels. OP-1 proved to be safe and effective for the treatment of tibial nonunions at 2 years after the operative procedure, with the benefit of lack of donor site morbidity (**Fig. 7.4**) [**60,61**].

FIG. 7.4 Radiographs taken at follow-up of a 34-year-old male patient treated with OP-1 after a closed, comminuted tibial fracture sustained in a motor vehicle accident. (A) Immediate postoperative radiograph. (B) Radiograph 9 months after treatment with OP-1. (C) Radiograph 24 months after treatment with OP-1. Source: Reproduced with permission from [**61**].



These studies and others have demonstrated the beneficial effect of using BMPs for accelerated healing of bone defects by recruitment of osteoblast and progenitor cells, promoting cell proliferation and differentiation, and accelerating matrix mineralization [**56,58,60–75**]. The clinical trial by Friedlaender et al. [**60,61**] conducted under a U.S. Food and Drug Administration (FDA)-approved Investigational Device Exemption has established the safety and efficacy of OP-1 for the treatment of tibial nonunions. Therefore, the FDA approved several BMP-based products for use in spinal fusion (OP-1™ Putty and InFUSE™ Bone Graft/LT-CAGE™), fractured bone (OP-1™ Implant), and periodontal defects (INFUSE® Bone Graft).

One question concerning the use of recombinant BMPs in stimulating bone healing in animals or humans remains unanswered: Why are large doses (supraphysiological) required to have an osteoinductive efficacy? The effective doses are orders of magnitude greater than the endogenous amounts of BMPs during normal bone repair or in normal bone remodeling. Presumably, the answer may lie in the combined action of various other factors giving rise to a synergistic response required for maximal efficacy of BMP-mediated osteoinduction.

FGF

The most abundant members of the FGF family are FGF-1 (acidic) and FGF-2 (basic). Both members are present and active in cartilage and bone and have been shown to be mitogenic for endothelial cells, fibroblasts, chondroblasts, and osteoblasts [**76–79**].

During the early stages of fracture healing, including angiogenesis and chondrocyte and chondroblast activation, FGF-1 and FGF-2 have been identified as influential players. This has been demonstrated by in vivo studies that showed increased bone formation after exogenous administration of FGF-1 and FGF-2. Their effects were shown to be mediated by TGF-β and prostaglandins because the production of these factors is likewise regulated by FGFs [**80,81**]. FGF-1 and FGF-2 under some circumstances are also able to stimulate bone resorption [**81**].

In vivo studies have shown that exogenously provided FGF stimulates proliferation of osteoblastic cells and that local injection of FGF-1 can promote calvarial bone formation [**82**]. In rabbits, percutaneous application of FGF-2 increased bone formation and bone mineral density [**83**]. Exogenous FGF-1 and FGF-2 are thought to act by increasing the recruitment of osteoblast precursor cells, which differentiate into osteoblasts. TGF-β increases FGF-2 mRNA in cells; thus, some of its cellular effects could be related to its regulation of other growth factors [**84**].

FGF also plays an important role during fracture repair. Ueno et al. demonstrated that FGF-2 is expressed in granulation tissue after fracture, suggesting that FGF could be stimulating cell migration and angiogenesis at early stages of fracture healing [**85**]. Increased levels of FGF-1, FGF-2, and FGF-18 have also been found during callus formation in a distraction osteogenesis model [**86**].

Exogenously provided FGF-2 has also been shown to accelerate bone repair. The effect of local injection of recombinant human FGF-2 (rhFGF-2) on the healing of segmental 3-mm tibial defects in rabbits was investigated by Kato et al. [**87**]. After osteotomy and subsequent fixation by an external fixator, each animal in the treatment group received either 0, 50, 100, 200, or 400 μg of rhFGF-2 at the fracture site. Injection of the growth factor increased the volume and mineral content of the regenerated bone at the defect in a dose-dependent manner. Significant effects were observed at concentrations of 100 μg or greater, as assessed by X-ray, dual-energy X-ray absorptiometry (DEXA), and histological evaluation at 5 weeks. It was concluded that a single local injection of FGF-2 is capable of stimulating the healing of segmental defects.

Zellin et al. found an increased number of osteocytes at newly formed bone sites in transosseous rat mandibular defects [**88**]. Three different doses of rhFGF-2 (10 ng, 100 ng, and 1 μg) were delivered in an absorbable collagen sponge carrier. The higher (1-μg) dose decreased bone formation whereas the lower doses had a mild stimulatory effect on osteogenesis after 24 days [**88**]. A more recent study combined a collagen sponge carrier with an outer microporous poly(lactic acid-co-glycolic acid-co-ε-caprolactone) membrane for the treatment of mandibular defects in beagle dogs. The study showed that although the presence of bFGF resulted in increased volume of new

bone, there was no effect of the presence or absence of bFGF on the density of regenerated bone [**89**].

Overall, the potential of FGFs in improving proliferation of osteoblasts, osteoid formation, and bone union has been demonstrated. However, there is no consensus on the effect of FGF on osteoblastic differentiation and bone mineral density [**83,87–93**]. The results of these studies suggest that FGF-2 shows potential to be used in the future as an adjunct to bone healing. Similar to TGF-β, the effects of the FGFs on increasing bone formation or induction are modest compared with those of the BMPs.

PDGF

PDGF is found in higher concentrations in platelets and vascular endothelial cells, although it is also present in other cell types. PDGF synthesis is often increased in response to external stimuli, such as low oxygen tension, thrombin, or stimulation by other growth factors.

The main function of PDGF is to act as a chemotactic factor. It is released by platelets and monocytes in fracture callus and sites of injury, and it induces MSC recruitment and proliferation, thus recruiting reparative cell populations [**94,95**]. PDGF is mitogenic for osteoblasts in vitro [**96**] because this is its primary effect on bone cells. Local application of recombinant human PDGF-BB has shown promise to promote bone formation via its mitogenic effect on osteoblasts in animal models and clinical studies [**97,98**]. PDGF containing product GEM 21S™ has been approved by the FDA for applications in periodontal defects. Likewise, AUGMENT® BONE GRAFT, which contains β-tricalcium phosphate as a carrier of PDGF-BB, has been approved for ankle fusion surgeries in several countries, including Canada, Australia, and New Zealand.

In a study on the effect of PDGF on tibial osteotomies of rats, Nash et al. [**99**] found that PDGF-BB has a stimulatory effect on fracture healing. Radiographically, there was a clear increase in callus density and volume in the treated tibias when compared with the untreated group. Histologically, the PDGF-BB-treated tibias displayed a more advanced stage of osteogenic differentiation. However, no significant increase in biomechanical strength was observed.

Although PDGF acts only via its chemotactic and mitogenic effects on MSCs, osteoblasts, and endothelial and inflammatory cells, it has been used successfully in the clinic for fracture healing.

IGF

Two IGFs have been identified thus far: IGF-I and IGF-II. Although IGF-II is the predominant form in bone, IGF-I is more potent than IGF-II and has been localized to healing fracture sites [**100**]. IGFs are found in multiple tissues, but they are abundant in the bone microenvironment, acting in an endocrine, paracrine, and autocrine manner to regulate bone formation [**101**]. IGF-I and IGF-II stimulate osteoblastic cell proliferation and type I collagen expression, and interference with IGF function by use of blockers such as IGF binding proteins (IGFBPs) to the ligands inhibits these effects [**102–104**].

Along with growth hormone, IGF is actively involved during fetal skeletal development and plays a major role in the repair and remodeling of the adult skeleton. IGF expression is increased in cells of the growth plate, healing fracture callus tissue, and developing ectopic bone tissue [**100,105–109**].

The half-lives of systemically administered IGFs are relatively short because of their small size. In vivo, IGFs are bound to larger protein complexes known as IGFBPs. Six IGFBPs have been identified (types I–VI) with types 2–6 being produced by osteoblasts. IGFBPs have been shown to modulate IGF activity. For example, IGFBP-3 and IGFBP-5 potentiate IGF stimulation of osteoblasts, whereas the other IGFBPs inhibit IGF activity. Bagi et al. administered rhIGFBP-3 and rhIGF-I alone and in combination in ovarectomized, osteopenic rats. The two agents in combination were more effective than either agent alone for increasing bone formation in osteopenic rats [**110**]. IGFBP-5 is unique in that IGFBP-5 alone or in combination with IGF-I or IGF-II can bind to hydroxyapatite and hence serve as a route for incorporation of IGFs into mineralized bone matrix [**111**].

Previous studies have shown that systemically administered IGF can augment bone repair. Using a rat calvarial critical defect model, Thaller et al. evaluated the role of IGF-I in stimulating intramembranous bone formation [**112**]. Bone healing in rats with continuous systemic administration of recombinant IGF-I (rIGF-1) via a subcutaneous pump was compared with saline-treated controls. It was observed that in the rIGF-I-treated group, repair commenced at approximately 1 week and the critical-size calvarial bone defects were completely obliterated by 6 weeks; in the control group, the surgical defects remained at 8 weeks. These results indicated that IGF-I does enhance the healing of intramembranous bony defects [**112**].

In another study, Thaller et al. examined the influence of rIGF-1 on standardized, critical-sized calvarial defects in 25 adult male streptozotocin-induced diabetic rats [**113**]. Their results showed that IGF-1 exerted a potentiating effect on the repair of bony defects in diabetes-induced rats. Because diabetic patients have an increased frequency of infection, delayed scar formation, and poor bony union, the results of this study suggest the possible usefulness of IGF-I in the diabetic population and those with clinically documented problems in bone healing [**113**].

It is interesting to note that IGF-I has also been reported to increase osteoclast recruitment and formation, thus having a stimulating effect on osteoclastic bone resorption [**114**]. IGF-I is thought to be released from bone during the resorption phase and function to increase osteoblast precursors [**115**]. During the remodeling cycle, resorption is balanced by an equal amount of bone formation. Therefore, bone formation and bone resorption are coupled, and IGFs play a role in the mediation of both of these processes.

IGFs have an important role in bone remodeling, reducing inflammation, and increasing matrix deposition, but the effects depend on interactions with IGFBPs [**116–119**]. Therapeutic potential for bone healing exists for IGFs, but the role needs to be better defined by further studies, keeping in mind the drawbacks that have been

associated with free systemic IGF levels such as hypertension, headache, hypoglycemia, fatigue, and dyspnea [**120**].

PTH

PTH is secreted by the parathyroid gland and is known to increase calcium concentration in the blood. It acts by stimulating bone resorption and thus releasing calcium from bone. However, it is well established that intermittent exposure to exogenous PTH has an anabolic effect on bone [**121**]. In fact, its anabolic use is well established for a peptide (from amino acid 1 to 34 [PTH(1–34)]) derived from PTH. This peptide is the only anabolic drug that has been approved for the treatment of postmenopausal osteoporosis. In the area of regeneration of damaged bone, early studies were performed in a rat tibial fracture model using 60 or 200 μg PTH(1–34)/kg body weight per day, in which the factor was administered subcutaneously every day. The study demonstrated a dose-dependent improvement in the ultimate load and callus volume at late time points [**122**]. High efficacy of PTH was also demonstrated for a rat calvarial defect model in a similar dose range [**123**]. However, the dosage used in these studies was very high, Alkhiary et al. attempted to reduce this dose in a closed femur fracture model in which they used 5–30 μg PTH(1–34)/kg body weight per day. This low-dose treatment also led to improved bone mineral content and newly formed bone volume [**124**].

In recent studies, PTH was also shown to be effective in improving host integration of graft and callus formation in healing of critical-sized defects when used in conjunction with allografts or inorganic scaffold [**125,126**]. The improved integration of allografts has been attributed to a decrease in peripheral angiogenesis, mast cell accumulation, and fibrosis in presence of PTH [**127**].

The efficacy of PTH has also been demonstrated in models with poor bone healing properties. For example Kim et al. showed that PTH(1–34) significantly improved mechanical strength and callus formation during fracture healing in ovarectomized rats (model for postmenopausal osteoporosis) [**128**]. This was attributed to proliferation of osteoprogenitor cells and elevated osteogenesis by blocking adipogenesis occurring because of deficiency of estrogen [**129**]. Although intermittent systemic exposure to PTH has been the standard method of administration for PTH, continuous localized release has also shown success in animal studies. In one such study, poly(ethylene glycol) (PEG) hydrogel was covalently functionalized with RGD and PTH(1–34) peptides, and this hydrogel was used for the treatment of mandibular defects with a diameter of 1.5 mm. The hydrogel containing PTH-derived peptide supported faster bone healing in a canine model [**130**].

Although PTH has demonstrated widespread success as a bone anabolic factor, its use is not without the risk of serious complications. A study in rats showed that PTH(1–34) treatment for 2 years led to the development of osteosarcoma in a dose-dependent manner [**131**]. Because the results in rats were not predictive of the clinical outcome in humans (as supported by clinical trials), FDA approved the drug

(Teriparatide [intermittent PTH]), but with a condition that these findings be notified in a black-box warning in the product insert.

Wnt

Wnt proteins are secretory proteins that participate in signal transduction pathways via cell surface receptors and are recognized for their central role in embryonic development of limb [**132**]. However, the role of Wnt proteins is not limited to development of skeleton. It has been demonstrated that several Wnts and their receptors are expressed at high levels in healing bone, indicating their role in regeneration of bone [**133,134**]. Wnt proteins act either in a β-catenin-dependent (canonical pathway) or independent manner (noncanonical pathway), both of which contribute to the healing of bone. The role of the noncanonical pathway has been thought to be more relevant in the earlier phases of bone healing, in which it probably supports chondrogenesis and formation of primary cartilage [**135**]. Whereas, the canonical pathway was shown to be involved in the later stages, that is after osteoblastic commitment. It was demonstrated that upregulation of activated β-catenin specifically in osteoblasts led to a dramatic increase in the healing of bone in mice by stimulating osteoblast proliferation [**136**].

Because of the significant involvement of Wnt signaling in bone healing, several studies have explored the therapeutic potential of Wnt proteins in augmenting bone regeneration. In one such study, the authors delivered Wnt3a (canonical pathway) protein via liposomes at the site of skeletal injury in mice. The addition of Wnt3a not only promoted proliferation of the skeletal progenitor cells but also accelerated the differentiation, leading to faster healing of the bone [**137**]. In another study, it was shown that brief exposure of Wnt3a via liposomal vehicles to peri-implant tissue led to rapid osteogenic differentiation and improved osseointegration of the implant [**138**]. Pre-exposure of Wnt3a to grafts before implantation has also been shown to restore osteogenic competency to syngeneic bone grafts taken from aged animals. Wnt3a pre-exposure led to significantly reduced cell death in the autograft and rapid osseous regeneration [**139**].

In addition to direct use of Wnt proteins, pharmacological inhibitors of intracellular blocker of Wnt signaling (glycogen synthase kinase-3β [GSK-3β]) and extracellular antagonists (Dickkopf [DKK], Sclersostin, Wnt inhibitory factor-1) have also been used to improve bone healing. One example of this is lithium, which can competitively inhibit GSK-3β, leading to stabilization of β-catenin and upregulation of canonical Wnt signaling. On the basis of this, lithium chloride has been explored for augmenting bone regeneration. In a recent study, lithium chloride was used for enhanced midpalatal suture expansion. The study demonstrated that lithium promoted β-catenin signaling and osteoprogenitor proliferation, which together promoted rapid midpalatal expansion [**140**]. Another study showed that strontium could upregulate β-catenin expression and improve osteogenesis in vitro and bone formation in vivo [**141**]. Inhibitors of the extracellular antagonists of Wnt signaling have also drawn attention; most often, humanized neutralizing antibodies for these antagonists have been

synthesized. Glantschnig et al. generated immunoglobulin (Ig)-Gs against DKK protein, and introduction of these antibodies into naïve mice led to significant improvement in trabecular bone volume and structure. Furthermore, it increased both trabecular and cortical bone mineral densities in a dose-dependent fashion, indicating that bone tissue moved into more active anabolism [**142**]. The aforementioned studies demonstrate the potential of targeting the Wnt signaling pathway either by using Wnt proteins or other molecules. However, this area is still emerging, and results from future studies may better decide the applicability of these agents in therapy.

Hh Proteins

Hh proteins are key regulators that play a crucial role in development, pattern formation, and cell proliferation. This family in vertebrates consists of three proteins: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh). Although the protein was discovered in the invertebrate *Drosophila,* its vertebrate homologs play a crucial role in chondrogenesis and limb development [**143**]. In fact, early reports suggest that their role is so crucial that mice lacking Shh fail to form the vertebral column, ribs, and distal limb elements [**144**]. Later, it was shown that Ihh is also crucial for endochondral bone development, and mutants lacking Ihh fail to regulate chondrocyte maturation and osteoblast development [**145**]. At the molecular level, it has been shown that the presence of Shh abolishes adipogenic differentiation and significantly enhances BMP-mediated osteogenesis. This response was at least partly mediated via SMAD-dependent upregulation of BMPs [**146**]. In another study, the anabolic effects of Shh were demonstrated in organ culture. It was concluded from this study that Shh treatment led to a significant increase in endochondral ossification via BMP- and Cbfa-1-dependent pathways [**147**]. Further, the role of Hh signaling has unambiguously been shown in a mouse bone autograft model in a study by Wang et al. In this study, the authors deleted Smoothened (Smo) protein (the receptors of Hh signaling) and observed that this led to reduced osteogenesis of periosteum-derived stem cells. Furthermore, there was also a 50 % reduction in periosteal bone callus formation, indicating the role of Hh signaling in adult bone repair [**148**].

The involvement of Hh signaling in bone repair prompted research for the use of Hh proteins in therapeutic applications. In one such study, Shh gene-transduced cells were delivered via alginate/collagen matrices into calvarial bone defects in rabbits. The study demonstrated that cells carrying the Shh gene supported significantly higher bone generation as compared with control cells. Further, this treatment did not seem to adversely affect any other tissue on necropsy [**149**]. In addition to individual treatment, combinations of Hh proteins with other growth factors have also been explored. Song et al. co-transduced cells with Shh and basic FGF (bFGF) before implantation in calvarial defects. The study demonstrated that the combination of the two factors showed synergistic effects on new bone formation [**150**]. However, in another study involving the use of β-TCP-based scaffold in conjunction with BMP-2 or N-Shh (N-terminal fragment) or both for the treatment of critical-sized defects in rat femur

showed contrasting results. They observed that the stiffness of explants of N-Shh-containing constructs were lower than β-TCP control. Furthermore, based on histological examination, they concluded that addition of N-Shh delayed the bone healing response; however, it led to a higher amount of cartilage becoming ossified [**151**]. Thus, the effects of Hh proteins when used in conjunction with scaffolds and growth factors are not completely elucidated. With the few studies in animal models, it is difficult at this juncture to predict the form and combination in which Hh proteins may be useful for bone regeneration.

Angiogenic Factors

Osteogenesis is closely co-related to and dependent on angiogenesis in the fracture callus [**13**]. In native wound healing, angiogenesis occurs because of secretion of VEGF from hypertrophic chondrocytes, as a result of which blood vessels invade the primary cartilage template followed by endochondral ossification [**10**]. However, angiogenesis in synthetic bone grafts remains a major hurdle in bone tissue engineering; hence, angiogenesis in bone grafts continues to be a topic of active research [**152**].

Angiogenesis is critical because the newly formed vessels are responsible for transportation of nutrients, gases, hormones, and waste products [**153,154**]; furthermore, progenitor cells are recruited along with new vessels, thus helping in regeneration. In addition, it has been demonstrated that the endothelial cells of blood vessels produce growth factors such as IGF-I and BMP2, which in turn mediate proliferative and osteoinductive effects.

Several growth factors are known to assist in angiogenesis, including VEGF, FGFs, PDGF, IGFs, TGF-β, and BMPs [**152,155–158**]. The most crucial factor is VEGF, which acts by enhancing the proliferation, migration, and morphogenesis of endothelial cells into capillary-like structures [**155**]. Use of VEGF is further beneficial because of its role in bone repair, in which it promotes migration and differentiation of osteoblasts [**159,160**].

Most other factors like FGFs, PDGF, and BMPs indirectly enhance angiogenesis by stimulating osteoblast proliferation and upregulation of VEGF expression. IGFs are also known to enhance angiogenesis by stimulating proliferation of endothelial cells. Although other factors have been shown to play a role in angiogenesis, VEGF has been relatively well explored because of its direct and crucial role in angiogenesis [**161**].

In a study performed by Geiger et al., the authors demonstrated that a gene-activated collagen matrix carrying plasmid DNA encoding VEGF could effectively accelerate healing of large segmental defects. They also showed that after 6 weeks of implantation, VEGF-carrying matrices had a 2- to 3-fold increase in the number of blood vessels as compared with the control group [**162**]. Because bone regeneration has a significant involvement of other osteoinductive factors, VEGF has also been used in conjunction with these factors. In one such study, the authors fabricated scaffolds that enabled the release of VEGF and BMP-2 in a sequential manner. Their results indicated that although VEGF alone could not induce bone formation, it significantly

enhanced the vascular invasion into the grafts. Furthermore, they demonstrated that a combination of VEGF and BMP-2 performed much better in terms of bone formation and vascularization as compared with BMP-2/VEGF alone in ectopic implants [**163**].

Platelet-Rich Plasma

Platelets are reservoirs of several biologically active proteins, including growth factors that enable faster healing of injuries. Platelet-rich plasma (PRP) is a fraction of blood isolated by centrifugation procedures to enrich platelet content. PRP enables accelerated healing by promoting cell migration, proliferation, and differentiation at the site of injury [**164**].

Thus, PRP acts as a cocktail of bioactive molecules that are stored as alpha and dense granules; the cocktail includes [**165,166**]

- Cell adhesion molecules: fibrinogen, fibronectin, and vitronectin
- Growth factors: PDGF, IGF-1, and Epidermal growth factor (EGF)
- Angiogenic factors: VEGF, angiopoietin-2, and FGF-2

Most of these factors can be released from the granules upon activation of platelets, thus making platelet activation a critical step before injection. Although several protocols for activation of PRP have been reported, some of the more widely used protocols are as follows:

- *Exogenous thrombin:* This method of activation results in bolus release of growth factors, with most of the growth factor being released within the first few hours [**167**].
- *Calcium chloride:* Ca^{2+} ions enable the conversion of prothrombin to thrombin, which results in the formation of a fibrin matrix with activated platelets trapped in it. This method results in gradual release of growth factors over a period of 6–7 days [**164**].
- *Collagen I:* Collagen type I has been reported to successfully activate platelets; thus, it has been used in conjunction with PRP [**168**].

It has been reported that PRP enhances fracture healing by playing a crucial role in chemotaxis, cell proliferation, cell differentiation, and bone formation [**169–172**]. It further enhances healing indirectly by initiating angiogenesis. Recruitment and proliferation of endothelial cells by PRP is one of the major strategies by which it initiates angiogenesis [**173**]. Because of its beneficial effects and ease of isolation, PRP was first used by Marx in 1996 for fracture healing [**174**]. In this clinical study, autografts were supplemented with autologous PRP, and the results showed that there was a significant increase in maturity and consolidation in the group of patients that were co-administered PRP. It was also observed that patients administered PRP showed a significant increase in trabecular bone density [**174**].

Although PRP has shown exciting results in some studies and has been established as a safe procedure because of its autologous nature, there are conflicting reports

in the literature regarding its usefulness in the healing process. For example, a recent clinical study used cancellous allografts with or without PRP for the treatment of edentulous ridge defects. The results of the study indicated that PRP enhanced bone regeneration and horizontal bone gain [**175**]. However, in another study, no additional benefit was observed when human-derived PRP was used in conjunction with a collagen carrier for the treatment of a long bone defect in sheep [**176**]. Likewise, there have been reports of PRP being ineffective for the treatment of intrabony defects for guided tissue regeneration in the clinical setting [**177**]. These differences may be due to the disparity in platelet concentration used in PRP, the protocol for activation of PRP, and the protocol for administration of PRP. Thus, in the current scenario, it is difficult to draw conclusions on the usefulness of PRP in fracture healing. More controlled clinical trials need to be performed before it can be successfully used in the clinic.

MODES OF GROWTH FACTOR INCORPORATION IN BONE GRAFT SUBSTITUTES

In the past, significant advancements have been made in understanding the science of growth factors in terms of their mode of action, effective concentration, and interactions with cells and biomaterials. It has been demonstrated that natural bone healing involves a cascade of events in which various soluble factors act in a highly orchestrated manner. This regulated expression of different factors for effective healing has led to the need of precise temporal control over the presentation of soluble factors for successful translation of growth factor therapy to the clinic. A study that clearly demonstrated this fact involved a comparison between sequential and simultaneous release of BMP and IGF. The results unequivocally showed that sequential delivery of these factors performed significantly better over their simultaneous delivery in terms of upregulation of alkaline phosphatase activity and matrix mineralization [**178**]. Hence, controlled presentation of growth factors has drawn much attention and, as a result, various methods have been explored. The methods have broadly used the following physicochemical phenomena for controlling the release kinetics of growth factors: diffusion of factor, charge interactions, covalent interactions, degradation of polymer, and the osmosis wetting phenomenon [**15,179**]. On the basis of these phenomena, the different approaches pursued for growth factor delivery include (1) physical encapsulation of growth factor in the delivery vehicle, (2) ionic complexation and affinity binding of growth factor into or onto the matrix, (3) covalent binding of growth factor with the polymer substrate, and (4) growth factor delivery through gene delivery.

Physical Encapsulation

Physical encapsulation involves the mixing of a growth factor with carrier material (e.g., synthetic/natural polymer solutions) before solidification/crosslinking. In this

method, the growth factors show a slow release profile and the release kinetics are governed by the diffusion of the soluble factor and degradation kinetics of the carrier material. Hence, the release kinetics of the growth factor can be controlled by altering the amount of the encapsulated growth factor, size, and geometry of the carrier device; the crosslinking density; and the material properties (molecular weight, susceptibility to degradation, mode of degradation, and swelling properties) [**180,181**]. Control of growth factor release kinetics obtained by virtue of physical encapsulation averts the unwanted cytotoxicity and inflammation caused due to the supraphysiological doses and ectopic bone formation seen in the case of direct administration of growth factors [**182**].

Physical encapsulation of growth factors in matrices can be accomplished by a wide variety of fabrication techniques, which include solvent casting and particulate leaching, freeze drying, phase separation, emulsion solvent evaporation, in situ polymerization, and gas foaming [**183**]. However, the bioactivity of the growth factors needs to be maintained during the process, which can be achieved by minimizing the exposure of growth factors to harsh environments such as high temperatures and solvent concentrations during the process of encapsulation [**181**]. In addition, the nature of the carrier and pH have implications in modulating the release kinetics or bioactivity of growth factors, leading to a differential bone healing response [**63**].

A combination of two or more fabrication techniques can also be used for sustained release of growth factors and to retain bioactivity. For example, gas foaming has been combined with particulate leaching to deliver bioactive growth factor in vivo that led to sustained release of factor and as a consequence resulted in improved tissue formation. To prevent initial burst release, growth factors can be encapsulated into the bulk polymer using techniques such as solvent casting that in turn can be incorporated into scaffolds using techniques such as gas foaming, resulting in sustained release of growth factor. A similar concept has been demonstrated in a study that made use of PLGA microparticles embedded in calcium phosphate cement implants for the delivery of rhBMP-2, which resulted in sustained release and consequent bridging of critical-sized defects in a rat model [**184**].

A special case of physical encapsulation is when stimulus-responsive release of the factors can take place. In this approach, different physical stimulus-sensitive polymers and biomimetic materials undergo reversible stimulus-dependent changes, enabling release of drug/growth factor under physiological conditions [**180**]. Stimulus-responsive release-based delivery systems release the growth factors in response to external stimuli such as temperature, pH, electric field, magnetic field, light, ultrasound, solute concentration, enzyme, etc. [**15,181**]. In one such study, a pH /thermosensitive block copolymer has been used for encapsulating human MSCs and rhBMP-2. Subcutaneous injection of this polymer solution into mice resulted in formation of ectopic bone with high alkaline phosphatase activity and mineralization [**65**]. In another study by Lutolf et al. [**185**], the authors used protease-sensitive collagen-mimicking synthetic hydrogels to deliver rhBMP-2 in rat cranial defects. They used

poly(ethylene glycol) (PEG)-based hydrogels crosslinked with MMP-sensitive ligands that led to enhanced release of rhBMP-2 in the presence of MMPs. A similar concept has also been used for the delivery and on-demand release of VEGF for angiogenesis [**186**].

Changes in ionic concentration, temperature, pH, light, and electric fields can also trigger the release of growth factors upon stimulation; hence, they can be used in growth factor delivery systems [**181**]. Nevertheless, the application of such system is not well explored in bone tissue engineering, although they possess the potential to be used as growth factor release matrices.

Ionic Complexation and Affinity Binding

Some studies use the ionic- and affinity-based interactions between growth factors and the matrix for their controlled presentation. These approaches derive inspiration from the natural presentation of growth factors by the ECM, in which it presents growth factors in a controlled manner through ionic- and affinity-based interactions. Further, it has been shown that the ECM also has the ability to modulate growth factor activity, improve proteolytic stability, and initiate differential signaling. For example, it has been demonstrated that although free growth factor-receptor complexes are rapidly endocytosed, an ECM–growth-factor-receptor complex physically prevents such endocytosis, leading to constitutive signaling [**187,188**].

Previous studies have shown that growth factors can be adsorbed on the surface of matrices via direct charge-charge interactions between growth factors and the matrix or via indirect interactions through intermediates such as heparin, plasmin, gelatin, or their mimics [**189–191**].

One such study has demonstrated sustained BMP-2 release from heparin-BMP-2 complexes at the fracture site to enable enhanced bone formation [**192**]; however, heparin has been associated with disadvantages such as internal bleeding because of the strong anticoagulant properties of heparin [**193**]. To overcome this limitation of heparin, a bioinspired approach that mimics heparin while eliminating its deterioratory effects has been attempted by using alginate sulfate. Similar to heparin, alginate sulfate demonstrated affinity-based high growth factor binding activity while circumventing the possibility of internal bleeding. The authors demonstrated the potential of alginate-sulfate-containing hydrogels to present TGF-β1 and BMP-4, which showed sustained release of the growth factor leading to enhanced chondrogenic and osteogenic differentiation of human MSCs, respectively [**191,194**].

Furthermore, synthetic sulfated colominic acid, another heparin mimic, has also been shown to potentiate the mitogenic activity of FGF without any cytotoxicity, suggesting its possible use as an FGF potentiator in bone healing [**195**]. In another study, sulfonated silk fibroin was shown to protect the potency of FGF-2, leading to enhanced signaling activity in human MSCs [**196**]. Taken together, the aforementioned studies demonstrated the potential of affinity-based strategies not only in controlled release of growth factors but also in the modulation of growth-factor-mediated signaling.

Covalent Binding

Growth factors can also be tethered to matrices via covalent binding, which leads to prolonged presentation of growth factors to cells. Furthermore, covalent tethering of growth factors provides an ability to precisely control the spatial distribution (e.g., establish gradients of growth factors) and amount of these factors in the matrix. Covalent binding of TGF-β2 with bovine dermal collagen using a PEG linker has been shown to have long-lasting response in vivo as compared with free TGF-β2 in collagen gels [**197**]. Likewise, in another study, Pohl et al. used surface-bound rhBMP-2 via self-assembled monolayer and demonstrated that covalently bound BMP-2 led to enhanced SMAD 1/5/8 activation and osteogenic phenotype in comparison to control [**198**]. The osteoinductive potential of covalently bound BMP has also been demonstrated on plasma-treated metallic implants in which the presence of covalently bound BMP-4 led to spontaneous osteogenic differentiation of MSCs [**64**].

Covalently bound synthetic mimics of various growth factors have also been shown to increase bone regeneration. For example, presentation of synthetic BMP-2 derived peptide covalently tethered to alginate hydrogels showed enhanced bone restoration in rat tibial bone defects [**199**].

However, a major issue associated with the covalent binding of growth factors is the potential loss of bioactivity of the bound factors because of the possibility of blockage of active sites during the process of immobilization. Hence, greater control over the process of immobilization of growth factors is desirable for increasing the efficacy of covalently bound growth factor-based systems.

Growth Factor Incorporation through Gene Delivery

The high cost and poor in vivo stability of growth factors are major limitations in the translation of direct growth factor delivery in the clinical setting [**200**]. An alternative to this is the delivery of a gene encoding the growth factor of interest using a localized gene delivery approach [**15**]. Different types of viral vectors such as adenoviral, adeno-associated, retroviral, and lentiviral vectors have demonstrated relatively better transfection efficiency; however, the safety issues associated with them remain a major bottleneck for their clinical use. In contrast, nonviral vectors are generally considered to be safe; hence, they have higher potential for clinical translation. Furthermore, they offer "easy to manipulate" gene delivery systems with higher stability and less immunogenicity. However, the low transfection efficiency associated with nonviral vectors must be overcome before they can be successfully translated to the clinic [**201**].

Reduced levels of gene transfer and cellular expression have been seen in the case of bolus delivery of plasmid DNA in vivo, probably because of the low residence time of plasmids in tissues [**202**]. To increase tissue residence time, polymeric gene delivery systems have been used [**203**]. In one such study, PLGA matrices loaded with plasmid DNA showed sustained release of plasmid from matrices, leading to high transfection of cells. They further showed that delivery of plasmid encoding PDGF led to upregulation of matrix deposition and angiogenesis in neo tissue [**204**].

To overcome the difficulties arising because of low transfection efficiency, poly (ethyleneimine) condensed plasmid encoding BMP-4 was delivered to rat cranial defects. The results demonstrated a 4.5-fold increase in osteogenesis along with increase in osteoid and mineralized tissue density [**66**].

In addition to these, there have been multiple other studies that show the effectiveness of nonviral delivery systems in stimulating bone formation [**205–209**]. Thus, it can be concluded that nonviral vector-based gene delivery systems have the potential for clinical use provided issues such as low residence time of plasmid in the tissue, degradation of the delivery system in vivo, and its interactions with the microenvironment are properly addressed.

STRATEGIES USING SINGLE AND MULTIPLE FACTORS FOR AUGMENTING BONE REGENERATION

Sampath and Reddi in an in vivo bioassay separated BMP from the insoluble demineralized bone matrix, which enabled the use of this factor as a therapeutic molecule for augmenting bone fracture regeneration [**47**]. Later, mass production of BMP and other factors using recombinant DNA technology enabled their use in research and therapy. Various growth factors (BMPs, TGF-β, FGFs, IGFs, PDGF, VEGF, etc.) have been screened since then for their ability to induce accelerated fracture healing. Most of the early studies concentrated on the local application of single growth factors for bone regeneration. However, soon it was understood that a more sustained release of the factors was desirable as compared with a single bolus delivery. Subsequently, many systems have been developed for controlled administration of these factors. **Table 7-1** summarizes the growth factors that have been explored as single therapeutic molecules along with their overall biological effects and the methods of incorporation used.

Early attempts on growth-factor-assisted bone regeneration focused on the use of BMPs; however, systems with improved delivery kinetics and scaffold architecture are still being developed. In a recent study, a hybrid scaffold consisting of an outer tube made of electrospun polycaprolactone mesh and inner alginate hydrogel with rhBMP-2 was developed for guided bone regeneration (GBR). The results from this study indicated that the construct enabled effective bridging of critical-sized segmental bone defects with osseous tissue [**71**]. Although osteoinductive molecules such as BMP have shown success in enabling the osteogenic differentiation of MSCs, they have little or no contribution toward vascularization in the newly formed bone, which necessitates the use of other factors such as VEGF. It has been demonstrated unequivocally that VEGF promotes vascularization. In one such study, GBR procedures delivered VEGF via an injectable alginate hydrogel into critical-sized rat calvarial defects. Controlled release of VEGF from alginate hydrogel led to increased blood vessel density as compared with no VEGF and bolus delivery of VEGF. Increased angiogenesis consequentially resulted in significantly improved bone regeneration [**210**].

TABLE 7.1 Summary of Growth Factors Used for Augmenting Bone Regeneration along with Method of Incorporation and Their Major Biological Effects

Class	Growth Factor	Method of Incorporation	Major Biological Effects	References
Inflammatory	IL-1	Localized injection of IL-1β	Small increase in endochondral ossification	**[26]**
	TNF-α	Localized injections of TNF-α	Increased fracture callus mineralization	**[23]**
	SDF-1α	Micro-osmotic pump	MSC chemotaxis, increased angiogenesis, and fibrotic response	**[211]**
Proliferative and Osteoinductive	TGF-β	Physical encapsulation/physical adsorption	Enhanced chondrogenic differentiation, osteoblast proliferation, and increased bone formation	**[37–44]**
	BMP	Physical encapsulation/affinity binding/covalent binding/stimulus-responsive material/plasmid mediated delivery	Maximum osteoinductive effect, recruitment of progenitor and osteoblast cells, promote cell proliferation and differentiation, accelerated matrix mineralization	**[56,58–60,62–75,228–242]**
	IGF	Physical encapsulation/ionic complexation/systemic administration (subcutaneous)	Reduced inflammation, increased matrix deposition, improved bone formation, and reduced bone resorption	**[112,113,116–119]**
	FGF	Localized injection/physical encapsulation	Proliferation and differentiation of osteoblasts, improved osteoid formation, improved bone formation, increased/no effect on bone mineral density, higher bone union	**[83,87–93]**
	PDGF	Physical encapsulation	Chemotactic and mitogenic effect on cells of mesenchymal and endothelial origin, accelerated bone formation and calcification, upregulated VEGF expression, and enable faster fracture healing in diabetic, geriatric, and osteoporotic model	**[97-99,243–245]**
	PTH	Subcutaneous systemic injections	Increased callus formation, elevated osteogenesis and blocking adipogenesis, increase bone mineral density, decreased peri-implant fibrosis	**[122–130]**
	Wnt	Localized delivery of recombinant Wnts via liposomes	Promote chondrogenesis and primary cartilage formation (noncanonical pathway), promote osteoblast proliferation (canonical pathway), improve survival of aging autografts	**[137–139]**
		Systemic delivery of nonprotein-based pharmacological stimulators of Wnt signaling		**[140–142]**
	Hh	Physical encapsulation/physical adsorption/viral gene delivery	Enhance BMP-mediated osteogenesis, abolish adipogenesis, increased endochondral ossification, delayed healing response	**[149]**
Angiogenic	VEGF	Covalent binding/affinity binding/physical encapsulation/adsorption/plasmid-mediated delivery	Increased infiltration and proliferation of endothelial cells, improved angiogenesis and bone regeneration	**[162,210,246–248]**

Although osteoinductive and proliferative factors are the most explored among different classes of factors that have been used for promoting bone regeneration, cytokines and factors that are part of the inflammatory cascade cannot be neglected. In a recent attempt, stromal cell-derived factor-1α (SDF-1α) was delivered through a micro-osmotic pump in a PLGA scaffold. The study demonstrated that incorporation of SDF-1α led to reduced inflammatory and fibrotic response and a concomitant increase in stem cell recruitment and angiogenesis in the implanted scaffolds [**211**].

Although single-factor delivery has seemingly shown exciting results in vitro and in vivo, a major limitation that prevents its translation to the bedside is the need to administer supraphysiological doses of growth factors for desirable results. This not only makes the treatment extremely expensive, but it is also disadvantageous because of the undesirable effects of such large doses—namely ectopic bone formation, risk of abnormal bone formation, and life-threatening cervical swellings [**182,212,213**]. Furthermore, regardless of how potent the effect of an individual factor may seem, it cannot mimic the native healing process, which is regulated by a highly orchestrated crosstalk between several growth factors and cytokines. Therefore, much focus has been directed toward the use of combinations of growth factors. Growth factors used in combination systems may interact synergistically or antagonistically to support or counteract each other. This interaction not only depends on the combination of growth factors used but also on their dosage, ratio, sequence of release, release kinetics, and experimental system. The effect of dosage/ratio was clear from recent studies that demonstrated that when scaffolds containing BMP-2 were supplemented with low doses of FGF-2, it facilitated bone regeneration, leading to a synergistic effect. However, when the amount of FGF-2 was increased, it led to inhibition of BMP-2 activity and poor bone regeneration [**214–216**]. The inhibitory effect at higher concentrations of FGF may be attributed to the dominance of its mitogenic effects over the BMP-induced differentiation response. Furthermore, this interaction could be explained by the dose-dependent increase in the expression of the inhibitory SMAD (SMAD6) by FGF, which led to an eventual decrease in bone formation [**214**].

Likewise, the sequence of administration of the factors also plays a crucial role in regulating their overall effect. For example, when IGF was used in combination with BMP-2, it was observed that although sequential delivery of BMP followed by IGF resulted in increased alkaline phosphatase activity and matrix mineralization, simultaneous delivery was plagued with counteracting effects, resulting in no enhancement in activity as compared with the controls [**178**]. Thus, it becomes extremely relevant to design systems with appropriate control over the dosage, sequence, and release kinetics of the growth factors. A summary of the various growth factor combinations used for augmenting bone regeneration, modality of administration, method of incorporation, and their overall biological effects have been listed in **Table 7-2**.

TABLE 7.2 Summary of Growth Factor Combinations Used for Augmenting Bone Regeneration along with Modality of Administration, Method of Incorporation, and Their Major Biological Effects

Factor I	Factor II	Modality of Administration	Method of Incorporation	Major Biological Effects	References
IL-6	PTH	Sequential PTH → IL-6	Localized injection at fracture site	Accelerated bone regeneration	[**249**]
SDF-1	BMP-2	Simultaneous	Physical encapsulation/adsorption	Synergistic effect	[**6,250,251**]
IL-11	BMP-2	Simultaneous	Physical encapsulation/adsorption	Synergistic effect	[**252,253**]
PGE2	BMP-2	Simultaneous	Localized injection	Enhanced regeneration in aged rats	[**254**]
TGF-β	BMP-2/7	Simultaneous	Physical encapsulation/adsorption	Enhanced bone formation	[**255–259**]
				Nonsignificant	[**260**]
IGF-1	BMP-2	Simultaneous	Physical adsorption	Significant increase in osseointegration	[**261**]
IGF	TGF-β	Simultaneous	Physical encapsulation/adsorption, localized injection	Significant increase over individual factors	[**262,263**]
				No increase as compared with TGF-β	[**264,265**]
FGF	BMP-2/6	Simultaneous	Physical encapsulation/adsorption	Synergistic effect at low dosage of FGF	[**9,214–216,266**]
		Sequential FGF → BMP-2	Physical encapsulation	Inhibitory effect	[**8**]
PDGF	BMP-2	Sequential PDGF-BB → BMP-2	Physical encapsulation and gene-mediated delivery	Enhanced bone regeneration in vivo	[**7**]
		Simultaneous	Affinity binding	Inhibitory effect of PDGF on healing	[**267**]

(Continued)

TABLE 7.2 (Continued)

Factor I	Factor II	Modality of Administration	Method of Incorporation	Major Biological Effects	References
PDGF	IGF-1	Simultaneous	Physical encapsulation	Synergistic effect	[**268**]
BMP-2	N-Shh	Simultaneous	Physical encapsulation/adsorption	Higher proportion of cartilage ossifying but delayed healing response	[**151**]
Shh	bFGF	Simultaneous	Gene transduced cells	Synergistic effect	[**150**]
Ihh	BMP-2	Simultaneous	Gene transduced cells	Synergistic effect	[**269**]
VEGF	BMP-2	Sequential VEGF → BMP-2	Physical encapsulation, gene-mediated delivery	Significant increase in bone formation	[**163,270,271**]
		Simultaneous	Physical encapsulation	Synergistic effect	[**272,273**]
PDGF	VEGF	Sequential PDGF → BMP-2	Physical encapsulation/adsorption	Enhanced bone formation	[**274**]
PRP	BMP-2	Simultaneous	Physical encapsulation	Synergistic effect	[**275**]
				Nonsignificant	[**276**]

CHALLENGES ASSOCIATED WITH GROWTH-FACTOR-BASED THERAPIES

Although much success has been obtained in growth factor therapy in many preclinical studies, its widespread and off-label use in patients has not been approved and may not be approved in the near future because of multiple challenges. Several issues need to be clarified before the wider acceptance of growth factors as therapeutics. These issues include but are not limited to immunogenicity, risk of cancer associated with certain growth factors, and cost-to-benefit ratio.

The risk of immunogenicity of recombinant proteins has been a very important issue that is most often detected during clinical trials or after product launch. Although in the case of rhBMP-2 and OP-1 immune response has been reported only in a small subset of patients, further studies need to be performed for a wider set of patients and dosage patterns [**217–219**]. In addition, new complications arising because of recombinant proteins have been reported in the case of other proteins, such as recombinant erythropoietin (EPO), in which the immune reaction cross-reacted with endogenous EPO, leading to pure red cell aplasia [**220**]. The possibility of such complications is still unknown for growth factors being used for bone healing. Furthermore, issues such as safety of these factors in pregnant women, developing fetuses, and on multiple dosing is yet to be clarified. The use of rhBMP-2, especially for spinal fusion, has been challenged because of a postoperative acute inflammatory response that has been reported as a consequence of rhBMP-2 treatment. In a case study, it was reported that the use of rhBMP-2 led to serious adverse effects such as massive neck swelling including pharyngeal tissue. The patient had to be admitted to an intensive care unit and intubated, and parenteral steroids were administered to decrease swelling [**221**]. Because of the risk of such unforeseen complications, the FDA has cautioned the off-label use of recombinant growth factors.

Another crucial challenge is to overcome the risk of cancer, which has been associated with several growth factors for some time. In an animal study involving rats, it was observed that prolonged exposure of PTH to rats for 2 years led to the development of bone neoplasia in a dose-dependent manner [**131**]. Although the study is not predictive of the response in humans, risk is associated with PTH therapy, because of which its use beyond 2 years is not advised. In addition, several other factors such as IGF and FGF have also been associated with risk of tumorigenicity because of their strong ability to promote mitogenic response in cells. A study by Toniolo et al. showed that higher circulating IGF-1 levels correlated with increased risk of premenopausal breast cancer [**222**]. Likewise, plasma IGF-1 levels have also been associated with increased risk of prostate cancer in men [**223**]. Growth factors such as FGF not only play a role in mitogenic stimulation but also promote epithelial-to-mesenchymal transition and possibly promote metastasis [**224**]. The risk increases further in elderly patients, making the problems worse because this is the group that most often needs anabolic factors to augment bone fracture healing. Such problems make it imperative

that more thorough studies be done to evaluate safe dosage and mode of delivery to minimize the risk of cancer in patients taking growth-factor-based therapeutics.

Lastly, it is very important for any new or emerging technology to be economically viable. The economic assessment of a new health-care technology is based on clear criteria of cost versus benefit. The new treatment modality is categorized as (1) "better and cheaper," (2) "worse and more expensive," or (3) "better but more expensive" in comparison with the standard treatment [**225**]. Although the decision-making for treatments falling in the first two categories is relatively easy, a much more complex decision process is involved for those classified in the last category. As of now, clinically successful growth-factor-based bone graft substitutes will most often classify into the third category. Computing the cost benefit for these technologies encompasses several factors, such as clinical benefits, cost of equipment, hospital time, unemployment costs, and cost of caretakers outside of the hospital [**226**]. Although growth-factor-based therapy adds to the direct costs of treatment, they may significantly improve clinical benefits and reduce cost by avoiding secondary surgeries, autograft donor site complications, and faster healing time, which reduces the unemployment costs. Garrison et al. have reported that although BMP treatment for open tibial fractures may be cost-effective, the cost-effectiveness ratio is sensitive to the price of BMP and the severity of open tibial fractures. Hence, in this case, the cost-effectiveness may be improved by reducing the cost of BMP and using it only in extremely severe cases. Furthermore, according to their economic evaluation, the use of BMP is unlikely to be cost-effective in spinal fusion [**227**]. Thus, there is an impending challenge to not only develop newer and better growth-factor-based grafts but also to make them economically more viable.

SUMMARY

Growth-factor-based bone graft substitutes have shown strong potential in preclinical studies. Several products have also been tested clinically and have demonstrated therapeutic potential. Areas of application for these new technologies include acceleration of fracture healing, treatment of nonunions, enhancement of spinal fusion, treatment of periodontal defects, and treatment of significant bone loss. Comprehensive strategies for therapeutic applications combine concepts of tissue engineering and growth factor delivery mechanisms for cellular expression toward desirable bone healing effects.

Several BMP-7/OP-1-, BMP-2-, and PDGF-based products are already approved by the FDA for their therapeutic use in spinal fusion (OP-1™ Putty and InFUSE™ Bone Graft/LT-CAGE™), fractured bone (OP-1™ Implant), and periodontal defects (GEM 21S and INFUSE® Bone Graft). Furthermore, PDGF-based AUGMENT® BONE GRAFT has been approved for use in foot and ankle fusion surgeries and distal radius fractures in Canada, Australia, and New Zealand. However, there are other growth factors and cytokines that are actively being investigated for similar purposes.

Use of single factors is bedeviled by the requirement of supraphysiological doses to obtain desirable effects. Such high dosages have led to complications such as

abnormal/ectopic bone formation, hypersensitive reactions, and suppression of signaling responses due to induced expression of antagonists. Dual/multiple growth factor delivery seems to provide an alternative by which the factors interact and act synergistically, thereby reducing the overall dose requirement. Furthermore, dual factor delivery is advantageous because of its ability to promote two or more diverse functions such as mineralization and angiogenesis, leading to overall accelerated fracture healing. However, it is critical to screen and identify not only the appropriate growth factor combinations but also the dosage/ratio, sequence, and release kinetics. Few in vivo studies have been performed in this direction; however, more systematic studies are required to understand the interaction of growth factors and their underlying mechanism. Concurrently, there is also a need to develop improved delivery systems to precisely control the spatiotemporal release of growth factors. Collective development on all of the aforementioned fronts will ensure faster translation to clinical applications.

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Chapter 8 | Bone Morphogenetic Proteins in Human Bone Regeneration: Successes and Challenges

Pedro Alvarez-Urena,[1] Arun R. Shrivats,[1] Amy M. Donovan,[1] Bruce Doll,[2] and Jeffrey O. Hollinger[1]

INTRODUCTION

One cannot tell the story of bone morphogenetic proteins (BMPs) without first considering the marvelous structure that is human bone. Bone has several properties that make it unique. Highly noteworthy is the intrinsic ability to restore form and function without scarring. Restoration of form and function defines regeneration.

A logical question to pose is, "What is special about bone that permits it to regenerate?" The biological cascade of regenerative osteogenesis is highly complex. Consequently, the answer to the epochal question of why bone can regenerate is commensurately multifaceted. A consortium of cell phenotypes, soluble biological signals, and biomechanical cues collectively integrate in time and space. The consequence of the dynamics of this symphony of elements produces a masterpiece: regeneration.

As tissue engineers, we often painstakingly deconstruct a complex composite tissue, identify the individual elements, and use these elements to attempt to reconstruct a functional tissue engineered product. A similar tactic was exploited with bone; however, the target for discovery focused on a single biological factor that functioned as "the master switch" for regeneration.

Retrospectively, a single target approach trivializes the precise dynamic interactive composite that blends the cells, signaling molecules, biomechanics, space, and time required for bone regeneration. Despite the daunting complexity to discover the master switch for bone regeneration, this goal became the relentless passion for Marshall R. Urist, M.D., and a legion of dedicated workers. Urist's tireless commitment to identify and isolate a factor from bone that would promote its regeneration was heroic, and the outcome of the effort led to the identification, cloning, and expression of human recombinant BMPs.

The notion that bone contained a substance to induce its regeneration has been the inspiration for noteworthy bone pioneers. In 1889, Senn treated osseous defects

[1] Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA
[2] Navy Medicine Research and Development, U.S. Navy, Frederick, MD

using decalcified bone implants [1]. In 1947, Lacroix speculated that a substance within bone possessed properties to incite bone formation in a new location [2]. He termed this substance *osteogenin*. In his pioneering *Science* article in 1965, Urist demonstrated that demineralized bone implanted intramuscularly in a nonbony site induced new bone formation; he termed this process *autoinduction* [3]. This legendary discovery on bone healing and several other significant contributions to fundamental bone biology guided a spirited cadre of dedicated disciples [4]. In 1971 issue of the *Journal of Dental Research*, Urist and Strates coined the terms that would be forever linked to Urist: BMP and *osteoinduction* [5].

The identification of soluble factors within the insoluble demineralized bone matrix was a formidable challenge. The breakthrough in the identification and characterization was accomplished by Hari Reddi and Kuber Sampath using the dissociative extractant guanide hydrochloride [6]. Reddi's epochal work guided the unveiling of BMPs as proteins consisting of approximately 120 amino acids with 7 canonical cysteine-rich residues forming a cysteine knot in the core of the protein [7,8].

The meticulous characterization by Reddi and colleagues revealed BMP homology to members of the transforming growth factor-β (TGF-β) family of proteins; thus, it was logical to house BMP in the TGF-β superfamily. Identification of the BMP amino acid sequences heralded the purification and cloning of the BMPs [9]. Wozney and associates isolated and cloned BMP-2A and -2B (later termed BMP-2 and -4, respectively) [7], and Ozkaynak et al. cloned and expressed BMP-7 (osteogenic protein [OP-1]) and BMP-8 [10] shortly thereafter.

The emphasis for this chapter will be bone regeneration with a focus on recombinant human BMP (rhBMP) as the pivot point. We will underscore BMP and BMP biology. However, the chapter authors first want to provide a short history of BMP to honor the dedicated workers who transformed orthopedic practice for bone regeneration.

It is not practical to assay all clinical options that could involve rhBMP; a book, rather than a chapter, would be appropriate for such an approach. This chapter will discuss contemporary rhBMP biology, biomaterials that may be exploited to deliver rhBMP for clinical applications, regulatory issues with rhBMP, and clinical challenges.

BMP BIOLOGY

Subclasses, Receptors, and Signal Transduction Subclasses of BMP

To date, approximately 20 BMP family members have been isolated, characterized, and categorized into several subclasses on the basis of structure and function. BMPs-1 to -7 are expressed in skeletal tissue; BMPs-2, -4, -6, and -7 are the most readily detectable in osteoblast cultures. BMP-2 and -4 are similar in structure and are highly conserved among species. BMP-2 and -4 function in osteogenic differentiation, and dysregulation of their signaling pathways has been identified in osteochondral

pathologies including heterotopic ossification (HO) and fibrodysplasia ossificans progressiva. BMP-5, -6, -7 (also known as OP-1), and -8 (OP-2) form a subclass of BMPs for which the role extends beyond osteogenesis. For example, it has been noted that deficiencies in embryonic BMP-7 levels in mice lead to mortality as a result of renal failure [11]. True to their name, the ability of BMPs-5, -6, and -7 to induce the osteogenic differentiation of cells has been demonstrated [12–14].

Receptors and Signal Transduction

BMP signals are mediated by type I and type II serine/threonine kinase receptors; these receptors are part of the overall TGF-β signaling pathway and are specific to BMP and activin ligands. Upon ligand binding to a type II receptor, a heterodimer complex is formed with a type I receptor. The kinase belonging to the type II receptor activates the type I receptor and initiates the signaling transduction cascade. There are three type I receptor variants activated by BMPs: activin receptor-like kinase (ALK)-2, ALK3, and ALK6. The BMP type II receptors include BMP receptor II (BMPRII) and activin receptor II (ActRII).

Upon BMP ligand binding, type II receptors are phosphorylated and subsequently recruit and phosphorylate a type I receptor, beginning the transduction of an intracellular signal. The first agents in this signaling cascade are the mothers against decapentaplegics (Smad) proteins; receptor-regulated Smads (R-Smads) are docked with type I receptors and are phosphorylated upon formation of the heterodimeric type I-type II receptor complex. The Smads function in BMP and TGF-β signaling cascades. Key agents in the BMP pathway are Smads-1, -5, and -8, whereas Smads-2 and -3 transduce TGF-β signals. Once the Smad-1/5/8 proteins are released from the cell membrane, they conjoin Smad-4 (known as a co-Smad) to form a heterodimeric complex that translocates into the nucleus. There, the complex recruits transcription factors, co-activators, and co-repressors as per the instructions conveyed by the BMP ligand. Depending on the specific ligand and the signals being transduced, there may be several nuclear effects that modulate gene expression and cell fate.

The complex signaling biological process raises the question, "How do different BMP ligands achieve signal specificity?" The precise answer is not known; however, we believe that not all BMP type I and type II receptors are identical. Rather, they have different binding affinities for different BMP/TGF-β ligands. For example, for type I receptors, ALK3 only binds to BMP ligands, whereas the ALK2 receptor can bind to BMPs and activins. On the type II side, BMPRII will bind BMP ligands whereas the ActRII receptor will also bind to activin ligands [15].

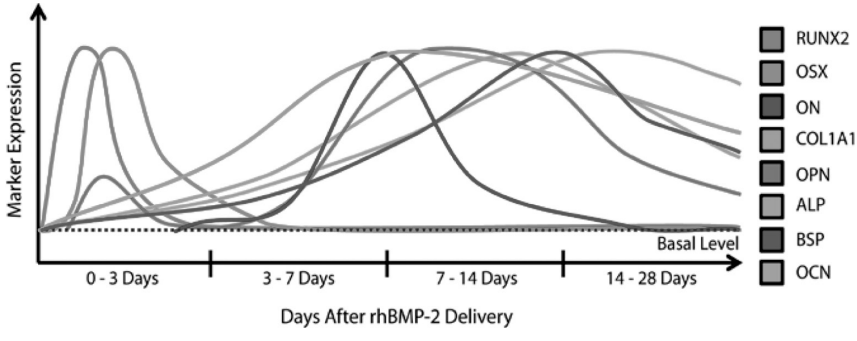
In addition to the canonical Smad-based BMP signaling pathways, BMP ligands upregulate mitogen-activated protein kinase (MAPK) and extracellular regulated kinase (ERK) pathways. These pathways independently regulate downstream targets and act interdependently with the Smad pathway. They activate genes such as alkaline phosphatase (ALP), osteocalcin, and type I collagen [16].

Although the BMPs have potent effects related to osteogenesis, they may also have effects on nonosteogenic cell phenotypes. In this chapter, we focus on the BMP-osteogenic partnership because it represents their greatest clinical potential. BMP-2/4/6 and -7 are most frequently linked to osteogenic activity. BMP-2/4/6 and -7 activate the R-Smad/Smad-4 complexes, which subsequently translocate to the nucleus to promote transcription of *RUNX2* and *OSX*. The *RUNX2* and *OSX* genes encode for the corresponding Runx2 (runt-related transcription factor 2) and Osx (osterix) transcription proteins; these two proteins are the master regulators of osteogenic differentiation.

The transcription and translation of *RUNX2* and *OSX* trigger expression of key osteoblast protein markers and drive osteoblast lineage progression. Upregulated proteins include type I collagen, ALP, osteopontin, osteonectin, and bone sialoprotein. The expression profiles of these key markers have been studied [**17–25**] and are summarized in **Fig. 8.1**.

The bone morphogenetic process that follows BMP induction occurs in a tightly regulated, multistep cascade of signals that mimic embryonic osteogenesis. Key steps include chemotaxis, mitosis, and cell differentiation [**26**]. In response to exogenous rhBMP administration, endochondral ossification may ensue; this includes chondrogenesis antecedent to osteogenesis. Chondrogenesis and osteogenesis occur with predictable cell phenotypes neatly calibrated in packets of time. Specifically, after exogenous rhBMP administration to a recipient, chondrogenesis is evident by 7 days. The hallmark cells for chondrogenesis are chondrocytes. By 9 days post-rhBMP stimulation, the hypertrophic chondrogenic milieu is invaded by vascular fingers as

FIG. 8.1 Osteogenic differentiation markers after rhBMP-2 delivery. Early markers include *RUNX2* and *OSX*. Osteoblast markers expressed at later hours include ON, *COL1A1*, OPN, ALP, BSP, and OCN. *RUNX2*, runt-related transcription factor 2; *OSX*, osterix; ON, osteonectin; *COL1A1*, type I collagen; OPN, osteopontin; BSP, bone sialoprotein; OCN, osteocalcin.
Source: Included with permission from Shrivats et al. [105].



angiogenesis begins. ALP is denoted as an "early" bone marker; by 4–7 days post-rhBMP, ALP is detectable with a crescendo at days 10–12. Osteocalcin is referred to as a "late" bone marker with an activity peak at approximately day 28 [**27**].

However, it has also been reported that exogenous BMP can induce intramembranous ossification (i.e., bone formation without chondrogenesis) in the healing of stabilized fractures [**28**]. BMPs can stimulate bone formation alone by recruiting osteoprogenitor cells and directly inducing them to differentiation to an osteoblast lineage [**28**].

Regulation of BMP Signaling

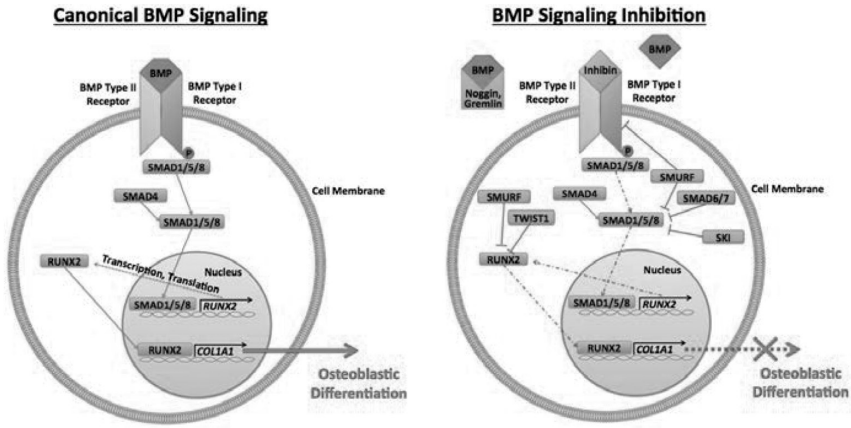
BMPs are robust promoters of bone formation; consequently, it is physiologically necessary that BMPs are tightly regulated. A meticulous choreography using BMP antagonists has been engineered to control BMP activity. Approximately twelve BMP antagonists have been identified; the antagonists titrate BMP activity to maintain bone homeostasis [**13**]. Bone homeostasis (homeostasis: the balance between formation and resorption) is a dynamic and intricate web of cellular, hormonal, and biomechanical cues. There are tightly modulated physiological and biomechanical feedback loops (i.e., positive and negative) to balance bone formation and resorption. We will underscore in this chapter the role for BMPs and their antagonists in the process of bone homeostasis.

Typically, BMP antagonists act in two manners: (1) binding the BMP ligand rendering it inactive, thus preventing BMP ligand interaction with its receptors, and (2) competitively binding BMP receptors, thus preventing BMP ligand attachment (**Fig. 8.2**). In addition to extracellular BMP antagonists, there are intracellular mechanisms to regulate BMP signaling; included in these mechanisms are Smurf, Twist, and Ski proteins [**29,30**]. However, to provide a focused, clinically relevant review of BMP antagonists and their use, we will eschew intracellular methods to shed a greater spotlight on true antagonists of BMP ligands and their receptors.

Noggin is the most well-known and characterized BMP antagonist (i.e., anti-BMP molecule). Noggin is a 222-amino-acid polypeptide that was the first BMP antagonist to be identified [**31**]. It functions by binding BMPs-2, -4, and -7; thus, it may function in the clinic as an inhibitor of vasculogenesis and osteogenesis. Moreover, noggin may prevent the pathology HO [**32,33**]. Noggin expression increases in response to BMP-2 stimulation in myoblasts—a negative feedback mechanism. In addition, silencing of noggin using RNA interference results in an acceleration of BMP-induced osteoblastic differentiation [**34**]. The effects of BMPs are mirrored by noggin in an inhibitory manner. Thus, the clinical scope of noggin is predominantly as an antiossification agent.

Chordin was originally identified in studies analyzing the products of the Spemann organizer [**35**]. Chordin is a 105-kDa protein composed of 941 amino acids and has a strong binding affinity to BMP-2 and -4 [**36**]. In developmental processes, chordin appears to share several similarities with noggin. They both function as

FIG. 8.2 Major components of the BMP signaling pathway leading to osteoblast differentiation and the points of interference of key inhibitors. The *COL1A1* gene (encoding type I collagen) is one of many genes activated downstream of *RUNX2* (encoding runt-related transcription factor 2). Other key markers include osterix, ALP, osteocalcin, osteonectin, and bone sialoprotein. The precise mechanisms by which each of these factors become activated has not yet been comprehensively elucidated. Red dashed lines indicate processes and mechanisms in the BMP signaling pathway that may be reduced by the use of inhibitors.
Source: Modified with permission from Shrivats et al. [**106**].



dorsalizing agents in the developing embryo by binding ventralizing TGF-β proteins. Noggin and chordin share the same phenotypic effect; however, there are key differences between the two. Noggin has an amino acid sequence length of 222, which is roughly one quarter of the length of chordin. There are no homologies in the sequences of noggin and chordin, and evidence suggests that they represent independent parallel signaling pathways. Expression of chordin is regulated by BMP-1, which is a metalloprotease that in hindsight was a misnomer: BMP-1 does not induce bone morphogenesis [**37,38**].

Noggin and chordin bind BMP ligands and prevent BMP ligand-receptor interactions and subsequent signaling cascades from occurring. However, the strength of inhibition differs between them. Noggin is a potent inactivator of BMPs; on the other hand, chordin does not fully inactivate BMP ligands. Chordin causes a reduction, rather than a complete abrogation, of BMP signaling. As such, therapeutic effects (i.e., abrogation of BMP signaling) sought by the delivery of chordin may lack potency in achieving desirable clinical outcomes [**39,40**].

Gremlin belongs to the deadenylating nuclease (DAN) family of proteins, which includes DAN, cerberus, and sclerostin. This family has no sequence similarity to

noggin or chordin; DAN may function in conjunction with other BMP antagonists [**13**]. Gremlin, also termed *downregulated by v-mos* (DRM), is a 20.7-kDa glycoprotein originally isolated from *Xenopus* embryos as an anti-BMP dorsalizing agent [**41**]. Gremlin binds to BMPs-2, -4, and -7 and prevents interactions with BMP receptors. Homozygous null mutations of Gremlin in mice models leads to severely affected limb development and to morbidity [**42**]. Conversely, overexpression of gremlin in mice also leads to upregulated bone resorption, leading to osteopenia and impaired bone formation [**43**]. As such, the importance of gremlin in regulating BMP activity during development and the subsequent remodeling processes cannot be understated [**42,44**].

Other notable BMP antagonists include fetuin, follistatin, and sclerostin. In brief, fetuin binds to TGF-β and BMP ligands; as such, therapeutic administration of fetuin may inhibit aspects of the TGF-β signaling pathway that are crucial for normal physiological functions [**45**]. In addition to inhibiting activins, follistatin may inhibit members of the BMP-5/6/7 subclass, although it does not have significant effects on the BMP-2/4 subclass [**46**]. Finally, sclerostin is not technically a BMP inhibitor; rather, it regulates bone formation through the Wnt (originally coined from the *Drosophila* melanogaster wingless gene corresponding to *int-1* [**47**]) signaling pathway. In terms of potential therapeutic applications to control pathological BMP-induced HO, these BMP antagonists are not as therapeutically compelling as noggin, chordin, and gremlin [**48**].

CLINICAL ISSUES WITH rhBMP-2

Engineering a system to deliver rhBMP remains an epochal challenge for safe, effective, and predictable clinical use. In this section, we present the likely reasons that contribute to challenges for rhBMP delivery systems. We also provide some exciting options to conquer the challenges.

In 2002, after a review of published reports and the safety data on rhBMP-2 and rhOP-1 submitted to the U.S. Food and Drug Administration (FDA) and to the European Agency for the Evaluation of Medicinal Products, Poynton and colleagues concluded neither reproductive toxicity nor adverse clinical effects had been associated with rhBMP-2 and recombinant human OP-1 (rhOP-1) (rhBMP-7) [**49**]. Systemic and local toxicity on organs had not been observed in human or animal studies, and the FDA and the European Agency for the Evaluation of Medicinal Products conceded that no human safety data were available [**49**].

Focused upon the 13 original industry-sponsored rhBMP-2 publications regarding safety and efficacy, including reports and analyses of 780 patients receiving rhBMP-2 within prospective controlled study protocols, the authors of the industry-sponsored publications indicated no rhBMP-2-associated adverse events [**50**].

In 2002, the FDA approved Infuse® for spinal fusion in patients with a degenerative disease affecting the lumbar-sacral vertebrae. Since 2002, Medtronic claimed that Infuse® had been used to treat more than 500,000 patients [**51**]. Reports of complications associated with Infuse® accumulated in the literature and prompted the scrutiny

of a congressional investigation and the medical profession [**50,52–54**]. Senators Baucus and Grassley directed an investigation into Medtronic and the Infuse® product. The Baucus-Grassley report in October 2012 outlined numerous questionable practices conducted by Medtronic and some of the clinicians who used the product [**55**]:

- Medtronic prepared an expert's remarks to the FDA advisory panel meeting before Infuse® approval. At the time, the expert was a private physician. Subsequent to the testimony, the same physician was hired as a vice president at Medtronic in 2007.
- Medtronic's influential role in authoring and substantively editing articles on the efficacy of Infuse® was not disclosed in the published articles.
- Medtronic paid approximately $210 million to physician authors of Medtronic-sponsored studies from November 1996 through December 2010.

Carragee and colleagues conducted a comparative review of FDA documents and subsequent publications that revealed unpublished adverse events and inconsistencies regarding Infuse® [**50**]. Level I and Level II evidence from FDA summaries and published data suggested study design bias in the original clinical trials. An increased risk of complications and adverse events were associated with patients receiving Infuse® for off-label (i.e., non-FDA approved use) use in spinal fusion. The authors estimated that the risk of adverse events associated with Infuse® was 10–50 times higher than the original estimates reported in the industry-sponsored peer-reviewed publications [**50**].

BMP AND BONE REGENERATION

BMP functions as a chemoattractant for osteoprogenitor cells, and it promotes proliferation (i.e., mitogenesis) of osteoprogenitor cells and osteoblast-lineage progression. The clinical outcome of this molecular osteogenic cascade is bone formation.

Administration and localization of BMP to a clinical site to promote an osteogenic regenerative outcome requires a delivery system. Before we underscore how complex the concept of BMP delivery is, let us review some of the biomaterials used to deliver BMPs.

BMP Delivery Systems

Clinically, rhBMP-2 and -7 (rhOP-1) have been therapeutically administered in combination with type I xenogeneic (bovine) collagen, β-tricalcium phosphate (β-TCP), and calcium hydroxyapatite [**56–58**]. It is crucial for therapeutic effectiveness that the release kinetics and dosing of these molecules match the cellular and biological osteogenic cascade.

At the clinical site of administration, BMP initiates the recruitment of mesenchymal stromal cells within 5 days [**59**]. The recruited cells are cued into a chondrogenic lineage within 7 days after implantation [**59**]. In addition, BMPs are angiogenic; angiogenesis is a compulsory phase of osteogenesis. During endochondral osteogenesis, new

vasculature presages chondrocyte hypertrophy, subsequent calcification (by osteoblast-lineage progression), and woven bone formation [**60**]. In addition, the osteoblasts vacate their niche on the bone surface and are replaced with osteoclasts (derived from blood-borne monocytes), which resorb bone. As a consequence of a complex cellular and coordinated osteoblast-osteoclast coupling, woven bone is remodeled to yield lamellar bone and bone marrow elements [**60**]. However, for remodeling to occur during osteogenesis, the delivery system for BMP must match the cellular, mechanical, and biochemical synchrony of the bone wound-healing cascade. What is meant by synchrony is discussed in the next section on the logic for a clinical delivery system for BMP.

Why Is a Delivery System Necessary for the Therapeutic Effectiveness of BMP?

The logic for a BMP delivery system (i.e., carrier) is profound. Without a properly engineered biological carrier, BMP is ineffective. Clinical performance criteria for a delivery system include biocompatibility, biodegradability, intrinsic porosity, surface properties to support cell attachment, sterilizability, mechanical properties that match bone in weight-bearing cases, clinical convenience, and osteoconductivity. Moreover, the delivery system must localize the BMP to the clinical administration site to preclude off-target BMP effects. Lastly, the BMP delivery system must biodegrade at the clinical implantation site in synchrony with new bone formation. Biodegradation that is too rapid may result in soft tissue prolapse whereas biodegradation that lags behind the bone formation rate will impede osteogenesis.

Example Delivery Systems

RhBMP-2 and -7 have been combined with xenogeneic (bovine) type I collagen, β-TCP, hydroxyapatite, synthetic polymers (e.g., poly(lactic-co-glycolic acid)), xenografts, autografts, allografts, and bone-derived extracellular matrix. The preferred rhBMP carrier for the clinic has been type I bovine collagen [**61**]. Type I collagen marketed as Helistat® (Integra LifeSciences, Plainsboro, NJ) has FDA approval for specific, defined orthopedic applications. It is noteworthy that type I collagen makes up more than 90 % of the organic matrix of bone [**62–64**]. Furthermore, rhBMP-2 has an affinity for collagen; this may be explained by the electrostatic interactions between rhBMP and collagen [**65**]. The combination product of bovine type I collagen with rhBMP-2 is marketed by Medtronic-Sofamor Danek (Memphis, TN) as Infuse®. This product will be described more comprehensively in the chapter.

There are major challenges in designing a delivery system for rhBMP. The challenges include dosing; temporal and spatial calibration of rhBMP-2 release with osteogeogenesis; and limiting edema, inflammation, and ectopic bone formation.

RhBMP must be delivered to the clinical site at a sufficient dose to produce a predictable, desired therapeutic outcome. Moreover, the therapeutic rhBMP dose must be delivered in temporal calibration to the dynamics of the osteogenic cascade. Temporal calibration means the delivery of the rhBMP at the precise instance in time when vasculo-osteogenic phenotypes will be available to bind with rhBMP. The precise

definition has not been elucidated unequivocally for the period of time that rhBMP must be delivered to produce the desired clinical outcome between the responding cell phenotypes and the rhBMP. Furthermore, the delivery system must localize the rhBMP for the appropriate packet of time at the site of administration to achieve the desired outcome and minimize the migration of the rhBMP. The migration of rhBMP from the site of administration may result in bone formation in soft tissue; this phenomenon is referred to as ectopic bone formation. Once the delivery system has performed these fundamental roles that are not perfunctory, the delivery system must be neatly and efficiently removed from the clinical application site through physiological biodegradation. The biodegradation of the delivery system must be sufficiently effective to not block bone formation. Moreover, the biodegrading delivery system must remain biocompatible (specifically, as an intact unit and throughout the biodegradation process), and when biodegradation is complete, regenerated bone replaces the delivery system.

Recent clinical reports on Infuse® have noted ectopic bone formation, also described as heterotopic bone formation (i.e., HO) [**66**]. We posit that ectopic bone formation sequela may be a consequence of an ineffective delivery system that does not contain or localize the rhBMP to the site of administration. For example, the consequence of delocalization of the rhBMP-2 from the collagen at the clinical application site is that rhBMP migrates to skeletal muscle envelopes that surround the bone, and pluripotential cells in the skeletal muscle bind with rhBMP and differentiate to osteoblasts. The osteoblasts produce ossicles in the muscle—the ectopic bone. Ectopic bone formation from rhBMP-2/collagen (i.e., Infuse®) spine fusion procedures may result in symptomatic compression of the spinal nerve roots and the unintended fusion of nearby spine segments [**67**].

Infuse®: The Clinical Product

To date, the only FDA-approved rhBMP-2-containing product is the Medtronic/Sofa-Danek product Infuse®. Infuse® includes rhBMP-2 and type I collagen from bovine Achilles tendon. Infuse® is approved by the FDA as a medical device under the designation premarket approval (PMA) for single-level anterior lumbar interbody fusion (ALIF) used in combination with the LT-Cage® (Medtronic Spinal and Biologics) lumbar tapered fusion device. For ALIF procedures, Infuse® is only approved by the FDA for the treatment of degenerative disc disease (DDD) at a single level from L2 to S1. In addition, in ALIF procedures, Infuse® may be used with the LT-Cage®, INTER FIX™ threaded fusion device, or INTER FIX™ RP fusion device. In 2004, Infuse® was also cleared by the FDA for use in acute, open tibial shaft fractures, stabilized by intramedullary (IM) nail fixation (within 14 days after the initial fracture) [**68**]. In 2007, Infuse® was cleared by the FDA for bone void filling in the sinus area to place endosseous dental implants in the upper jaw and in extraction sites before implant placement [**69**]. However, Infuse® has been applied to many non-FDA-approved procedures for posterior-lateral spine, tibia, and craniofacial applications [**50,53,54,70-74**].

Infuse® applications for non-FDA-approved clinical procedures have resulted in reports of adverse medical events, including inflammatory cyst formation, adjacent vertebral body osteolysis, ectopic bone formation, cancer, uncontrolled bone growth, and male retrograde ejaculation [**50,52,54,71,72,75,76**].

OP-1 Putty®

OP-1 Putty® (Stryker Biotech, Hopkinton, MA) received FDA approval under the Humanitarian Device Exemption (HDE) in October 2001 as an alternative to autograft in recalcitrant long bone nonunions [**77**] and in April 2004 as an alternative to an autograft for patients who require single-level posterolateral (intertransverse) lumbar spine fusion (PLF) [**78**]. The HDE emphasizes application for either compromised patients (e.g., osteoporosis, smoking, diabetes, geriatric) or those with a previous spinal fusion that failed (i.e., fusion site became a pseudoarthrosis).

OP-1 Putty® is composed of the OP-1 implant (rhBMP-7/type I bovine collagen), carboxymethylcellulose sodium (CMC; a biodegradable polymer), and sterile saline solution. CMC is a water-soluble thickener that creates a malleable putty when added to the rhOP-1/collagen composition, greatly improving the surgical handling characteristics of the blend.

OP-1 Putty® has been used in combination with Calstrux® (β-TCP; Stryker Biotech). The rationale for adding the CMC to the Calstrux® product was to improve surgical handling (i.e., localize the product at the site of surgical implantation). Allegedly, CMC increases β-TCP/collagen viscosity and cohesiveness to provide a more clinically suitable medium for surgical manipulation [**79,80**]. However, the combinatorial outcome may affect rhBMP-7 release kinetics with CMC functioning as a diffusion barrier [**79,81**]. Therefore, it was expected that the CMC would allow the protein to remain in place during the beginning of the bone formation process. Complications of OP-1 Putty® have been reported and include autoimmune reactions and hypersensitivity [**82**].

Calstrux

Calstrux® is a 510(k) FDA-cleared bone void filler that is manufactured by Stryker Biotech and consists of β-TCP. Reports indicate that Calstrux® has been used in non-FDA-approved procedures in combination with rhOP-1 [**82,83**]. The consequences of the non-FDA-approved clinical applications in patients include edema, pain, indurated tissue at the site of the operation, and neurological sequelae [**84**]. Moreover, reports suggest that the Calstrux®/OP-1 combination migrates from the surgical implant site [**85**], increasing the risk of ectopic ossification.

CLINICAL APPLICATIONS

The goal of spinal fusion is to achieve a solid bone mass to maintain appropriate disc spacing. When the spinal vertebrae fail to fuse after rhBMP treatment, this outcome is termed a *pseudoarthrosis*, or the failure to produce a single, solid fusion. A revision surgery is necessary to correct the pseudoarthrosis and produce the desired vertebral bone fusion (**Table 8.1**).

TABLE 8.1 FDA Regulatory Summary rhBMP-2 and rhBMP-7 (rhOP-1)

Application		BMP	Device/ Additions	Specifications	FDA Approval Path, Date, Applicant
Spinal	ALIF	Infuse® Bone Graft	LT-CAGE™	DDD, may have spondylolisthesis or retrolisthesis, L2–S1 vertebrae, 6 months failed nonoperative treatment	PMA July 11, 2002 Medtronic Sofamor Danek USA, Inc.
	PLIF	OP-1 Putty®	n/a	Alternative to autograft, compromised patients	HDE April 7, 2004 Stryker Biotech
		Infuse® Bone Graft	MASTER-GRAFT®, Posterior Fixation System	Symptomatic, posterolateral lumbar spine pseudoarthrosis, ≥2 levels, compromised patients	HDE October 10, 2008 Medtronic Sofamor Danek, Inc.
Traumatic	Recalcitrant long bone nonunions	OP-1 Implant®	n/a	Alternative to autograft, autograft use unfeasible, failed alternative treatments	HDE October 17, 2001 Stryker Biotech
	Acute, open tibial shaft fractures	Infuse® Bone Graft	IM nail fixation	Appropriate wound management, apply 14 days after initial fracture	PMA April 30, 2004 Wyeth Pharmaceuticals, Inc.
Oral/ maxillofacial	Sinus augmentation, alveolar ridge augmentation	Infuse® Bone Graft	n/a	Alternative to autograft for sinus augmentations, localized alveolar ridge augmentation for extraction socket defects	PMA March 9, 2007 Medtronic, Inc.

n/a: not applicable.

Posterolateral Lumbar Fusion

Spinal fusion procedures attempt to reclaim vertebral disk space lost to DDD. The FDA issued an HDE to Stryker Biotech for OP-1 Putty® on April 7, 2004 for spine fusion applications. The HDE authorized OP-1 Putty® for use as an alternative to autograft in compromised patients (examples include individuals with osteoporosis, smokers, and diabetics) requiring revision posterolateral (intertransverse) lumbar spinal fusion for whom autologous bone and bone marrow harvest are not feasible or are not expected to promote fusion. The effectiveness of OP-1 Putty® for this indication had not been demonstrated at the time of approval.

OP-1 Putty® is a two-component system, or "unit," comprising a vial containing 1 g of bovine collagen and OP-1 and a second vial containing 230 mg of CMC. The package insert recommends a total of 7 mg of OP-1 to be administered per bilateral posterolateral revision fusion. Furthermore, no more than two vials of bovine collagen/OP-1 are recommended per application.

During surgery, the recipient vertebrae are debrided and decorticated to allow direct contact between the OP-1 Putty® and viable bone. The benefit of the OP-1 Putty® may be diminished without adequate vascularity. The OP-1 Putty® package insert also warns that localized ectopic or heterotopic bone formation may occur outside of the treatment site, and care must be taken to minimize or prevent this complication by meticulously localizing the treatment administration. Any irrigation and surgical manipulations to the site should be completed before implantation of the device, and adequate hemostasis should be provided to ensure that the material remains at the surgical site. The putty is packed into the prepared site, and soft tissues are closed around the defect containing the OP-1 Putty® using sutures (**Fig. 8.4**). Surgical closure is critical for containment and maintenance of the putty in the area of fusion and any stray particles of OP-1 Putty® should be removed via irrigation of the field after closure.

The Infuse®/MASTERGRAFT® posterolateral revision device (Medtronic) was authorized as an HDE for the repair of symptomatic, posterolateral lumbar spine pseudoarthrosis in a subset of patients in October of 2008.

The Infuse®/MASTERGRAFT device is indicated to treat two or more levels of the lumbar spine. Similar to OP-1 Putty®, the subset of patients identified to benefit from the Infuse®/MASTERGRAFT device included patients with diabetes mellitus and smokers for whom autologous bone or bone marrow harvest were not feasible. Autologous bone and bone marrow harvest are often not feasible for patients with compromised vasculature.

The Infuse®/MASTERGRAFT® posterolateral revision device comprises Infuse® bone graft and MASTERGRAFT® granules as well as a supplemental posterior fixation system. Under the HDE, all three components of the system must be used in combination. The MASTERGRAFT® granules are ceramic granules of 15 % hydroxyapatite and 85 % β-TCP. The MASTERGRAFT® granules are placed onto the collagen sponge of the Infuse® Bone Graft/LT-Cage® lumbar tapered fusion device or Infuse® bone graft on its own. The HDE was withdrawn at the request of the sponsor, Medtronic Sofamor Danek, Inc., in March 2010.

Anterior Lumbar Interbody Fusion

Medtronic Sofamor Danek, Inc., received an FDA PMA (P000058) for the Infuse® Bone Graft/LT-Cage® lumbar tapered fusion device in July 2002. This device consists of two components containing three parts: the Infuse® rhBMP-2/absorbable collagen sponge (ACS) combination and a metallic spinal fusion cage. The biological rationale is that the Infuse® bone graft induces new bone formation whereas the LT-Cage® allows for localized application of the Infuse® bone graft, restores disc space height, and provides structural support during fusion.

This device is approved by the FDA for spinal fusion procedures in skeletally mature patients with DDD at one level from L2 to S1. To be considered for the device, patients must have undergone 6 months of nonoperative treatment before implantation of the device.

The Infuse® Bone Graft/LT-Cage® lumbar tapered fusion device is implanted via an anterior approach to the spine. After removal of the disk, two titanium LT-Cage® devices are filled with Infuse® bone graft and inserted between the vertebrae to be fused (**Fig. 8.5**). The biological rationale is that the Infuse® bone graft induces new bone formation whereas the LT-Cage® allows for localized application of the Infuse® bone graft, restores disc space height, and provides structural support during fusion.

It is possible that Infuse® was more clinically successful in ALIF procedures than in posterior lumbar interbody fusion (PLIF) procedures as a consequence of the anatomical and biomechanical differences between the sites. Anteriorly, there is less muscle, nerves, and vascularity in the spine than posteriorly. Biomechanically, the compressive load that the implant would be subjected to in an ALIF may be beneficial for bone formation. Studies suggest that intermittent nonhydrostatic compressive loads or octahedral shear stress can promote endochondral ossification, which would improve the efficiency of Infuse® [**86,87**]. Anatomically, the posterior spine is wrapped in musculature, the erector spinae muscles, which may be a locus for post-Infuse® HO. rhBMP-2 in Infuse® may potentially trigger inflammatory neuropeptides, such as substance P, when in contact with the dorsal root ganglion (DRG) [**88**]. In addition, in a PLIF, the implant may be subject to shear stress, which is not optimal for bone formation and could increase the risk of pseudoarthrosis [**87,89**].

Long Bone

In atrophic long bone (i.e., clavicle, femur, tibia, fibula, phalange, metacarpal, metatarsal, humerus, radius, and ulna) nonunions, the bone healing callus is absent or malformed. The absence of a bone healing callus may be due to vascular or metabolic causes. Vascular issues include inadequate blood supply from vascular injury, and metabolic causes include diabetes, smoking, and certain medications (e.g., nonsteroidal anti-inflammatory drugs, steroids, and anticoagulants). The application of BMPs to these nonunions is especially fitting because osteogenic capacity can be restored. With this rationale in mind, in October 2001, the FDA approved Stryker's OP-1 implant under the HDE program for recalcitrant long bone nonunions where autografting was not feasible and alternative treatments had failed.

Although osteoinductive, the OP-1 implant is biomechanically not sufficiently robust to support fixation without a shared loading/stabilizing adjunct (i.e., cast, instrumentation, etc.) in long bones. Fixation methods have been used in clinical trials studying OP-1 implants, and these include casts/braces, external fixation, IM rods, and internal plates.

The benefit of the OP-1 implant may be diminished without adequate vascularity in the surrounding tissue. The OP-1 implant package insert warns that localized ectopic or heterotopic bone formation may occur outside of the treatment site, and care must be taken to minimize or prevent this complication. Any irrigation and surgical manipulations to the site should be completed before implantation of the device, and adequate hemostasis should be provided to ensure that the material remains at the surgical site.

The most common adverse medical events associated with the OP-1 implant include, but are not limited to, fever, complications involving hardware, pain, nausea and vomiting, wound infection, local inflammation, rash, redness, and itching of the skin and wound.

In April 2004, Wyeth Pharmaceuticals, Inc., received a PMA (P000054) for Infuse® bone graft for tibial fractures. The device was approved for treating acute, open tibial shaft fractures that have been stabilized with intramedullary (IM) nail fixation after appropriate wound management. The Infuse® bone graft was indicated for skeletally mature patients in which graft application within 14 days of the initial fracture was specified. However, Infuse® bone graft was not approved if used in the vicinity of a resected or extant tumor and in patients with an active malignancy or undergoing treatment for one. Its contradictions also include patients with an inadequate neurovascular status (e.g., at high risk of amputation), an active infection at the operative site, or a compartment syndrome of the affected limb.

Sinus Lift and Alveolar Ridge Augmentation

On March 9, 2007, Medtronic Sofamor Danek, Inc., was given a PMA for the Infuse® bone graft. The FDA approval is for clinical use in the maxillary sinus to promote bone formation antecedent to insertion of endosseous[3] dental implants. It was also approved to increase bone in extraction sites before implant placement. As with all Infuse® bone graft applications, the rhBMP-2 and ACS components must be used as a system.

According to Wolff's law, a decrease of functional forces transferred to the bone after tooth loss causes a shift in the remodeling process toward bone resorption [**90**]. Osteoclasts are the hallmarks for resorption; increased osteoclastic activity results in low-density trabecular bone with a minimal cortical layer and poor stress tolerance [**91**]. Inferior bone height and low bone density are limiting factors for implant placement in the posterior maxilla and alveolar ridge [**92**]. The osteoinductive nature of BMPs allow

[3] Endosseous dental implants are inserted in the jawbone for dental applications. Some of the most common dental applications for endosseous implants are crowns, dentures, and fixed bridges.

for de novo bone formation at these sites originally undergoing bone resorption. In addition, rather than following a "graft consolidation gradient" in the presence of pharmacologically relevant doses of BMPs, new bone in the maxillary sinus is equally distributed throughout the augmented area [**93**]. This provides the necessary volume of bone for the mechanical and biologic support of endosseous dental implants [**94**].

The rhBMP-2 powder must be reconstituted with sterile water and applied to the ACS at least 15 min, but no longer than 2 h, before implantation. The size of the Infuse® bone graft kit should be selected to reflect the volume required at the implant site, and the graft is implanted after the implant site is prepared using standard surgical techniques (**Fig. 8.3**).

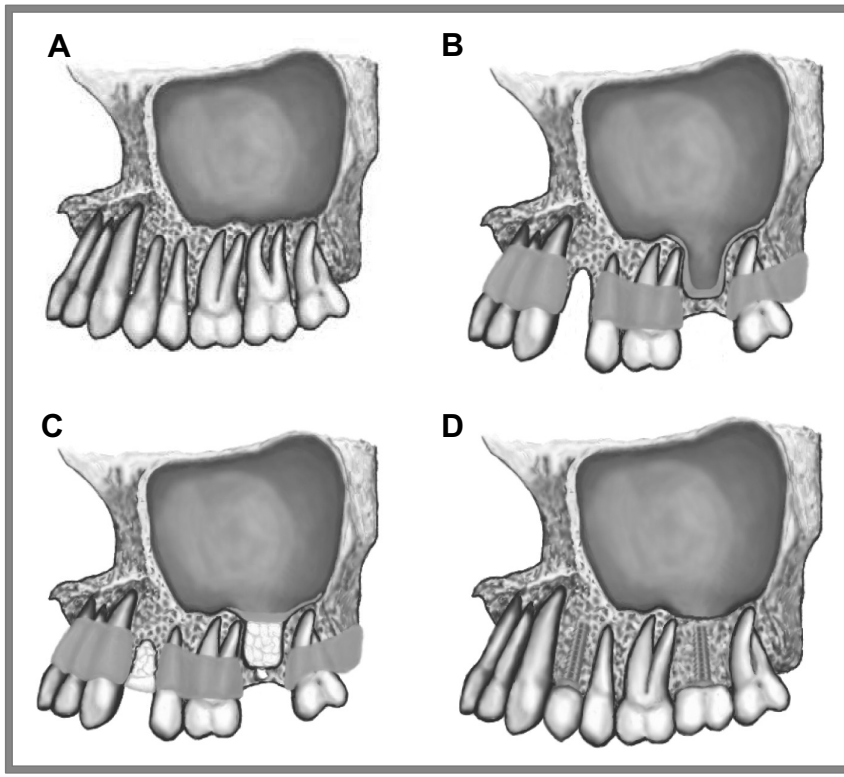
For sinus augmentation, the sinus membrane is elevated, and the Infuse® bone graft is cut into several pieces that are placed with an even distribution within the lower third of the sinus where bone formation is desired (**Fig. 8.3**). To prevent migration of the graft, irrigation should be precede implant placement; complete soft tissue closure must be achieved. For alveolar ridge augmentations associated with extraction sockets, the socket should be completely debrided after tooth extraction, and several perforations should be made in the socket wall to expose the marrow space [**95**]. The Infuse® bone graft is cut into small pieces to be loosely packed in the socket, and a large strip of graft is placed over the entire site before soft tissue closure. In a successful procedure, new bone formation occurs and completely replaces the Infuse® bone graft in sufficient volume for dental implant anchorage.

DELIVERY SYSTEM OPTIONS

There is a significant inadequacy in the clinical performance of the delivery systems used with rhBMP-2 and -7. The inadequacy is a consequence of several factors related to the physical and biological properties of the delivery system itself and its relationship with the rhBMP-2/-7. We have stated these specific deficiencies in previous sections throughout the chapter. Two unique possible considerations to address delivery system challenges for the rhBMPs are offered in this section.

One option is BMP-binding peptides (BBPs), which may reduce the inflammatory response of rhBMP-2 and rhBMP-7 [**96**]. These synthetic cyclic 19-amino-acid peptides are derived from the cystatin-like domain of an 18.5-kDa fragment of the bone matrix protein, SPP-24. The BBPs are synthetic peptides that may bind to rhBMP-2 and rhBMP-7, reduce inflammation, and enhance BMP-induced osteogenesis [**97–99**]. The question to pose is, "Why does this tactic affect the delivery system?" The answer is as follows: The inflammatory response to rhBMP-2 and rhBMP-7 is dependent on the dose [**96**]. Therefore, by incorporating a synthetic peptide into, for example, a collagen scaffold, tighter pharmacokinetic control over rhBMP release kinetics may result, thus requiring lower administrative rhBMP dosing [**96**]. Current delivery systems for rhBMPs release a supraphysiological dose [**100**], which could increase the risk of mutagenesis. Studies demonstrated that by using BBPs, the dosing of rhBMP can reduced from 70 % to 90 % while achieving the same osteogenic response [**96**]. A dose closer to

FIG 8.3 (A) Healthy alveolar ridge and maxillary sinus with teeth in place. (B) After tooth extraction, bone loss in the alveolar ridge or maxillary sinus or both can occur. (C) INFUSE™ Bone Graft placement for ridge augmentation and sinus lift. (D) The INFUSE™ Bone Graft is replaced with a sufficient volume of new bone for dental implant anchorage.



the physiological rhBMP dose will reduce the potential mutagenic risk and side effects such as soft tissue edema. In vivo studies have reported that delivering BBPs with rhBMP is as effective as larger doses of BMPs when using a collagen matrix [**96–99**].

A second strategy is biomimetic calcium phosphate coatings for BMP delivery systems in spinal fusion procedures [**101**]. The rhBMP is incorporated within the physical structure of the calcium phosphate. The logic here is to produce a biomimetic delivery system for rhBMP; the outcome intended will be physiological osteogenesis [**101**].

CONCLUSIONS

The rationale for BMP for bone regeneration is compelling. BMP is a potentiator for the osteogenic cascade: it initiates osteoblast-lineage progression and promotes

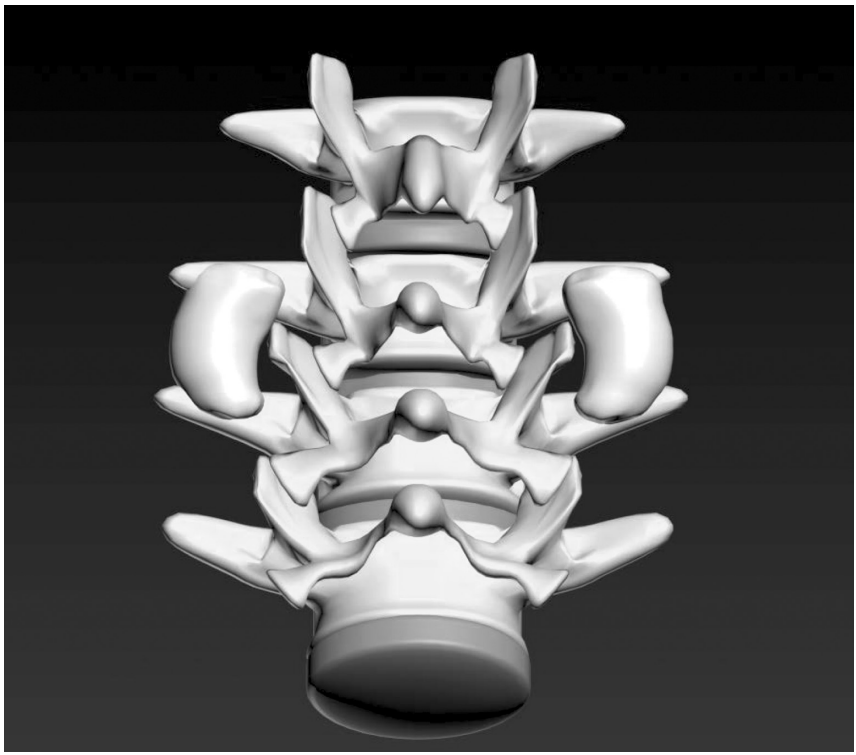
angiogenesis. As a consequence of this powerful biological role, it was a natural process that evolved into rhBMP-containing therapies.

RhBMP-2 is approved by the FDA as the product Infuse® for ALIF spinal fusion using a collagen sponge and metal cage. Infuse is approved by the FDA as a medical device rather than as a drug. The logic for a device approval by the FDA for the rhBMP-2/xenogeneic combination (i.e., Infuse®) seems odd. Infuse® is approved only for ALIF; however, non-FDA-approved PLIFs occur.

Because of the diffusion of rhBMP from the applied clinical site, milligram quantities of rhBMP-2 are required to produce a therapeutic outcome. The milligram dosing likely exceeds the physiological endogenous BMP, posing unwanted safety concerns. Moreover, the ALIF spine fusion cages that contain the rhBMP-2 include an rhBMP-2 dose of 1 mg/mL, and there are reports of using up to 8 mg per cage [**102**].

A dose that exceeds the physiological endogenous level may be termed *supraphysiological*. Supraphysiological doses of rhBMP-2 with Infuse®, improperly engineered delivery systems for rhBMP-2 and -7, and non-FDA-approved surgical applications have resulted in disturbing reports of clinical complications [**54,71,72,76,100**].

FIG. 8.4 OP-1 Putty use in posterolateral revision surgery.



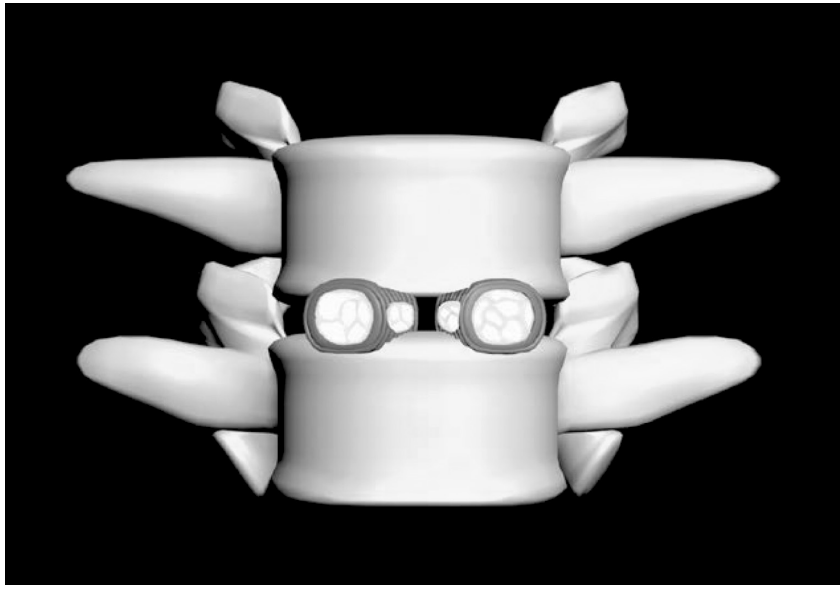
In addition, the insufficient data on the biomechanical effects of the spine on spine grafts have also raised serious concerns. Current studies suggest that ALIF is more stable in left torsion and right torsion compared with PLIF [**103**]. However, there is a lack of well-designed, controlled, clinical trials in which data establish the advantage of one interbody fusion technique compared with another [**104**]. This is particularly true when delivering an osteoinductive growth factor such as rhBMP-2 or rhBMP-7 into the spine. Therefore, future work in spine fusion should focus not only in improving clinical outcomes with enhanced techniques but also on improving the performance criteria of the current bone grafts to facilitate a positive outcome for patients.

Lastly, the intent for the content within this chapter was to emphasize the powerful and natural physiological role that BMP has in osteogenesis. It is only by respecting the power of that physiological role that logically designed therapeutics will evolve that will safely and predictably improve bone regeneration for patients.

ACKNOWLEDGMENTS

A special thanks to Ami Saroka for making the two spine-related figures (**Figs. 8.4** and **8.5**). The authors thank the Armed Forces Institute of Regenerative Medicine and the Defense Medical Research and Development Program for supporting this work.

FIG. 8.5 The LT-CAGE devices are packed with INFUSE™ Bone Graft before placement between vertebral disks.



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Chapter 9 | Synthetic Biomimetic Porous Polymer Scaffolds for Bone Regeneration

Ming Dang,[1] Jae Min Shin,[2] and Peter X. Ma[1,2,3,4]

INTRODUCTION

Bone defects resulting from tumors and traumas present a major health-care problem. Small bone defects are self-repairable because bone itself is a dynamic tissue, which is highly vascularized with a unique capacity to heal and remodel without leaving a scar. However, if fractures and defects are greater than a critical size, autogenous healing may result in malunion or nonunion; therefore, a graft material is needed to achieve complete repair [**1**]. The gold standard for a bone graft procedure is currently to use an autograft from the patient. Some disadvantages to this method are a limited graft supply, donor site morbidity, and multiple surgery requirements. Allografts from other human donors provide an alternative option, but their usage is also limited by the inherent risks of disease transmission and host immune response [**2**].

Bone tissue engineering has emerged as a promising new approach for bone repair. Compared with the traditional autograft and allograft procedures, a tissue engineering strategy has several advantages, including the abundant scaffolding materials, elimination of the surgery to harvest a bone graft, reduced risk of immune rejection, and pathogen transmission associated with allografts. The basic elements needed for tissue engineering include a scaffold, cells, and signaling molecules [**3**]. Optimization of these three factors is critical in promoting cellular function and tissue regeneration. Among these three key components, the scaffold plays a critical role in supporting cell adhesion, proliferation, osteogenic differentiation, and mineralized bone tissue regeneration in three dimensions (3D). A bone tissue-engineering scaffold should possess a multitude of properties to provide the required optimal 3D microenvironment (synthetic temporary extracellular matrix [ECM]) to facilitate bone tissue regeneration [**4**].

[1] Macromolecular Science and Engineering Center, University of Michigan, Ann Arbor, MI
[2] Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI
[3] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI
[4] Department of Materials Science and Engineering, University of Michigan, Ann Arbor, MI

Up to now, various materials have been proposed to fabricate scaffolds, including metals, ceramics, and polymers. Metals are frequently used as bone implant materials because of their excellent mechanical properties, but they are disadvantageous scaffold materials because of their general lack of degradability. Ceramics have also been investigated as bone regeneration materials. However, they have limitations in forming highly porous structures because of their brittleness [**5**]. In contrast, polymers have great design flexibility in chemical and physical structures, which can be tailored to meet the specific needs of a tissue-engineering scaffold. Therefore, polymers have received considerable attention and are widely used as scaffold materials for bone tissue engineering. In general, polymeric materials can be categorized as natural polymers (e.g., collagen and fibrin) and synthetic polymers (e.g., poly(glycolic acid) [PGA], poly(lactic acid) [PLA], and their copolymer poly(lactic-co-glycolic acid) [PLGA]). Many naturally derived polymers inherently possess certain biological recognition and the ability to interact with the host tissue. However, natural polymers also have several drawbacks, including possibly immunogenic response, variability associated with sources, and limited range of properties [**6**]. On the other hand, synthetic polymers have several advantages over natural polymers, such as the absence of immunological concerns and batch-to-batch uniformity. The chemical versatility and diverse processing methods of synthetic polymers enable a rational design of a scaffold with predictable structures and properties. Compared with the natural polymers, synthetic polymers usually lack biological cues. Thus, several strategies have been developed to incorporate biological cues into the polymers to create advantageously functional synthetic polymer scaffolds.

This chapter reviews the application of polymeric materials in bone tissue engineering, mainly focusing on the synthetic polymer materials and new advances in scaffold fabrication technologies using biomimetic approaches.

INTERCONNECTED POROUS SCAFFOLDS

Several desired features for a tissue-engineering scaffold have been identified [**7–11**]: (1) the material should be biocompatible and have suitable biodegradability; (2) the scaffold should possess interconnected pores of appropriate size to facilitate tissue integration and vascularization; (3) the scaffold should have sufficient mechanical integrity to maintain the predesigned tissue structure; (4) the scaffold should have appropriate chemical and physical structures on the pore surface for cell attachment, proliferation, and differentiation; and (5) the scaffold should ideally also be able to release soluble signals in a spatially and temporally controlled fashion.

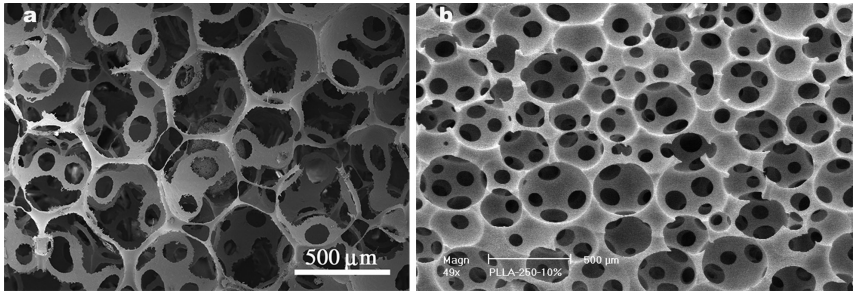
Biocompatibility and biodegradability are the fundamental and imperative requirements for tissue engineering scaffolds [**8**]. Scaffolds must be compatible with the host tissue without eliciting a negative immune response. The degradation rate also needs to match the neo tissue formation rate so the scaffolds can be totally degraded by the time the defect is completely repaired [**3**].

High porosity in a scaffold design is a desirable feature to support cell proliferation, tissue formation, and vascularization for most tissue engineering applications, including bone. In addition, adequate interconnectivity is beneficial not only for uniform cell seeding, but also for the diffusion of nutrients and oxygen and elimination of metabolic wastes from the scaffolds [**12**]. Various techniques, such as particulate leaching [**13,14**], gas foaming [**15,16**], emulsion freeze-drying [**17**], phase separation [**18**], and rapid prototyping (RP) [**19,20**] have been developed to fabricate interconnected porous scaffolds with varying degrees of success. Because high porosity and interconnected pores are well-recognized features for a scaffold and have been widely reported, this chapter will not focus on exhaustively reviewing the techniques to generate porosities. Interested readers can read the above-cited literatures and other related chapters in this book. Here, we would like to use the newer particulate-leaching techniques as examples to briefly illustrate the importance and methods of achieving the interconnected pore structures.

The particulate-leaching technique is the most widely used method to create porous scaffolds. The traditional particulate-leaching method involves mixing porogens (pore-generating materials) such as sodium chloride (NaCl) into the polymer solution [**13**]. After removal of the solvent, porogens are leached out to yield a porous scaffold. However, the interconnectivity between the pores is low and the size and the shape of the pores are difficult to control. Several techniques have been developed to generate a polymer scaffold with well-controlled spherical pores and interconnectivity. In one example, paraffin spheres were utilized as the porogen material and assembled into a negative replica to prepare a 3D scaffold with interconnected spherical pores [**12,21**]. Polymer solution was then casted into a paraffin sphere assembly. After removal of the solvent, the paraffin spheres were leached out with an organic solvent to obtain a porous structure (**Fig. 9.1a**). This method can advantageously control the shape and the size of the spherical pores as well as the interconnectivity by adjusting different assembly conditions (time and temperature of heat treatment for paraffin spheres). In another work, sugar spheres were used as an alternative porogen, which retained the advantages of achieving interconnected spherical pore structure (**Fig. 9.1b**) while allowing removal of porogen using water instead of organic solvents [**22**].

In the past decade, various RP techniques have been introduced such as stereolithography, selective laser sintering, solid free-form fabrication (SFF), fused deposition molding, and 3D printing [**23**]. Several studies have investigated the application of RP technologies for direct and indirect manufacturing of scaffolds from various materials [**24–29**]. These techniques enable the creation of highly customizable scaffolds of complex geometries. However, because of the resolution associated with the current RP technologies, it remains difficult to generate scaffold features at very small scales such as at the nanometre or even the lower micrometre scales. By integrating the RP technique with a polymer phase separation technique, our laboratory generated complex-shaped scaffolds with the nanofibrous (NF) feature to be detailed in *Mimicking the Physical Structure* [**30**].

FIG 9.1 Scanning electron micrographs of poly(α-hydroxy acid) scaffolds. (a) Porous PLGA scaffolds prepared using paraffin spheres with a size range of 420–500 μm (×50); (b) porous PLLA scaffolds prepared using sugar spheres with a size range of 250–425 μm ([dbar]×50). Source: Panel a reprinted with permission from Ma and Choi [**12**]. Panel b reprinted with permission from Wei and Ma [**22**].



Mechanical stability is another important parameter to consider when designing a scaffold [**31**]. Bone is a tissue under continuous mechanical stress, and the application of mechanical stimuli can enhance bone formation [**32**]. Thus, bone scaffolds should have sufficient mechanical stability to provide a suitable physical and mechanical environment for cell function and new bone formation. It is worth noting that the degree of porosity and interconnectivity can often influence other properties of the scaffold, such as the mechanical properties. Thus, these factors should be well balanced to meet the needs of bone regeneration. The rest of this chapter will focus on how to achieve desired scaffold pore surface properties and biological signal delivery capacity in porous scaffolds with special emphases on biomimetic approaches and recent advances.

MIMICKING THE NATURAL ECM

Naturally occurring ECM is known to play a critical role in regulating cell adhesion, growth, and differentiation. In addition to providing physical support and enabling diffusion of nutrients and metabolic products, an ideal scaffold should replicate certain advantageous features of the natural ECM. As our knowledge in bone ECM and material science progresses, there have been more attempts to incorporate biomimetic features into scaffolds. This would aid in achieving positive interactions between the scaffold and the cells, enhancing cell adhesion, growth, migration, and their differentiated function [**4**]. However, it is not practical or necessary to duplicate all features of the ECM in a tissue-engineering scaffold; one reason is that the therapeutic bone regeneration is an accelerated process that differs from the natural development and healing process. Moreover, the natural ECM from a mature tissue usually does not possess some of the advantageous features of the artificial scaffolds, including high

porosity and high interconnectivity, which are essential for the accelerated therapeutic regeneration. The high porosity and interconnected pore network design can facilitate cell seeding, nutrient supply, and metabolic waste removal, ultimately accelerating the bone regeneration process. Thus, an ideal biomimetic artificial scaffold should provide an optimal microenvironment for tissue regeneration by combining the advantageous features of natural ECM, synthetic materials, and the porous structural design.

For bone tissue engineering, the natural bone ECM is a source of biomimetic features for the scaffold design. Collagen is the major component of bone ECM, which is assembled into nanofibers ranging from 50 to 500 nm [**33**]. Numerous studies showed that the nanofibers promote osteogenesis [**34,35**]. The collagen fibers with nano-hydroxyapatite (HAP) crystals form a mineralized composite fibrous network, which gives bone its lightweight and superior mechanical properties [**36,37**]. To mimic the key characteristics of the complex natural bone ECM, there is extensive ongoing research efforts, including imitating the physical structure, chemical composition, and surface chemistry of the ECM.

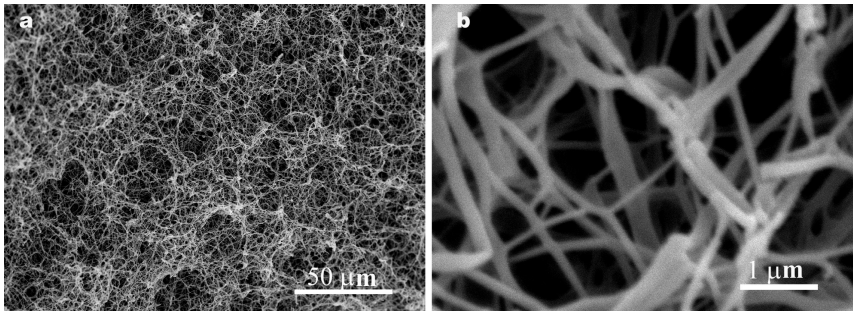
Mimicking the Physical Structure

NF scaffolds have been developed to mimic the structural features of the ECM. An NF polymer scaffold can be fabricated using self-assembly, electrospinning, and thermally induced phase separation (TIPS) techniques.

The self-assembly approach involves spontaneous organization of molecules into a well-defined structure such as nanofibers [**38**]. Various self-assembled molecules such as peptides [**39,40**] and block copolymers [**41**] have been designed to form a stable arrangement through preprogramed noncovalent interactions. However, these self-assembled NF scaffolds are currently limited in the form of hydrogels, which are often unable to provide a stable 3D porous structure for bone tissue engineering application. Electrospinning is the oldest but the most commonly used method to form nanofibers [**42**]. A typical electrospinning system includes a polymer solution or melt reservoir with a spinneret, a high-voltage electric field, and a grounded target collector. The polymer solution is drawn onto the collector under the electric field and the jet follows a whipping and a spiraling path, reducing the diameter during the travel to the ground collector. By adjusting the operating parameters, the resulting fibers can range from approximately 0.02 to 20 μm [**43**]. Electrospinning is a quick and a simple way to generate nanofibers from multiple types of materials ranging from natural polymers such as collagen [**44**], chitosan [**45**], and silk fibroin [**46**] to synthetic polymers such as polycaprolactone (PCL) [**47**], PGA [**48**], poly(L-lactic acid) (PLLA) [**49**], and PLGA [**50**]. However, it is difficult to create a 3D scaffold with designed pore shapes or complex geometries using this technique.

In a typical TIPS process, a homogeneous multicomponent system becomes thermodynamically unstable under certain conditions and tends to separate into a multiphase system (polymer-rich phase and polymer-lean phase) to lower free energy [**8**]. After removal of the solvent, the polymer-rich phase solidifies to form the polymer

FIG 9.2 Scanning electron micrographs of PLLA NF matrix prepared from a 2.5 % (w/v) PLLA/tetrahydrofuran (THF) solution at a gelation temperature of 8°C. (a) Original magnification of 500×; (b) original magnification of 20,000×. Source: Reprinted with permission from Ma and Zhang [**18**].



skeleton whereas the polymer lean phase becomes the void space. NF matrices have been created from synthetic biodegradable polymer by using a novel TIPS method. Poly (L-lactic acid) (PLLA) was dissolved and thermally induced to phase separate from the solvent when temperature was decreased [**18**]. After removal of the solvent by extraction, sublimation, or evaporation, a continuous 3D NF PLLA architecture was generated. The fibers formed in this manner had diameters ranging from 50 to 500 nm, similar to the dimensions of collagen fibers, and they had porosity in excess of 98 % (**Fig. 9.2**). Other synthetic polymers or natural polymers also have the ability to form NF matrices via TIPS. A series of biodegradable amphiphilic poly(hydroxyalkyl methacrylate)-graft-poly(L-lactic acid) copolymers were synthesized to allow for further conjugation with bioactive moieties via the functional groups grafted to the PLLA [**51**]. These copolymers could form nanofibers by TIPS and degrade faster than a PLLA homopolymer. A highly porous NF gelatin matrix has also been fabricated by TIPS using either ethanol/water mixture or methanol/water mixture [**52**]. Compared with the commercially available product gelatin foam (Gelfoam®) lacking the NF feature, the NF gelatin matrix generated using the TIPS method exhibited a much higher surface area and greater mechanical stability.

One major advantage of TIPS over other techniques is the capacity to integrate with other fabrication methods such as particulate leaching, SFF, and emulsion techniques. The combined techniques broaden the control over the 3D architecture from macro- to micro- to nanoscales. For example, when combined with the particulate leaching method, a interconnected porous 3D structure was generated in NF matrices [**22**] (**Fig. 9.3, a** and **b**). Through the reverse SFF technique, an NF scaffold with the predesigned shape of bone segment was precisely created using phase separation technique and a mold reconstructed from the computed tomography scans of the

FIG 9.3 Various NF scaffolds prepared using a phase separation method. (a) A scanning electron micrograph of a NF scaffold with interconnected spherical pore network prepared by integrating a phase separation technique with a particulate leaching technique, with an original magnification of 50×. (b) A scanning electron micrograph of the above scaffold with an original magnification of 10,000×. (c) An NF scaffold for human mandible segment reconstruction prepared by integrating a phase separation technique with a SFF technique (scale bar: 10 mm). (d) The NF pore wall morphology of the above scaffold taken at a higher magnification (scale bar: 5 μm). (e) A scanning electron microscopy image of an injectable NF hollow microsphere prepared by combining phase separation with an emulsion technique at a lower magnification. (f) A scanning electron microscopy image of the above NF hollow microsphere taken at a higher magnification to show the NF morphology. Source: Panels a and b reprinted with permission from Wei and Ma [**22**]. Panels c and d reprinted with permission from Chen et al. [**30**]. Panels e and f reprinted with permission from Liu et al. [**53**].

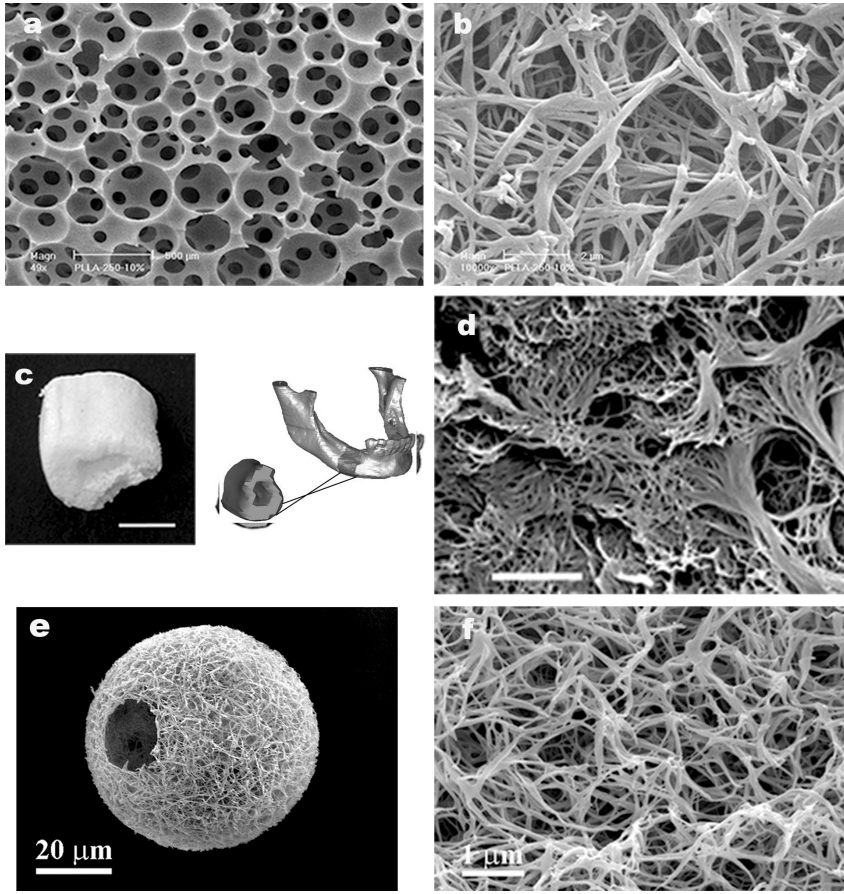
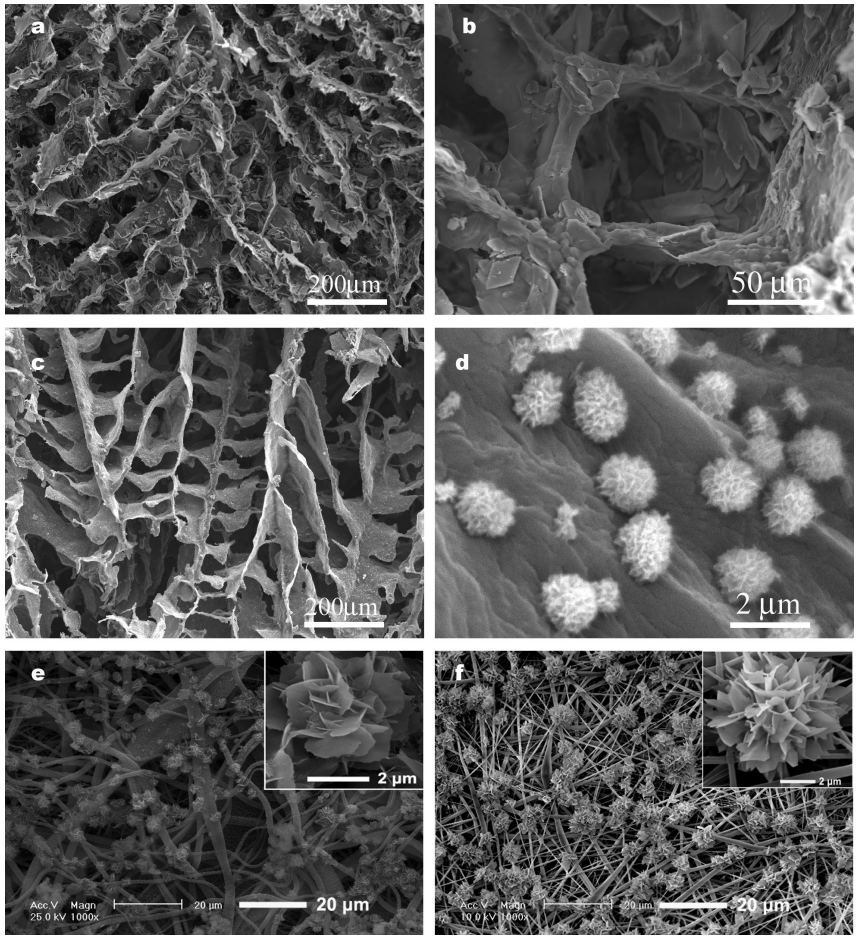


FIG 9.4 Scanning electron micrographs of various PLLA/calcium phosphate composite scaffolds. (a) A PLLA/HAP composite scaffold fabricated using a phase separation technique. (b) The above scaffold viewed at a higher magnification. (c) A PLLA/calcium phosphate composite scaffold prepared using a phase separation technique and a biomimetic process in an SBF. (d) The above scaffold viewed at a higher magnification. (e) A PLLA/calcium phosphate composite scaffold prepared using an electrospinning technique and an electrodeposition technique at 60°C and 3 V for 15 min. (f) A PLLA/calcium phosphate scaffold prepared under the same conditions as for the previous scaffold for 30 min. Source: Panels a and b reprinted with permission from Zhang and Ma [**57**]. Panels c and d reprinted with permission from Zhang and Ma [**59**]. Panels e and f reprinted with permission from He et al. [**62**].



same bone anatomical shape [**30**] (**Fig. 9.3, c** and **d**). Because the bone defects are rarely of a simple shape, this computer design technique allows customization to meet the specific requirements for each individual patient. Recently, a novel emulsion technique has been developed to prepare injectable NF microsphere scaffolds for irregular shaped defect repair [**53**] (**Fig. 9.3, e** and **f**). Star-shaped PLLA was emulsified into liquid microspheres and quenched with liquid nitrogen to induce the phase separation for the formation of NF matrix. Hollow microspheres with openings were obtained by using star-shaped PLLA while the linear PLLA was not able to generate the same hollow microspheres with openings. Compared with the traditional 3D porous scaffold, this new kind of injectable NF scaffold allows greater flexibility and convenience in repairing irregular shaped tissue defect and for minimally invasive procedures.

Synthetic NF scaffolds, which mimic the NF architecture of the natural ECM, are excellent tissue engineering scaffolds because of their unique properties, such as high surface-to-volume ratio, high porosity, and morphological similarity to the natural ECM. It has been found that NF scaffolds absorb 4.2-fold more human serum proteins than solid-walled scaffolds (control scaffolds with smooth pore morphology); therefore, they enhance osteoblastic cell adhesion [**54**]. The NF scaffolds also promoted osteoblastic progenitor cell (MC3T3-E1) attachment and enhanced the expression of the osteoblastic marker genes, indicating the increased level of differentiation of these cells. In addition, a significantly greater amount of mineral deposition was observed in the NF scaffolds than in the control (solid-walled) scaffolds. Calcium assay revealed 13-fold greater amount of mineral deposition in the NF scaffolds than in the control scaffolds [**55**]. These results demonstrated that the biomimetic NF polymer scaffolds enhanced cells' osteoblastic differentiation.

Mimicking the Composition

Another approach in creating biomimetic scaffolds is to mimic the chemical composition of the native ECM. Natural bone matrix is an inorganic/organic composite material consisting of collagen and minerals. HAP ($Ca_{10}(PO_4)_6(OH)_2$) crystals are dispersed in the NF collagen matrix and this natural composite has excellent mechanical and biological properties in the bone [**36**]. Approximately 60 wt % of bone is made of HAP and associated calcium phosphates; thus, it is natural that HAP and calcium phosphates (e.g., α-TCP, β-TCP) have been intensely investigated as a major component of scaffold materials for bone tissue engineering. As expected, calcium phosphates have an excellent biocompatibility because of their close chemical and physical resemblance to bone mineral. Numerous in vitro and in vivo assessments have reported that calcium phosphates, in all forms (powder, bulk, coating, or porous foam) and phases (crystalline or amorphous) support the attachment, differentiation, and proliferation of osteogenic cells (such as osteoblasts and mesenchymal stem cells) [**56**]. Therefore, calcium phosphates have been incorporated into many polymer matrices to fabricate inorganic/polymer composite scaffolds for bone tissue engineering.

One method to prepare PLLA/HAP and PLGA/HAP composite scaffolds is to combine blending and phase separation techniques (**Fig. 9.4, a** and **b**) [**57**]. These composites showed improved mechanical properties and osteoconductivity. HAP has also been incorporated into PLGA and PLGA/PCL polymer blends to produce composite scaffolds using the particulate leaching method [**58**].

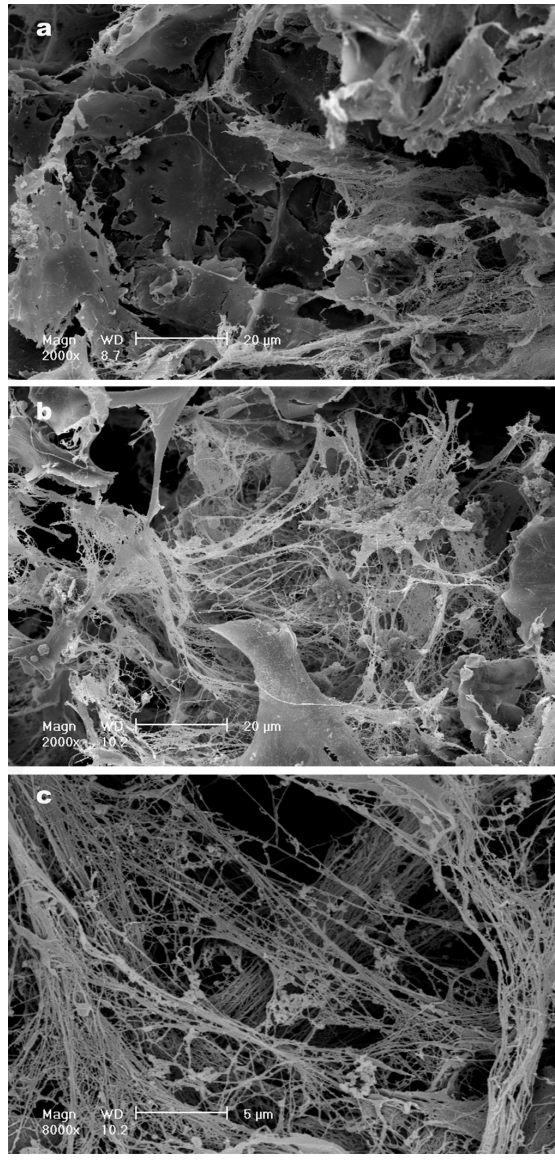
Rather than blending minerals into a polymer matrix, a biomimetic process has been developed to allow bone-like apatite nanoparticles to "grow" on the internal walls of the porous NF scaffold in a simulated body fluid (SBF) environment (**Fig. 9.4, c** and **d**) [**59,60**]. Many nanoparticles were grown on the internal surface of the scaffold after incubation in SBF and the macropores remained open as in the nontreated plain scaffolds. The particle size and the coverage of the surface can be controlled by the incubation parameters such as SBF concentration, incubation time, and pH value to achieve a desired composite structure. HAP was also successfully incorporated onto the gelatin scaffolds via a SBF method and the composite scaffolds were used to investigate the osteoblastic differentiation of MC3T3-E1 cells. The positive biological effect of HAP on osteogenic differentiation was shown by the enhanced bone marker gene expression: The genes encoding bone sialoprotein and osteocalcin in MC3T3-E1 cells on the HAP/gelatin scaffold were significantly upregulated compared with those in the cells on the unmodified gelatin scaffold [**61**].

Although the SBF method is a well-established method to deposit HAP onto the porous scaffolds, a faster method would be more desirable. Recently, a substantially fast and versatile process has been developed to deposit calcium phosphate via an electrodeposition technique (**Fig. 9.4, e** and **f**). A NF PLLA film was deposited on a metal electrode, followed by the calcium phosphate coating through electrodeposition [**62**]. High-quality minerals were achieved within 1 h, and the surface topography and chemical composition (Ca/P ratio) of the minerals could be tailored by processing parameters such as temperature, deposition time, and voltage. It was found that after 10 days in culture, the cell number was significantly higher on the mineralized NF PLLA films than on the unmineralized NF PLLA films. The alkaline phosphatase (ALP) contents of cells on mineralized scaffolds were significantly higher than that on unmineralized PLLA films after 7 and 14 days in culture, confirming that the presence of calcium phosphate on PLLA nanofibers enhances osteoblastic differentiation of the cells. This electrodeposition method has also recently been successfully performed to achieve rapid mineralization of a gelation matrix [**63**]. Therefore, the electrodeposition technique was demonstrated to be a rapid and an effective approach to mineralize scaffolds. More importantly, electrodeposition offers the potential to modulate the calcium release kinetics, which is largely determined by the crystal structure and the chemical composition (Ca/P ratio), and thereby to regulate the cell proliferation and differentiation (ongoing research in the Ma laboratory).

Mimicking the Surface Chemistry

In addition to mimicking the structure and composition, one approach is to mimic or reproduce the surface chemistry of the ECM because the scaffold directly affects the

FIG 9.5 Scanning electron microscopy images of PLLA scaffolds 4 weeks after cell seeding. (a) PLLA control. (b) PLLA scaffold surface-modified with gelatin using an entrapment method, followed by chemical crosslinking. (c) The surface-modified scaffold viewed at higher magnification. A significantly higher amount of collagen fibers and other cell secretions were deposited on the surface-modified scaffolds than on the control scaffolds.
Source: Reprinted with permission from Liu et al. [**68**].



cellular activity through surface interaction. As discussed earlier, a lack of biological recognition on the surface is a potential drawback of synthetic polymers; therefore, various modification methods have been explored to obtain more desirable surface characteristics of scaffolds for tissue regeneration application.

Bulk or surface modification can generally serve to obtain a desirable cell and material interface. In a typical bulk modification process, the functional groups or the biological recognition sites are introduced into the polymer backbone before the scaffold fabrication. For example, an Arg-Gly-Asp (RGD) peptide, a well-known sequence for cellular adhesion, was chemically grafted onto the lysine residue of poly (L-lactic acid-co- L-lysine) to enhance cell adhesion [**64**]. However, a potential drawback of bulk modification is that the process often results in the change of the properties of the matrix materials, and many of these bioactive sites may be buried inside of the pore walls rather than on the pore surfaces.

On the other hand, surface modification is performed after the scaffolds have been created. Therefore, this strategy will not significantly alter the architecture and properties of the scaffolds. Many techniques (e.g., partial hydrolysis [**65**], chemical vapor deposition [CVD] treatment [**66**], plasma treatment [**67**]) have been developed to modify the scaffold surface, but thus far most of them have only focused on a two-dimensional film or a very thin 3D scaffold.

Gelatin is derived from collagen by hydrolysis and has almost identical composition to that of natural collagen. Because gelatin is a denatured biopolymer, the selection of gelatin as a scaffolding material can avoid the concerns over possible immunogenicity and pathogen transmission associated with collagen. An entrapment method has been developed to enable gelatin to be physically immobilized on the surface through the entanglement with the molecule chains of the scaffold matrix [**68**]. A prefabricated PLLA 3D scaffold was immersed in a gelatin solution in a solvent mixture (e.g., dioxane and water) in which the PLLA matrix swelled but was not dissolved, allowing the gelatin molecules to penetrate to a certain degree. Then, the scaffold was moved to a nonsolvent of the PLLA, which caused the matrix PLLA to shrink, resulting in the entrapment of the gelatin. MC3T3-E1 preosteoblasts were seeded on the gelatin-modified scaffolds and were cultured for 4 weeks. It was observed that the surface modification significantly improved cell attachment (**Fig. 9.5**). In addition, cell numbers on the surface-modified films and scaffolds were significantly higher than those on the control films and control scaffolds. One advantage of the entrapment method is that it does not require functional groups on the matrix materials as long as proper solvent system is selected. Another advantage of the entrapment method is that it can be used to modify scaffolds with any porous geometry and morphology.

The electrostatic layer-by-layer self-assembly process has also been used to incorporate gelatin onto the surface of 3D NF scaffolds [**68**]. An interconnected porous 3D PLLA scaffold was first fabricated and was then activated with poly(diallyldimethylammonium chloride) (PDAC) to induce a positive surface charge. After being washed with water, the activated scaffold was then immersed in a solution of negatively charged

biomolecules (e.g., gelatin). Alternately immersing the scaffold into the two different solutions created multiple layers of biomolecules on the surface of the scaffold. This process is simple and can be performed in aqueous solutions, providing a controlled way to regulate the charge types and the thickness of the layers within the 3D scaffold.

As discussed previously, mimicking the natural collagen matrix with the gelatin-modified porous scaffolds combines the advantages of synthetic and natural materials. The synthetic PLLA provides the mechanical strength and controllable degradation rate whereas the gelatin material promotes cell adhesion and proliferation. In modifying complex 3D scaffolds, these methods are not limited to gelatin and can be applied with other bioactive molecules such as proteins, peptides, and growth factors.

Nanostructures on material surface have recently been shown to enhance scaffold performance because of their significantly increased surface area and roughness compared with the conventional material surface. Therefore, nanostructures have been used to increase cell adhesion and viability on porous scaffolds [**69**]. Nanophase ceramics such as nano-HAP have been demonstrated to promote mineralization in bone tissue engineering [**70**]. In addition to nanophase ceramics, nanophase metals, such as nanophase titanium and their alloys, were demonstrated to significantly enhance osteoblast adhesion compared with conventional metals [**71**]. Carbon nanotubes (CNTs) on scaffold surface were reported to accelerate ectopic bone formation [**72**]. However, many publications reported the adverse health effects of CNTs [**73,74**]. Therefore, further evaluation of the long-term effects of such nanomaterials is needed for their safe use in the field of tissue engineering.

MIMICKING THE SOLUBLE FACTOR MICROENVIRONMENT

In addition to the insoluble signals transmitted from ECM to cell (e.g., through matrix morphology and surface chemistry), soluble signals such as growth factors, hormones, and small molecules are important in cell function and tissue formation [**75**]. At any time of bone regeneration or fracture healing, multiple growth/differentiation factors are functioning in a coordinated manner. One of the research fronts in the tissue engineering field is to design bioactive scaffolds that can elicit a controlled action or reaction or both in a physiological environment. Scaffolds capable of delivering biological signals have the ability to regulate cells through the soluble signaling molecules and thereby regulate cellular activity. Considering the high cost, short half-life, and cells' sensitivity to biomolecule concentration, a biomolecule-releasing scaffold should be carefully designed. While maintaining the biological activity in vivo, such scaffolds should be able to release the desired dosage of the biomolecules in a temporally and spatially controlled fashion.

Biological factors have been directly incorporated inside of the scaffold matrix by several methods such as emulsion freeze-drying [**76,77**] and polymer-protein coaxial

FIG 9.6 BMP-7 releasing macroporous and NF PLLA scaffolds for bone regeneration. (a) In vitro release kinetics of rhBMP-7 from PLGA nanospheres immobilized on NF PLLA scaffold pore surfaces. (b) A scanning electron micrograph of the PLGA nanospheres immobilized on an NF PLLA scaffold at a lower magnification. (c) The above scaffold at a higher magnification showing the immobilized nanospheres on the surface of the internal pore walls. (d) Fibrous tissue formation in a control scaffold without rhBMP-7 after subcutaneous implantation in a rat for 6 weeks. (e) Fibrous tissue formation in a control scaffold presoaked in a rhBMP-7 solution after subcutaneous implantation in a rat for 6 weeks. (f) Significant bone formation in the rhBMP-7 nanosphere-containing scaffolds after subcutaneous implantation in a rat for 6 weeks (Panels d–f are hemotoxylin and eosin staining images with an original magnification of 100×).
Source: Reprinted with permission from Wei et al. [**82**].

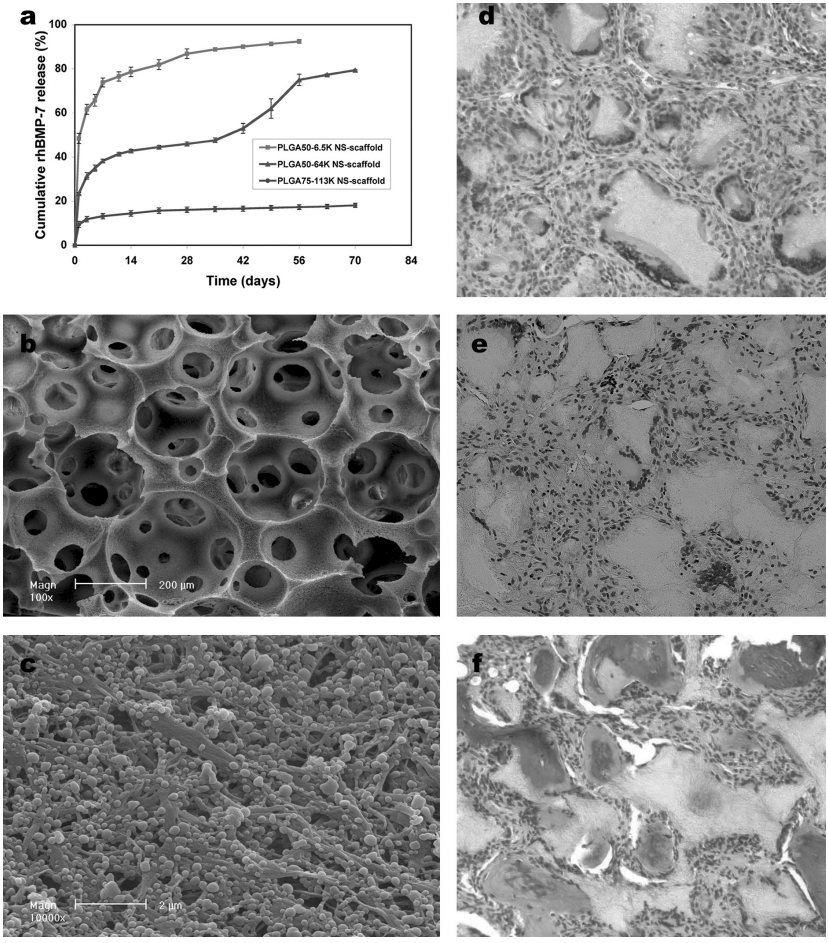
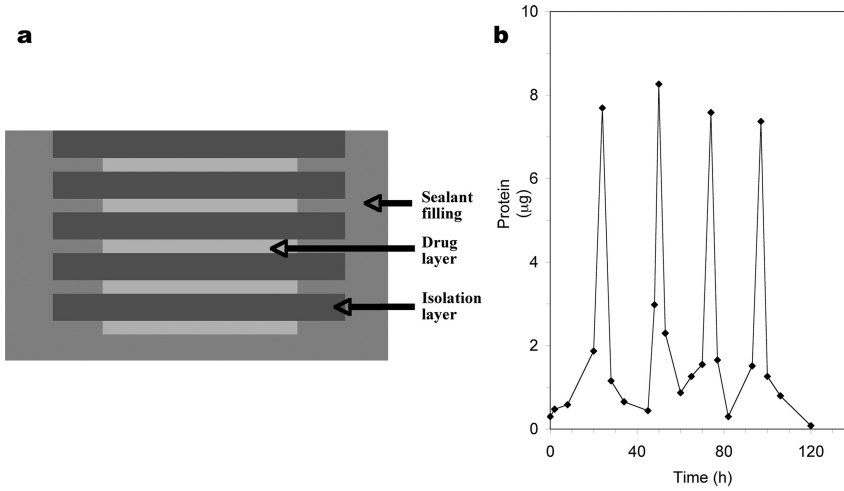


FIG 9.7 A pulsatile drug releasing system. (a) Schematic cross-sectional view of the device, showing alternately stacked polyanhydride layers and drug-containing layers. (b) Pulsatile release profile of BSA from the layered device. BSA, bovine serum albumin.
Source: Reprinted with permission from Liu et al. [**86**].



electrospinning [**78**]. The control over the release kinetics is limited when these techniques are used because the protein release is dependent on the bulk degradation of the scaffold. There have been also some attempts to coat the surface with biological factors by certain coating techniques [**79**], but poor ability to control the release kinetics and loss of bioactivity are two major shortcomings.

Polymer microspheres or nanospheres have been widely used to encapsulate various biomolecules to retain the bioactivity and to achieve controlled release [**80**]. Bone morphogenetic proteins (BMPs) are the most commonly used growth factors to induce osteoblast differentiation in bone tissue engineering [**81**]. PLGA nanospheres encapsulating recombinant human bone morphogenetic protein-7 (rhBMP-7) were prepared by using a double emulsion method [**82**]. Sustained or continuous delivery of rhBMP-7 from days to months could be easily achieved through varying the PLGA molecular weight and PLGA copolymer LA/GA ratio [**83**]. Subsequently, by using a postseeding technique, the rhBMP-7 nanospheres were immobilized uniformly on the scaffold via tight attachment to the surface of the matrix (**Fig. 9.6, c** and **e**). Scaffolds functionalized with rhBMP-7 nanospheres without cells were evaluated in a rat subcutaneous implantation study. After 6 weeks, histological results indicated that the sustained delivery of the rhBMP-7 from the nanosphere-functionalized scaffold actively induced ectopic bone formation while only fibrous tissue was formed within the blank scaffold or the scaffold with passively absorbed rhBMP-7. This technique

allows the 3D structure and the properties of the scaffold to be separated from the drug release system. Therefore, it provides greater flexibility and versatility in engineering a biomolecule delivery system and a scaffold architecture that can be independently achieved. Bone is a highly vascularized tissue; thus, bone regeneration should benefit from angiogenesis and corresponding blood vessel formations. Platelet-derived growth factor (PDGF) is a multifunctional growth factor that has been shown to play important roles in inducing vascularization and postnatal tissue repair. Using a similar technique as discussed above, PDGF-releasing microspheres incorporated in a porous 3D scaffold have been shown to promote angiogenesis in vivo [**11,84**].

For some drugs, intermittent (pulsatile) delivery is preferable to continuous delivery. Parathyroid hormone (PTH) is a typical example. The pulsatile delivery of PTH can improve bone microarchitecture whereas continuous exposure to PTH results in bone resorption [**85**]. An implantable device has been fabricated by alternately stacking polyanhydride isolation layers and PTH-loaded alginate layers [**86**]. Multipulse release of PTH was achieved while retaining the desired biological activity (**Fig. 9.7**). Because of the surface erosion property of the polyanhydrides, by adjusting the chemical composition and thickness of the isolation polyanhydride layer, the duration between the two adjacent pulses and the release profile can be adjusted. This device appears promising as release of multiple drugs can be achieved by loading different drugs in the same layer or in different layers based on a preprogrammed time sequence.

Although growth factor or protein delivery was used as an example, the approaches described above can be easily adapted to deliver other kinds of biological signals such as small molecules, DNA, and small interfering RNA (siRNA). The techniques discussed above provide a platform to engineer 3D scaffolds with controlled-release capacity. The release kinetics (continuous or pulsatile manner) as well as the spectrum of the biological factors (single drug or multiple drugs) and their release sequence can all be designed and programmed to regulate cellular activity and tissue regeneration.

PERSPECTIVE

Bone tissue engineering has progressed tremendously in the last 2 decades and has benefited significantly from the development of novel and advantageous scaffolds. Tissue engineering scaffolds have evolved from simply the physical supporting materials to biomimetic and bioinstructive materials, which better regulate cell function to promote bone regeneration.

Life sciences (including stem cell biology, genomics, proteomics, and so forth) are evolving dramatically and have substantially expanded the knowledge base for tissue regeneration. The rapid advancement of stem cell biology brings more and more cell types (e.g., new stem cells such as iPS cells) into the bone regeneration field and increases the complexity along with the new opportunities.

The biomimetic scaffold designs have greatly benefited from the knowledge of the natural extracellular environments around the regenerative cells. However, more systematic and quantitative studies are needed to uncover the underlying mechanisms of cellular interactions with their microenvironments and to establish the relationships between new tissue formation and the three key components (scaffold, cells and biological factors) in tissue regeneration.

Development of new and better scaffolds will remain as a centerpiece in tissue engineering research. The paradigm has been shifting from "doing no harm" to "eliciting a beneficial response" and to "programming tissue regeneration." A scaffold is not only used to provide a passive physical structure but it is also becoming an active participant in the bone regeneration process. With increasing knowledge about the extracellular microenvironments of stem cells and specialized cells along with the understandings of the mechanisms of their functions, the biomimetic scaffolds will evolve from blindly mimicking the ECM to more rationally mimicking and designing the ideal scaffolds. The future scaffolds will potentially be able to respond to and interact with cells in a more dynamic fashion in which they regulate cell's fate and function at the gene, molecular, nanometre, and micrometre scales.

In summary, bone tissue engineering has high potential to overcome the limitations of the current bone grafting therapies. Biomimetic scaffolds are receiving considerable attention as attractive substitutes for bone grafts. The increasing knowledge of stem cell biology and bone biology and the fast-evolving materials science will substantially advance this field. More advanced scaffolds integrating the biomimetic and rational design approaches will further accelerate the translation from tissue engineering research to clinical applications.

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Chapter 10 | Synthetic Bone Graft Substitutes: Basic Information for Successful Clinical Use

Barbara D. Boyan,[1] David J. Cohen,[1] and Zvi Schwartz[1,2]

INTRODUCTION

Bone grafting techniques are used in many fields of surgery, including orthopedic and trauma surgery, periodontal surgery, and maxillofacial surgery. The need to replace bone lost as a result of chronic disease, infection, or trauma is clear. In response to this need, the field has expanded considerably since synthetic substitutes were first used to supplement or replace autologous or allogeneic bone grafting. The number of choices continues to increase as new materials are developed, but in many clinical situations, the rationale for selecting one material over another material remains unclear. This is in part due to the lack of information on basic bone biology as it applies to implant materials in general and to a particular clinical application. The purpose of this review is to introduce the various synthetic bone graft materials currently available, discuss some of the new materials under development, and provide a biological rationale for the outcome when these materials are used clinically in orthopedics.

The current accepted gold standard in bone grafting is autograft because of its physical and biological properties as well as the fact that it is resorbed over time, allowing replacement with host bone. Although transmission of infection is minimal, autograft brings its own problems of donor site morbidity and limitation of supply [**1**]. The most common bone graft substitute in clinical use is allograft, including frozen, freeze-dried, and demineralized. Cadaveric allograft bone has the advantage of being osteogenic, albeit to a lesser degree than autograft, and there is no donor site morbidity. Cadaveric allograft is not in limitless supply, and the tissue processing it must undergo to reduce the risks of transmission of infection can cause changes in its structural integrity and its biological activity. Tissue banks distribute allografts, and there is lack of uniformity in the products of individual banks [**2**]. Thus, there has been limited standardization of material when performing experimental or clinical work

[1] Virginia Commonwealth University, Richmond, VA
[2] University of Texas Health Science Center at San Antonio, San Antonio, TX

TABLE 10.1 Osteoinductive, Osteoconductive, and Osteogenic Materials Used As Bone Graft Substitutes

Osteoinductive	Osteoconductive	Osteogenic
DBM[1]	Ceramics	Autografts
BMPs[2]	Hydroxyapatite	Allografts
	Deproteinized bone	Xenografts
	Coral-derived products	MSCs[3]
	Calcium phosphates	Growth factors
	Calcium sulfates	Cytokines
	Polymer scaffolds	Enamel matrix proteins
	Bioglass	Attachment peptides[4]

[1]Demineralized bone matrix, demineralized freeze-dried bone allograft (DFDBA).

[2]Bone morphogenetic proteins including BMP-2, BMP-4, and BMP-7 (osteogenic protein-1, OP-1).

[3]Mesenchymal stem cells, marrow stromal cells, and osteoprogenitor cells.

[4]Peptides such as the arg-gly-asp (RGD) sequence in fibronectin; the attachment proteins themselves.

Notes: In clinical practice, two or more of these materials may be used in combination. Generally, all of these materials are used with any available autologous bone and, in some cases, with autologous bone marrow. Osteoinductive materials cause bone to form in tissues that would otherwise not support bone formation; osteoconductive materials provide a substrate that supports the migration of osteoprogenitor cells, their proliferation and differentiation into osteoblastic cells, and extracellular matrix synthesis and physiological calcification by these cells. Osteogenic materials permit bone formation to occur in an orthotopic site to a greater extent than would be expected in the presence of an osteoconductive substrate.

using human material. This, coupled with the limits to supply of bone grafts, has stimulated the development of synthetic bone graft substitutes.

The ideal synthetic bone graft substitute material would be osteoinductive, osteoconductive, able to bear weight, resorbable in a predictable manner, biologically acceptable, and have a proven safety profile with no adverse local or systemic effects. As yet, the perfect material does not exist, although many materials address one or more of these features (**Table 10.1**). These descriptive terms have evolved as our understanding of bone biology has developed. They are now used in very specific ways in the current literature and have been codified in ASTM Standards **F2529-13** and **F2721-09**.

Osteoinduction is a specific term describing the ability of a material to cause bone to form in a tissue that would otherwise not form bone. This can only be shown in a heterotopic site because it is not possible to distinguish osteoinduction from osteogenesis in an orthotopic site. However, materials that are osteoinductive tend to enhance or promote osteogenesis in bone defects over the bone formation that would occur in the absence of the material. Bone morphogenetic proteins (BMPs) are classic examples of osteoinductive agents; implantation of BMPs in muscle tissue results in

formation of a complete ossicle consisting of cortical and trabecular bone and bone marrow. When BMPs are implanted in an orthotopic site, bone formation occurs more effectively. Demineralized bone matrix (DBM) also has osteoinductive characteristics, which are hypothesized to be due to the presence of BMPs.

Clearly, if BMPs are present in DBM, they are also present in autograft and allograft. However, unless autograft and allograft are demineralized, they are not osteoinductive by the definition above; rather, they are osteoconductive and potentially osteogenic. Osteoconduction is the ability to support new bone formation via the in-growth of new host bone into/onto a scaffolding material. This may be a naturally occurring scaffold such as an organized hematoma or autograft or a biomaterial such as synthetic polymeric foam. Agents that enhance osteogenesis but are not themselves osteoinductive can be thought of as osteogenic. These agents have the ability to enhance heterotopic bone formation by an osteoinductive agent or to enhance osteoconduction in an osseous site.

Different properties are required of bone graft materials in different clinical situations. Bone graft substitutes for use in long-bone diaphyses may need to be weight-bearing but mandibular grafts may not. Contained metaphyseal grafts may require different properties than grafts that bridge cortical defects. Some grafts will be placed with supportive internal or external fixation whereas others will be placed in a defect with no additional structural support. The site of graft placement will determine the functional properties expected of the graft material. Although the clinical use may differ, all bone grafts are expected to perform one function in all sites—that of re-creating a bone-like material in an area of bone that has been damaged by either trauma or chronic disease.

BIOLOGY OF WOUND HEALING

All bone grafts are placed in a wound, and it is appropriate here to review briefly the events occurring in a fresh, soft-tissue wound. In most tissues, wound healing is a reparative process rather than a regenerative one and results in a scar composed of fibrous tissue. The phases of wound healing provide an environment in which fibrous protein synthesis is facilitated. The first phase of healing includes hemorrhage, ultimately producing a hematoma. As the fibrin polymerizes, the edges of the wound become more closely approximated. Vasoconstriction also occurs. Platelets adhere to exposed endothelium and to the hematoma. Substances released from the damaged tissue and from the platelets themselves mediate platelet aggregation. The coagulation and complement cascades are initiated, and platelets release mitogens for endothelial cells and fibroblasts, such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) as well as other cytokines, chemokines, and regulatory agents.

In the second phase, vasodilatation occurs with a consequent increase in local blood flow. Chemotactic agents attract polymorphonuclear leucocytes (PMNs) and endothelial permeability increases. The PMNs release proteolytic enzymes, which

assist in remodeling of the hematoma. They also release chemokines and cytokines that modulate responses of the cells migrating to the hematoma. Circulating monocytes are attracted and are activated, becoming macrophages, which phagocytose the necrotic cell debris and produce factors to stimulate fibroblast proliferation and angiogenesis.

During the third phase of healing, the fibroblasts produce proteoglycans and structural proteins including collagen. Angiogenesis proceeds by capillary budding, and the wound edges contract. The wound is filled with vascular granulation tissue, in which fibroblasts proliferate and protein synthesis continues. Wound strength increases with the increase in collagen, and the fourth phase, that of scar formation, is entered.

In a wound in which healing conditions are compromised by low oxygen tension due to poor blood supply or by chronic pre-existing disease or drug effects, the healing process is slowed and the risk of infection increases. In wounds with infection or the presence of a continuing tissue irritant, chronic inflammation may supervene, and the process of inflammation will continue alongside the healing response.

BONE HEALING

Healing in a primary bone wound involves similar stages to healing in other wounds, with the initial formation of a hematoma, followed by an inflammatory reaction and PMN infiltration. The clot is invaded by macrophages and chemotactic agents attract marrow stromal cells and stimulate angiogenesis. Marrow stromal cells contain a few mesenchymal stem cells (MSCs), which have the ability to differentiate into various cell types depending on the local environment and regulatory factors. In addition, some of the marrow stromal cells are already in the osteochondral lineage, and in a bone environment they have the potential to differentiate into chondrocytes or osteoblasts, depending in part on the relative stability of the bone and oxygen tension. In sites that are stable and well vascularized, most of the cells will become osteoblasts, whereas in sites that are mechanically unstable and less well vascularized, the cells tend to become chondrocytes. Some of the marrow stromal cells may also differentiate into adipocytes, which is why regions of fat are frequently found in histological sections of healing bone.

MSCs are fibroblastic in appearance, as are many of the uncommitted or partially committed populations of marrow stromal cells. It is these cells that actually migrate to the wound site. If conditions are not optimal for bone or cartilage formation, then the cells may differentiate along a default pathway and become fibroblasts. Nonunion results when this occurs. Fibrogenesis also is seen next to implant materials that are not osteogenic or osteoconductive, when there is an excessive immune response, and when infection is present.

As indicated above, when the ends of the bone are in close approximation and the bone is mechanically stable, osteochondroprogenitor cells are able to migrate across the hematoma and directly form bone. After proliferation, these

cells differentiate into osteoblasts, which synthesize and then calcify osteoid via a mechanism that involves matrix vesicles. Those osteoblasts that are surrounded by calcified osteoid become osteocytes. This rapidly forming bone is termed *woven bone* because it lacks structural organization. After it undergoes remodeling, it is replaced by lamellar bone, including Haversian canals. This process takes varying lengths of time depending on the site and whether the bone is in mechanical function. Bone healing and remodeling generally require at least 6 months, and this may be longer in complicated or large wounds.

If the wound site is mechanically unstable, then callus formation occurs. In this situation, osteochondroprogenitor cells that have migrated to the wound site proliferate and differentiate into chondroblasts, which then synthesize cartilage matrix. This cartilage is similar to the cartilage found during embryonic bone formation and in the growth plate. It undergoes endochondral differentiation, resulting in calcified cartilage. Once the cartilage matrix is calcified, it is remodeled by chondroclasts (osteoclasts that resorb calcified cartilage), leaving the newly formed underlying bone. The calcified cartilage serves as a scaffold for new bone formation and it acts as an internal fixation device. As a result, wounds and defects that heal by callus formation are mechanically stiffer during healing than those that heal by primary bone formation.

Growth factors play important roles in fracture healing. These factors are released by platelets aggregating at the wound site and by the injured bone itself. In addition to BMPs, they include TGF-β, PDGF, insulin-like growth factors (IGF-I and IGF-II), and acidic and basic fibroblast growth factors (FGF-1 and FGF-2) [**3**]. FGFs play a particular role in angiogenesis and act as mitogens for osteoblast precursors.

The design of graft substitutes must take into account the fact that whichever material is used, it will be placed in a wound, often a wound in compromised tissue or where there has been a long-standing disease process. There will inevitably be interaction between the graft material and the local host tissue, which may affect the ability of the tissue to form living bone on the graft and to incorporate the graft. Therefore, experimental studies should take into account the differing environments in which the substitute compounds will be placed. The effect of age on the host bone and its ability to form new bone because of poorer vascularity or lower numbers of marrow stem cells and MSCs [**4,5**] must be considered, as should the effect of pharmacological agents such as steroids, anti-inflammatory drugs, and chemotherapeutic agents. The presence of significant host disease (e.g., diabetes, neoplastic conditions, infection, and vascular disease) may all have a detrimental effect on bone formation [**6**], and models or clinical studies must be designed to address the specific questions posed by each of these circumstances.

BIOLOGY OF BONE GRAFTING

The gold standard in bone grafting is autograft. One of the reasons for this is the fact that autograft is osteogenic in addition to being osteoconductive. However, it can also be stated that no bone graft substitution procedure can be undertaken without

some degree of local host bone debridement and preparation; therefore, there is always some autograft present at the graft site, even when synthetic substitutes are being used.

The synthetic bone graft substitutes include ceramics, polymers, and substances such as calcium sulfate and deorganified coral or bovine bone. These materials are osteoconductive rather than osteoinductive. Methods already exist to alter the properties of these materials to enhance their osteoconductivity and these will be outlined. These substances can also be used with various growth factors to enhance the biologic activity of the local host tissues, resulting in increased bone formation. Moreover, the addition of specific factors such as the BMPs can cause the materials to become osteoinductive. These materials are increasingly being used in combination with each other, with or without the addition of osteogenic factors, either from the host tissues or manufactured by recombinant techniques. Bone marrow cells may be added to these graft materials.

For new bone to form at the site of bone grafting, whether autologous or allogeneic bone grafts or synthetic bone graft substitutes are used, cells must be present that have the potential to become bone cells. These cells must be able to follow the osteoblastic lineage cascade, synthesizing and calcifying osteoid, and going on to become bone lining cells or osteocytes within the newly formed calcified matrix. These multipotent cells are present in adult human bone marrow [**7,8**]. Early experiments with rat bone marrow cells used with ceramic scaffolds show that they are capable of osteogenesis [**9**], even after culture expansion [**10**], and of healing bone defects [**11**]. This ability depends on local factors, including the presence of growth factors and other peptides [**12,13**], and on the oxygen tension of the local environment [**14**]. Other factors affecting their osteogenic potential include age and gender of the animal. The number of osteogenic cells is decreased in older rabbits and in humans, particularly in females [**4,5**]. Surface characteristics of any bone graft substitute material influence the adherence and differentiation of marrow stromal cells [**15,16**]. It is possible to alter these surfaces chemically by the addition of various proteins (e.g., fibronectin or laminin [**17**], peptides, or growth factors [**18**]) to promote cell attachment, proliferation, and osteoblastic differentiation.

SYNTHETIC BONE GRAFT SUBSTITUTES

Ceramics

Ceramics are highly crystalline structures formed by heating nonmetallic mineral salts to high temperatures in a process known as sintering. Many ceramics are known to be biocompatible and are used in orthopedics in various applications. These include the resorbable ceramics such as tricalcium phosphate (TCP), ceramics with highly reactive surfaces such as bioactive glasses, and ceramics with surface chemistries that do not react with biological fluids to an appreciable extent [**19**]. The least reactive ceramics (e.g., alumina and yttrium-stabilized zirconia) are in use in arthroplasty

components whereas the more reactive ceramics have been used as bone graft substitutes in the form of granules, porous blocks, and cements.

Bone growth behavior varies on different bioceramic materials. This is due in part to the morphological characteristics of the bone graft substitute. Granule size can alter the particle packing characteristics and affect bone in-growth through the resulting interstices [**20**]. The temperatures at which porous ceramics are sintered can affect biological response by altering the chemical and topographical features of the material surface [**21**]. Crystallinity (crystal size and perfection) also influences cell and tissue response by affecting the adsorption of serum components to the surface and ultimately the ability of osteogenic cells to attach, proliferate, and differentiate [**22**].

Ceramics can be modified to improve the tissue response. Porous ceramics have been shown to provide an osteogenic platform for bone marrow stromal cells [**10,11**], and the attachment of these cells is enhanced by fibronectin and laminin treatment of the ceramic surfaces [**17**]. Surface chemical modifications also influence cell reactions to ceramics [**23**].

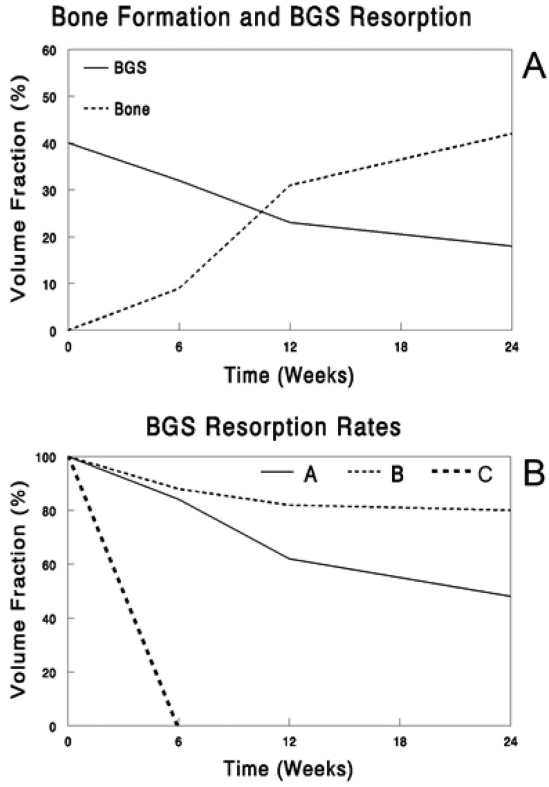
Hydroxyapatite

Hydroxyapatite is one of the families of calcium orthophosphate molecules, and it is one of the most biologically compatible substances used as a bone graft substitute material. Although synthetic hydroxyapatite materials share similarities with the mineral phase of bone, they are very different. Bone mineral is highly carbonated and exists as very small, plate-like crystals in a three-dimensional matrix in dynamic arrangement with proteins and other extracellular matrix constituents. Moreover, it contains numerous ion substitutions that alter the chemical and physical characteristics of the crystals; how they interact with the organic phase of the extracellular matrix; and, ultimately, the material properties of the bone. In contrast, synthetic apatites tend to be homogeneous in composition, with larger and more perfect crystals. Although organic constituents adsorb on the surface, they are generally not structural components of the biomaterial and do not modify the physical properties of the bone graft substitute.

Hydroxyapatites have been used in particulate form for over half a century [**24**]. Porous blocks prepared by sintering [**25**] enabled clinicians to use these materials to restore physical structure. Although this was an important advance, these materials tend to be highly crystalline and are resorbed very slowly—over decades rather than years. Other calcium phosphates are designed to be more soluble, such as TCP, but as a group, resorption is still relatively slow—certainly longer than the time needed to synthesize and remodel bone.

A bone graft substitute should ideally resorb as new bone is synthesized and remodeled (**Fig. 10.1**). If a ceramic implant remains after bone healing is complete, then it has the potential to alter the material properties of the bone and its mechanical resistance to stress. Most hydroxyapatite implant materials are osteoconductive, but when large blocks are used, even if they are highly porous, the ability of osteoprogenitor cells

FIG. 10.1 Relationship between BGS resorption and new bone formation. (A) Bone graft substitutes are ideally replaced by new bone. (B) To accomplish this goal, the BGS formulations vary in their rate of resorption. Even when designed to resorb over the average healing period for most bone defects, many BGS materials do not meet this goal and may remain in the tissue for relatively long periods of time, in some instances for the lifetime of the patient. In such instances, it is important to show that the retained material does not negatively affect the mechanical properties of the bone. It should be noted that even autograft and allograft might remain within newly formed bone for relatively long periods of time. BGS, bone graft substitute.



to migrate throughout the implant may be compromised, and fibrous connective tissue may result. To overcome these problems, hydroxyapatite and other calcium phosphates may be used as composites with a more resorbable material, such as collagen or a synthetic biodegradable polymer [**26–29**].

Pore size and porosity are also important characteristics of hydroxyapatite bone graft substitutes. Pore size was initially thought to be the most important factor [**30**]. No in-growth occurred with small pore sizes, and fibrous tissue formed with pore sizes

from 15 to 40 μm, whereas osteoid formed with pores of approximately 100 μm. Pore sizes of 300–500 μm were thought to be ideal because vascular in-growth could occur. Pore size may be less critical than the presence of interconnecting pores [**31**]. Interconnecting pores prevent the formation of "blind alleys," at the bottom of which is found low oxygen tension, which prevents osteoprogenitor cells from following the osteoblast lineage cascade, and they instead differentiate into cartilage, fibrous tissue, or fat [**32**].

There is recent evidence from animal studies that some synthetic hydroxyapatite materials may also be osteoinductive [**33,34**]. The observation of new bone formation in an ectopic site has been reported to occur particularly when specific geometric conditions are met. The packing of granules such that the interstices can support enrichment by critical growth factors appears to be important. Silicated hydroxyapatites have been shown to support heterotopic osteogenesis [**35–37**], indicating that surface chemistry may also be an important factor. Synthetic carbonated hydroxyapatite is not identical to the carbonated apatite of bone mineral, but it is an attractive bone graft substitute material because it is more rapidly resorbed than other forms of synthetic hydroxyapatite [**38**]. In vitro studies indicate that the amount of carbonate is a factor in osteoblast response to the material [**39**], but whether this is important in vivo is not known.

It is clear that ceramics are highly effective materials in many ways [**40–43**]. Porous hydroxyapatite ceramics have been used in the treatment of bone defects after curettage of benign bone tumors [**44**], in part because they are osteoconductive, but also because they provide space-filling structural support. However, a ceramic material that is also able to bear load has to date remained an elusive goal.

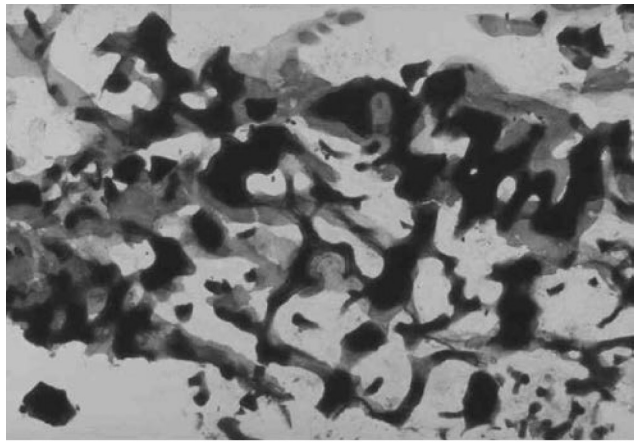
Coral-Derived Products

Coralline bone graft substitutes are derived from marine corals. Natural corals have a highly porous exoskeleton, which is similar in structure to cancellous bone. Coral has been used in its natural mineral form of calcium carbonate [**43**]. More commonly, the replamineform process is used to convert calcium carbonate to calcium hydroxyapatite [**45**].

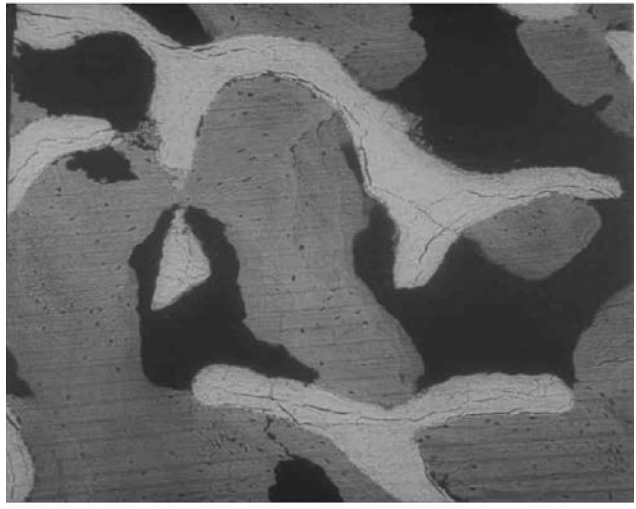
Hydroxyapatite is less resorbable than calcium carbonate, and hybrid forms of these compounds have been developed that have a core of calcium carbonate and a variable surface layer of calcium hydroxyapatite [**46**]. This allows the manufacture of a product with variable and, more importantly, predictable resorption rates. The hydroxyapatite layer resorbs slowly with bone formation, but the carbonate core, once exposed, resorbs rapidly, again allowing bone substitution.

Coral-derived bone graft substitutes are available in granular and block form. Depending on the type of material, the pore sizes may vary. Two average pore sizes have emerged as the most common, 200 and 500 μm, and studies have shown that the rate of vascular in-growth is comparable in both. One advantage of the coral-derived materials is that the pores are interconnected so that bone can grow throughout the interstices of the implants (**Figs. 10.2, A and B**). Coralline implants have been

FIG. 10.2 Bone formation within the pores of coral-derived bone graft substitutes. (A) Low-power micrograph of a human biopsy showing the ability of bone to grow throughout the interconnecting pores of an implant used as a bone graft substitute. The lack of blind alleys limits the formation of cartilage and adipose tissue. (B) Back-scatter electron micrograph showing the close inter-relationship of the newly formed bone (dark gray) and the bone graft substitute (pale gray) and soft tissue, including marrow and blood vessels (black).



A



B

used in lumbar spinal fusion, orbital reconstruction, and bone defect reconstruction in orthopedic oncology, in addition to the treatment of tibial plateau fractures and of distal radial fractures in conjunction with internal fixation [**40,41,47–49**]. These materials have been combined with growth factors and BMPs to enhance osteogenesis. When used without osteogenic factors, the coral-derived products are only osteoconductive.

Deproteinized Bone

Similar to the concept underlying the use of coral-derived materials as bone graft substitutes, bovine bone can be processed to remove the organic component, leaving the structural properties of the mineral intact. This is an attractive concept because the pore size and porosity of the resulting material is biologically compatible with normal bone. Deproteinized bone has been developed as an alternative to autograft or allograft using various processing methods. At lower temperatures, many of the physical characteristics of the bone mineral are retained, whereas at higher temperatures, the mineral becomes sintered hydroxyapatite. Recent studies have shown that bone processed at lower temperatures retains some organic material trapped within the mineral phase, including minute levels of biologically active osteogenic factors [**50**], which may contribute to the apparent clinical success of these bone graft substitutes. However, as with deproteinized bone processed at high temperatures, the attractive feature is the osteoconductive three-dimensional bone-like morphology.

TCPs

TCP was first used as a bone graft substitute in 1920 [**51**]. Whereas hydroxyapatite has a Ca:P molar ratio of 1.67, TCP has a Ca:P molar ratio of 1.5. TCP is less crystalline than hydroxyapatite; therefore, it is more soluble. Implants that contain TCP are both biocompatible and osteoconductive, but because of its relative solubility it is used in situations in which structural support is less important. TCP has been used in the treatment of large cancellous defects in pigs and in humans [**52,53**], and it has been used in spinal fusion mixed with allograft bone with results comparable to allograft alone [**54**].

α- and β-TCPs are high-temperature TCPs with a similar chemical composition to amorphous calcium phosphate but with increased crystallinity [**55**]. α-TCP is more soluble than β-TCP and is a major component in apatite cements. In addition to being more soluble than β-TCP, α-TCP has been reported to be more easily degraded in vivo. However, recent reports examining resorption in mini-pigs suggest that both forms of TCP degrade at comparable rates [**56**]. α-TCP bone graft substitutes can be obtained in block, granular, or powder form. β-TCP also has been used as a bone graft substitute as blocks or granules and is degradable by osteoclast activity [**31**].

Biphasic Calcium Phosphate

Biphasic calcium phosphate is a composite of hydroxyapatite and β-TCP. It is more rapidly degradable than hydroxyapatite alone. Biphasic calcium phosphate is

commercially available and has been used as a bone graft substitute in posterior lumbar fusion mixed with autograft [**57**]. Clinical results were good in 31 of 32 patients, and, in 3 patients, solid fusion was noted at second surgery for hardware removal.

Calcium Phosphate Cements

Calcium phosphate cements form on mixing one of a range of calcium phosphates with an aqueous solution, resulting in dissolution of the calcium followed by a precipitation reaction in which the calcium phosphate crystals grow and the cement becomes rigid. Brown and Chow invented the first calcium phosphate cement that could be constituted at room temperature from calcium phosphate powder and water in 1985 [**58**]. Modern formulations enable surgeons to directly inject the cement into the defect because the setup involves an endothermic rather than an exothermic reaction [**59–61**], limiting the potential for local tissue damage.

Calcium phosphate cements fall into one of two categories: apatite and brushite. Apatite cements form hydroxyapatite as an end product, although some have carbonates present and form carbono-apatites. They are more degradable than hydroxyapatite but still degrade slowly. Mechanical properties vary between the cements and depend to some degree on the composition. Porosity is similarly variable and has an inverse relationship with tensile strength. Mechanical strength increases over time in vivo. Apatite cements are biocompatible, and few inflammatory reactions have been reported. Brushite cements degrade to form dicalcium phosphate dihydrate (DCPD) and are more degradable than apatite cements. They resorb more quickly in vivo by dissolution and by osteoclast resorption. The mechanical properties of brushite cement decrease rapidly in vivo. However, as bone in-growth occurs, the mechanical properties of the healing bone increase. Brushite cements are biocompatible, although inflammatory reactions have also been reported [**62**].

Calcium phosphate cements have been used as carriers for growth factors, antibiotics, and BMP. As a group, the calcium phosphate cements are strong in compression but have low tensile strength, making them most suitable for treatment of fractures and defects that are not weight-bearing. Their use is reported in the treatment of fractures of the distal radius, the calcaneus, and the tibial plateau with or without fixation [**63–65**]. These materials are generally unsuitable for diaphyseal fractures.

Bioactive Glasses

Bioactive glasses are surface-reactive ceramics formed by melt or sol-gel techniques and are available in sintered porous bulk or particulate form. The surface of a bioactive glass takes part in a reaction with host tissue on implantation, involving dissolution of the surface of the glass and release of mineral ions. In vitro studies have shown that initial reaction of some bioactive glasses causes a local increase in pH [**66**]. Other studies have confirmed this and proposed that this alkalinization is beneficial to cell activity and hydroxyapatite production [**67**]. A calcium phosphate layer forms [**68**], and this layer is thought to enhance protein adsorption to the surface of the implant

[69] and to be involved in the surface reaction with host bone. Surface-treated bioglass has been shown to adsorb fibronectin more selectively than do hydroxyapatite ceramics [66]. Fibronectin is closely involved in the osteogenic function of bioglass ceramics [70]; this protein has been shown to increase cell attachment on bioactive glasses that have been pre-treated with calcium phosphate or hydroxyapatite surface layers [71]. The presence of both the calcium phosphate layer and serum proteins has been shown to influence the behavior of osteoblasts on the surface of the material [69].

Osteoblast responses to bioactive glasses are variable. In vitro studies show that the ionic products of glass dissolution increase osteoblast proliferation and upregulate many genes concerned with cell attachment, proliferation, and protein production in human osteoblasts [72–75]. It vitro studies also confirm that osteoblastic differentiation occurs in the presence of bioactive glasses [76]. Moreover, bioactive glass ceramics are osteogenic even in osteopenic bone [77], suggesting that they not only support osteoblast differentiation, but enhance it as well. Recent reports suggest that bioglasses may have some osteoinductive properties [78].

Porous melt-derived glasses resorb very slowly in vitro, but the recent sol-gel glasses, which have higher specific surface area, are much more resorbable while having similar osteoconductive effects and no loss of structural characteristics [79]. This allows replacement of the resorbed implant by new bone, thereby eliminating the concern that their retention in bone may compromise the mechanical properties of the tissue. Sol-gel derived bioactive glasses have several properties that make them attractive materials as bone graft substitutes. They may be used as carriers for proteins such as BMP-2 and TGF-β in the bone graft setting, and they appear to act synergistically with the growth factors [80–82]. One compound of this family has also been shown to have bacteriostatic properties [83].

Bioactive glasses have been studied for use in bone defects with good results in animal studies [84–86] and have been used clinically, particularly in restorative dentistry with varying results [87–89] and in craniofacial surgery [90]. These materials are biocompatible, and in many clinical situations are as effective as hydroxyapatite or autologous bone graft. However, some bioglass formulations are brittle and may form particular debris, contributing to the release of inflammatory cytokines [91].

Calcium Sulfate

Calcium sulfate is familiar to orthopedic surgeons as plaster of paris, but its use as a bone graft material was first described in the late 19th century, and over the ensuing years there have been reports of its use for this purpose [92]. Peltier described his experiences with this material in 1959 [93]; in 1978, he described the long-term follow-up of 26 patients with unicameral bone cysts, of whom 24 healed without complication [94]. Coetzee reported in 1980 on 100 patients in whom he had used calcium sulfate to treat cranial bone defects and concluded that it was a safe and effective substitute for autologous bone graft, allowing deposition of cancellous host bone while the graft substance resorbed [95].

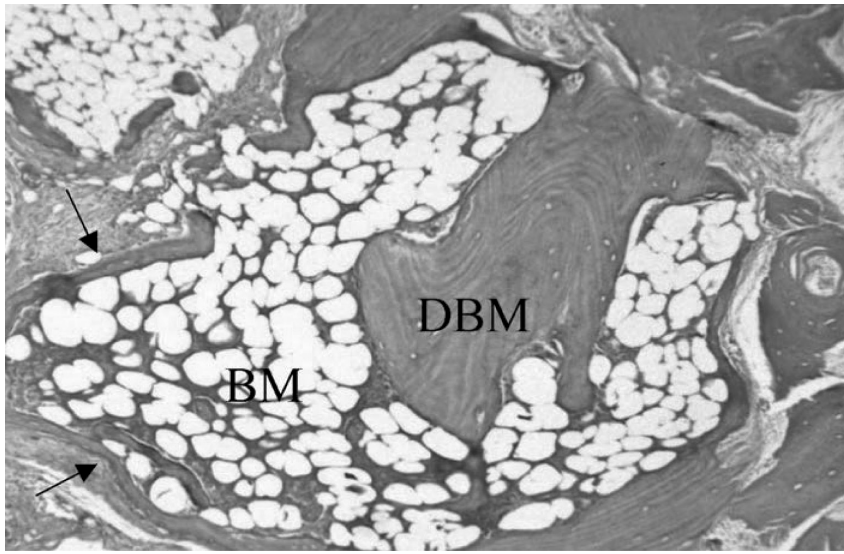
Calcium sulfate is a crystalline substance that is osteoconductive. The variable crystallinity of the early plaster of paris has been addressed in the manufacture of surgical-grade calcium sulfate, which has predictable structure and properties. This has been used in the form of pellets to treat bone defects with successful results. Kelly et al. [**96**] reported the use of calcium sulfate pellets alone or in combination with other substances in the treatment of 109 patients with bone lesions; radiographic and clinical follow-up showed that 88 % of defects filled with trabecular bone. Turner et al. [**97**] described a study of canine humeral models in which calcium sulfate tablets were implanted into large medullary defects and compared with autograft and no graft. The calcium sulfate-grafted defects compared well with the autografted defects, and histological examination confirmed the resorption of the calcium sulfate as the defects healed with new bone. In a sheep vertebral fusion model, in which calcium sulfate was compared with autologous bone graft, frozen allograft, coral-derived hydroxyapatite implants, and demineralized bone, there were no differences in the amount of new bone formed between the groups, and strength testing of the constructs was similar in autograft and calcium sulfate grafts [**98,99**].

Calcium sulfate has also been used as a bone graft expander with excellent experimental results in a canine spinal fusion model [**100**] and a canine femoral defect model [**101**] as well as excellent clinical results in the treatment of benign bone lesions [**102**] and human spinal fusion [**103**]. It has been used together with demineralized bone and with hydroxyapatite and bovine osteogenic protein-1 (BMP-7) [**104,105**]. In these studies, it was shown to be biocompatible and caused little inflammatory reaction, although this has been described [**106**]. However, its relatively rapid resorption means that it is not suitable for clinical applications in which structural support is required of the graft material. Therefore, it can only be used alone in contained, nonstructural defects or combined with fixation and other materials to enhance bone formation. It may well prove to be a good carrier for growth factors and BMPs in appropriate clinical settings.

Demineralized Bone Graft

Bone graft that has been demineralized is osteoinductive, on the basis of the definition that it can cause bone to form in tissues that would otherwise not form bone (**Fig. 10.3**; **ASTM F2529-13**). Dentists and oral and maxillofacial surgeons call this *demineralized freeze-dried bone allograft* (DFDBA) whereas orthopedic surgeons and neurosurgeons call it *demineralized bone matrix*. As a material, human DBM consists of cell remnants, extracellular matrix, and a small amount of residual mineral. Historical recognition of the value of DBM as a bone graft substitute dates from the time of Aristotle. More recently, Nicholas Senn reported on the use of cadaver bone that was "sterilized" using muriatic acid as a treatment for osteomyelitis in patients who needed bone graft during the U.S. Civil War in the 1860s. On the basis of the studies of Marshall Urist, Hari Reddi, and Julie Glowacki [**107–111**], the clinical use of DBM is now well accepted, particularly in situations in which the benefits of

FIG. 10.3 Photomicrograph of a retrieved implant that contained active DBM after 56 days of implantation in nude mouse calf muscle. Note the new bone formed (arrows), as well as BM and DBM particles. Sections were stained with hematoxylin and eosin; original magnification x20. BM, bone marrow.



osteoinduction are desired but the mechanical stability of the bone graft substitute alone is not required.

Exactly why DBM is osteoinductive is not well understood. This property is ascribed to the presence of active BMPs and their release from the mineralized matrix of bone during the demineralization step, but other factors may also play a role. When DBM is implanted in heterotopic sites, it attracts mesenchymal cells to the implant surface. There is some question as to whether the surface of the DBM is remineralized as an initial step because tissues that are implanted with DBM exhibit radio-opaque masses on X ray but histology may fail to show the presence of new bone [**112**]. If the DBM has retained its osteoinductive ability and a suitable responding cell population is present, then the mesenchymal cells will proliferate and differentiate into chondroblasts. The chondroblasts synthesize cartilage matrix and then undergo endochondral development, ultimately calcifying their extracellular matrix. These vascularized and osteoprogenitor cells are able to form bone on the calcified cartilage scaffold. The cartilage is ultimately replaced with marrow and marrow elements in the same manner as is seen in embryonic bone development. When DBM is implanted in an orthotopic site, endochondral ossification may be initiated, but it is

clear that direct induction of osteogenesis also occurs and in some situations may predominate.

Although allograft has all of the same components as DBM, it is not osteoinductive. Demineralization is an absolute requirement [**113**] to convey this property. If allograft is implanted in a heterotopic site, then it is resorbed [**114**]. However, if allograft is used orthotopically, it is very effective, more so than would be predicted by osteoconduction alone. This is likely due to the release of osteoinductive factors during osteoclastic resorption.

Most of what we know about osteoinduction has been learned from studies using rat and mouse DBM implanted in mesenchymal tissues of animals with compromised immune systems. Using these models, it is clear that the cascade of events is directed and timed in the same way in each experiment. When DBM is prepared from rats or mice with differing physiologies and ages, some variation exists, but for the most part, the DBM is prepared in a laboratory setting and is very reproducible in its size, shape, and composition. Human DBM is a very different story. Variability in the physiology of the donors is considerable. Donor age is negatively correlated with osteoinductive ability, but donor sex does not appear to be a factor [**115**]. Other issues also confound the problem. The length of time a donor has been dead can affect the osteoinductive properties of the DBM, as can the method of procurement [**116**]. Each bone bank has its own method for processing [**2**]. Although there are certain general processing steps, there is no agreed upon mandatory protocol. DBM is not only demineralized; it is washed, extracted with organic solvents, dried, cut, pulverized, sized, and sterilized by methods that are subtly unique to each bone bank. Thus, osteoinductive ability may vary as an intrinsic property of the donor bone and as a consequence of its preparation [**117**].

For many years, there was no agreed upon assay of osteoinduction. A consensus standard guide for determining the osteoinductivity of a material was recently established (**ASTM F2529-13**). This standard provides information on selection of an animal model as well as detailed instructions on how best to quantify relative osteoinductivity. It is unfortunate that it can be financially prohibitive for not-for-profit bone banks to test each batch of DBM for osteoinductivity in vivo. Although in vitro assays are under development, to date no in vitro assay has been shown in a peer-reviewed publication to be directly correlated with in vivo osteoinduction, although indirect correlations have been noted [**118,119**]. Thus, the sense of clinicians that DBM is frequently not more osteogenic than allograft is real.

Despite these drawbacks, DBM is an excellent material to use as a bone graft substitute because it is osteoconductive and, at the very least, it is osteogenic. DBM is provided to the clinician as a dried powder. Even when it is reconstituted in sterile saline, it has a tendency to float away from the defect site. To improve its handling characteristics, surgeons frequently premix DBM with autologous blood, allowing it to clot slightly before implantation. Commercial preparations have focused on sterility and shelf life in addition to handling characteristics. Currently, DBM has been

formulated with glycerol, calcium sulfate, hyaluronic acid, or a reverse phase polymer, and other possibilities are on the commercial horizon. Each of the carriers confers properties on the composite that differ from DBM alone. Some cause swelling of the particles and some provide a material with a putty-like consistency. By modifying the physical form of the DBM before mixing, the final product can be further manipulated to meet a clinical need. Although these modifications may add to the attractiveness of DBM in terms of use, it must be remembered that they also may reduce, or even destroy completely, the osteoinductive properties of the DBM. Even if the osteoinductive ability of the DBM is not negatively affected, the carrier may make the composite effectively nonosteoinductive by physically preventing attachment of the appropriate responding cell populations to the DBM.

DBM is itself an excellent carrier. It has been used effectively as a carrier for BMP, thereby enabling clinicians to make use of its excellent osteoconductive properties while ensuring that the implant is osteoinductive [**120**]. Active DBM can also be made more osteoinductive by addition of osteogenic materials such as proteins derived from porcine fetal enamel [**121**].

Polymers

Polymers are used in various surgical applications. Nonresorbable polymers include ultra-high molecular-weight polyethylene, used as a bearing surface in total joint arthroplasty, and polymethyl methacrylate, used as acrylic cement for implant fixation and filling of defects. These materials are not intended to be replaced with bone, although they may interface with bone tissue.

Biodegradable polymers have several different applications. Early uses included suture materials (polydioxanone, polylactide, polyglycolide) [**122,123**] and internal fixation devices, which resorbed, thereby theoretically avoiding the need for implant removal [**124**]. More recently, the use of bioresorbable polymers has expanded to include scaffolds for tissue engineering in various geometric forms, including porous membranes, porous blocks, and microspheres. These have been developed as carrier materials for cells and growth factors as well as other proteins [**125–131**], allowing local introduction of osteogenic substances to the area of the bone defect while providing a framework for in-growth of new host tissue.

Most of the bioresorbable polymers in use at present belong to the polyhydroxy acid family. This group of α-hydroxy acids includes poly(L-lactide) and poly(D,L-lactide) (PLA), polyglycolide (PGA), and copolymers based on PLA and PGA. Other polymers that have been studied include polypropylene fumarate (PPF), polycaprolactone, tyrosine-derived polymers, and polyanhydrides. When used as a bone graft substitute, either alone or as a composite, the polymers are constructed to form osteoconductive surfaces. The intent is for these materials to resorb as new bone forms, ultimately replacing the tissue-engineered medical product.

Many biodegradable polymers are delivered in particulate form so that they can pack irregularly shaped defects and to permit tissue in-growth through the interstices.

Particulate forms of the biodegradable polymers have a tendency to be lost from the defect site. To overcome this obstacle, several strategies have been used. One approach is to deliver the polymer in a resorbable gel, such as gelatin or fibrin. In addition, compounds have been engineered to allow injection into a bone defect and in situ polymerization rather than insertion of a preformed block [**132**]. This can involve the use of a photoinitiator, although this carries with it the potential for toxicity as the crosslinked particles degrade. Advances in click chemistry have made it possible to polymerize hydrogels without the use of a crosslinking agent [**133**], providing a new approach for delivery of osteogenic materials.

The concept of bioresorbability presumes that the synthetic bone graft substitute will be replaced by new bone as osteogenesis proceeds. The reality is very different. Some materials resorb over years; some never completely resorb. Thus, engineering materials so that their removal occurs in a reasonable period of time with only limited release of toxic byproducts is an important undertaking.

New technologies under development are taking advantage of polyethylene glycol (PEG) as a core component of porous polymeric scaffolds for bone. PEG is useful in this regard because it can be modified chemically to have various biologically active side chains [**134–136**]. Scaffolds constructed using polymers such as PEG are described as "nonfouling," meaning that proteins do not readily adsorb to their surface, thereby limiting cell attachment and migration. Thus, in addition to adding bioactive side chains, it is frequently necessary to add cell attachment peptides such as arginine-glycine-aspartic acid (RGD) [**137**]. In addition, to enable degradation of polymeric PEG, it is necessary to engineer crosslinks that are susceptible to enzymic breakdown via enzymes present (and active) at the desired sites [**138–140**]. The physiological consequences of released PEG monomer are not well understood. These polymeric systems are of necessity complex and their value as synthetic bone materials is yet to be established.

Vert et al. has outlined definitions for polymers based on their breakdown and their elimination or retention in the body, either locally or systemically [**141**]. The bioresorbable polyhydroxy polymers described above degrade by hydrolysis, resulting in formation of carbon dioxide and water, which are completely eliminated from the body. One consequence of hydrolytic degradation is the release of acidic products and a corresponding fall in the local tissue pH. As a result, local inflammatory reactions have been reported, and intraosseous cysts have been noted in bone in which resorbable polymer implants have been placed [**142**], leading to questions regarding the biocompatibility of polymer implants. In general, the normal buffering capacity of biological fluids is sufficient to compensate for the release of acid during degradation, and movement of these fluids ensures that byproducts of the degradation are diluted and are able to diffuse from the implant site. However, some polymeric materials undergo bulk degradation, causing the acid load to be greater than can be accommodated by dilution or physiological buffering. Other materials, particularly those that are more crystalline, degrade very slowly, causing the acid insult to become chronic.

Either of these two situations may affect tissue response. Fluid flow around polymers can also affect their degradation [**143**], suggesting that the local pH change may serve as a positive feedback mechanism, increasing the rate of degradation. It should be noted that in vivo and in vitro rates of degradation are variable and depend on the polymer composition, physical properties, and local environmental factors [**144**], including host site vascularity and degree of implant loading.

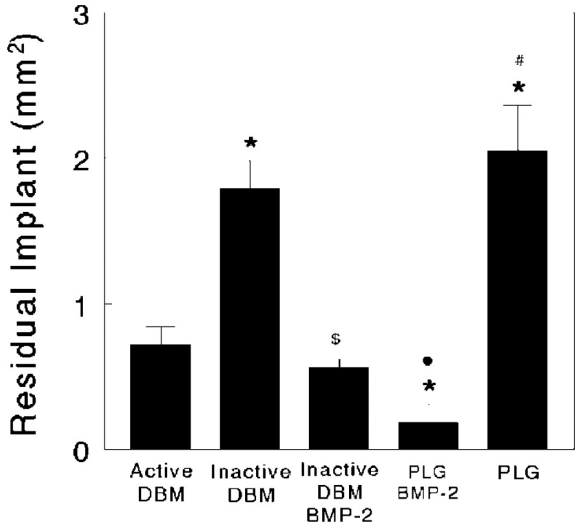
Acidic loading can be counteracted by incorporation of basic salts or calcium compounds such as hydroxyapatite or TCP into the polymer [**145,146**]. In addition to buffering properties, the incorporation of such substances into polymers allows the formation of composites that can be specifically designed to have predictable biomechanical properties and resorption rates.

Polymer scaffolds may be fabricated by several different methods, including casting, extrusion, molding, and solid free-form fabrication. The pore size and porosity are dependent on the method of manufacture of the scaffold [**147**]. The use of electrospinning to generate synthetic bone graft materials has provided new insights into the orientation of fibers on cell migration [**148**]. Other factors influencing the chemical, physical, and biomechanical properties of polymers and their composites include the chemical structure, morphology, composition, ratio of components, addition of low-molecular-weight components, presence of residual monomers, and polydispersity. In a rat study, it was seen that different calcium salts incorporated into the polymer scaffold had variable effects on local bone formation and inflammation, in addition to allowing porosity of the polymer composite to be controlled [**149**].

Much has been written on the pore sizes of ceramic bone graft substitutes and the relationship of pore size, porosity, and new tissue in-growth. It should be noted that the pore sizes of polymers engineered for use as scaffolds depends on the method of fabrication of the three-dimensional construct, but that many fall below the pore sizes of ceramic materials. However, bone growth has been noted in the smaller pores [**150**], and it is clear that overall porosity is considerably more important than simple pore size.

One of the most exciting and most studied areas of polymer science in relation to bone grafting is the use of polymer composites as carriers for osteogenic substances and cells. Several studies have reported on the kinetics of protein release from degrading polymers and shown that this is a predictable event. Polymers have been used as carriers for recombinant human bone morphogenetic protein 2 (rhBMP-2), and it has been shown that bone formation induced by a polymer/BMP-2 implant exceeds that of polymer alone [**128**]. It is interesting to note that BMP-2 also stimulates the degradation of polymer carriers (**Fig. 10.4**), perhaps because bone formation is enhanced [**128,132**]. The incorporation of VEGF and endothelial cells into polymer implants may be useful in improving the vascularity and incorporation of host bone growth [**151,152**]. By using various factors to modulate the differentiation of marrow stromal cells and MSCs, and by modifying the physical characteristics with calcium salts or by changing the characteristics of the polymer [**153,154**], degradable polymer bone graft substitutes

FIG. 10.4 Effect of BMP-2 on the degradation of bone graft substitutes. BMP-2 (5 μg) was added to DBM that had no osteoinductive ability or to PLG scaffolds and implanted intramuscularly in nude mice. The graph shows the area of residual DBM and PLG particles in comparison with osteoinductive DBM, inactive DBM, or PLG. Each measurement is the mean + SEM of eight implants harvested from four mice. *P < 0.05 vs. active DBM; #P < 0.05 PLG particles vs. inactive DBM or PLG particles plus rhBMP-2; •P < 0.05 PLG particles plus rhBMP-2 vs. DBM plus rhBMP-2; $P < 0.05 inactive DBM vs. inactive DBM plus rhBMP-2. PLG, poly D,L-lactide-co-glycolide.



have almost limitless potential, particularly if the application does not require load-bearing.

DISCUSSION

The work described in this chapter gives a snapshot view of the class of materials used as bone graft substitutes. These materials include calcium salts and calcium-based ceramics; synthetic polymers and biopolymers; and synthetic composites and biohybrid constructs of cells, proteins, and scaffolds. No single material will work optimally in all applications. By developing improved preclinical models, the ability to identify appropriate bone graft substitutes has moved forward considerably during the past decade. These studies have helped to define the parameters that must be met to determine if a bone graft substitute can be used effectively in humans. Depending on the nature of the defect and the health status of the recipient, there may be specific requirements for structural support, degradation rate, and addition of osteogenic components including growth factors and osteoprogenitor cells.

In vitro studies can be used to screen specific characteristics of potential bone graft substitutes, but it remains a necessity to test the effectiveness in vivo in an animal model. Studies using mice and rats have great utility in determining if a material will be biocompatible and if a material has clinical value in a bone defect.

To this end, the concept of a critical-size defect has been of considerable value. Critical-size defects are defects in bone that will not heal completely filled with bone if left untreated. When placed in the cranium or in a long bone, fibrous connective tissue and fibrocartilage fill the defect space, although there may be some bone healing at the margins. The critical-size defect can be used to assess the relative effectiveness of a material with respect to rate and quality of healing in comparison with the gold standard autograft, allograft, or another bone graft substitute.

However, for many applications in orthopedics, it is not necessary for a bone graft substitute to be as good as autograft. It is important that when used to extend autograft, it should not reduce the osteogenicity of autograft to any great extent. Among the most important functions of a bone graft substitute are to stabilize the hematoma that forms at a wound site and provide a structural support for cell migration and growth factor delivery. Thus, in some circumstances, these materials may enhance the osteogenic activity of autograft and allograft. Until appropriate standards are established and used, the maze of potential bone graft substitutes will unfortunately continue to confound the orthopedic, neurosurgical, reconstructive and maxillofacial surgery, and dental communities.

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Chapter 11 | Bone Graft Substitutes: A Regulatory Perspective

Tao Jiang[1, 2, 3] and Sergio J. Gadaleta[4]

INTRODUCTION

For more than 40 years, the orthopedic community, including academic, industrial, and government sectors, has been investigating the use of synthetic-, growth factor-, and cell-based therapies as bone graft substitutes, with the ultimate goal of reducing or eliminating the use of autograft in healing bony defects. Various synthetic bone graft substitutes, demineralized bone matrix (DBM), bone morphogenetic proteins (BMPs), allogeneic bone cells, and mesenchymal stem cells (MSCs) currently are legally marketed bone graft substitutes; however, each of these products has a distinct regulatory classification and, as such, requires a different regulatory path for approval before marketing.

The regulatory environment surrounding bone graft substitutes is complex and requires knowledge of device, biologic, and tissue regulations because these products are regulated by different centers under different regulations within the U.S. Food and Drug Administration (FDA). Synthetic bone graft substitutes, when indicated for filling bony voids or gaps that are not intrinsic to the stability of the bony structure, require premarket notification (also known as a 510(k)) before marketing. Bone graft substitutes incorporating morphogenetic proteins require premarket approval (also known as a PMA) and thus clinical studies to show safety and effectiveness before marketing. DBM, when provided without additives, is regulated as tissue for transplant; therefore, it does not require premarket notification or PMA before marketing. On the other hand, DBM with additives such as carrier medium requires premarket

[1] Institute for Regenerative Engineering, University of Connecticut Health Center, School of Medicine, Farmington, CT

[2] The Raymond and Beverly Sackler Center for Biomedical, Biological, Physical and Engineering Sciences, University of Connecticut Health Center, School of Medicine, Farmington, CT

[3] Department of Medicine, Division of Endocrinology, University of Connecticut Health Center, School of Medicine, Farmington, CT

[4] Becton Dickinson, 1 Becton Drive, Franklin Lakes, NJ

notification before marketing. The degree of regulatory control imposed by the FDA is different for each of the aforementioned products according to the product's classification and regulatory status. The Center for Device and Radiological Health (CDRH) oversees most medical devices regulated by FDA. CDRH also oversees PMA and oversight of all radiation-emitting devices, including animal devices. On the other hand, the Center for Biologics Evaluation and Research (CBER) regulates certain human devices that contain biological products.

The goal of this chapter is to provide the readers with a rudimentary review of the classification of medical devices, the mechanisms by which a manufacturer may commercialize a medical device, and the current regulatory status of various bone graft substitutes. Much of the text describing the FDA's programs such as premarket notification (510(k)), PMA, humanitarian device exemption, and product development protocols is taken directly from various publications available on the FDA's website and has been referenced as such. The readers are encouraged to review these documents in detail because they provide more comprehensive information regarding these topics.

CLASSIFICATION OF MEDICAL DEVICES

The Food, Drug, and Cosmetic (FD&C) act defines a medical device as "an instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent, or other similar or related article, including a component, part, or accessory, which is:

- Recognized in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them,
- Intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or
- Intended to affect the structure or any function of the body of man or other animals, and which does not achieve any of its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes..."

Medical devices may typically be classified into three general categories: Class I, Class II, and Class III. The classification of a device determines the type of application that must be submitted to the FDA before the product may be legally commercialized. Most Class I devices and a few Class II devices are exempt from the premarket notification (510(k)) requirements subject to the limitations on exemptions. However, these devices are not exempt from other general controls. A list of devices exempt from the premarket notification requirement may be found in the Title 21 of the Code of Federal Regulations (CFR) [1]. Medical devices categorized as Class III require the submission of a PMA application.

Medical devices categorized as Class I are devices for which general controls are sufficient to provide reasonable assurance of safety and effectiveness. General

controls consist of mandatory procedures to ensure proper registration, device listing, design and manufacture, labeling, and notification to the FDA before marketing the device. However, for certain Class I devices, general controls may not be sufficient to provide reasonable assurance of safety and effectiveness. These medical devices may remain Class I so long as they are not life-supporting, life-sustaining, and not for a use of substantial importance in preventing impairment of human health and do not present a potential unreasonable risk of illness or injury to the patient. General controls apply to all devices, regardless of class, unless specifically exempted by regulation. General controls contain requirements for device manufacturers or other designated persons to register their establishment with the FDA; list their devices with the FDA; comply with labeling regulation in 21 CFR Part 801, 809, or 812; submit a premarket notification to the FDA; and design and produce devices under the current Good Manufacturing Practices (cGMP). In 1990, FDA undertook the start of the revision of the cGMP regulation to add the design controls authorized by the Safe Medical Devices Act. FDA now requires design controls as part of the development process for regulated medical devices. The purpose of design controls is to ensure that medical devices be developed using a formal, documented engineering process. The Quality System Regulation (QSR, 21 CFR 820) requires design control procedures (21 CFR 820.30) to include provisions for design and development planning, design input, design output, design review, design verification, design validation, design transfer, design changes, and design history file [**2**]. **Table 11.1** provides a description of general controls.

Medical devices categorized as Class II are higher risk devices for which general controls alone are insufficient to provide reasonable assurance of safety and effectiveness; however, special controls are sufficient to provide reasonable assurance of safety and effectiveness. Special controls may include labeling requirements, mandatory performance standards, postmarket surveillance, patient registries, or guidance documents.

Medical devices categorized as Class III are devices for which insufficient information exists to ensure safety and effectiveness of the device solely through general or special controls. These devices, such as heart valves and pacemakers/implantable cardioverter-defibrillators, are judged to pose the highest potential risk. Moreover, these devices generally support or sustain human life, are of substantial importance in preventing impairment of human health, or present a potential unreasonable risk of illness or injury to the patient.

ROUTES TO MARKET

Manufacturers of medical devices are required to go through one of two evaluation processes before marketing a medical device: 510(k) or PMA. In certain instances, devices legally on the market before May 28, 1976, may not require either a 510(k) or PMA submission. These products, known as pre-amendment products, have unique regulatory status. Information regarding pre-amendment devices may be found in

TABLE 11.1 General Controls

General Controls	
Registration and listing	Section 510 of the FD&C act requires that U.S. device manufacturers and distributors register their establishments with the FDA on form FDA-2891. All manufacturers are required to list the generic type of devices they have in U.S. commerce with the FDA on form FDA-2892. Establishment registration and medical device listing should be submitted before commercial distribution. Requirements for establishment registration and medical device listing can be found in 21 CFR Part 807.20.
Labeling	All medical devices in U.S. commerce must be properly labeled. Device labeling requirements of the FD&C act are found in the following parts of Title 21: Part 801 General Device Labeling; Part 809, In Vitro Diagnostic Products; Part 812, Investigational Device Exemptions; Part 820, Good Manufacturing Practices; Part 1010, General Electronic Products. The FD&C act, Section 201, defines the terms *label* and *labeling* as they apply to medical devices as follows: Section 201(k) defines *label* as a "display of written, printed, or graphic matter upon the immediate container of any article…" The term *immediate container* does not include package liners. Any word, statement, or other information appearing on the immediate container must also appear "on the outside container or wrapper, if any there be, of the retail package of such article, or is easily legible through the outside container or wrapper." Section 201(m) defines *labeling* as "all labels and other written, printed, or graphic matter (1) upon any article or any of its containers or wrappers, or (2) accompanying such article." The definitions of label and labeling apply to devices held for delivery for shipment or for sale after shipment in interstate commerce. The term *accompanying* is interpreted liberally to mean more than physical association with the product. It extends to posters, tags, pamphlets, circulars, booklets, brochures, instruction books, direction sheets, fillers, etc. Accompanying includes labeling that is brought together with the device held for delivery for shipment or after shipment in interstate commerce.
Premarket notification	Most devices are cleared for commercial distribution in the United States by the premarket notification (510(k)) process. Most Class I devices are exempt from the 510(k) requirement by regulation. However, they are not exempt from other general controls, such as establishment registration and device listing. Before marketing a medical device, which is not exempt from the marketing clearance process, the manufacturer must submit a premarket notification (510(k)) or a PMA application to the FDA. The manufacturer cannot market the device unless the firm receives a marketing clearance letter from the FDA as stated in Section 513(I)(1)(A) or Section 515(d)(1)(A)(I) of the FD&C act.

the CFR. In addition, most Class I and a few Class II devices are exempt from the premarket notification requirement and, as such, may be commercialized without notification to the FDA.

Premarket Notification [3]

Most medical devices are cleared for commercial distribution by the premarket notification (510(k)) process. A premarket notification is a marketing application demonstrating that the medical device is as safe and effective as or substantially equivalent (SE) to a legally marketed device (i.e., predicate device) that was or is currently on the U.S. market and that does not require PMA.

If the Indication for Use statement of a new device is shared with another legally marketed device (i.e., a predicate device) that was or is currently on the U.S. market and that does not require PMA, the new device will require a premarket notification submission, before marketing, unless exempt from this requirement by regulation. However, it is important to fully understand the definition of a predicate device. A predicate device is

- A medical device that was legally marketed in the United States before May 28, 1976;

  OR

- A medical device that has been reclassified from Class II to Class I or II;

  OR

- A medical device found SE through the premarket notification process. The term *predicate device* only applies to devices undergoing review via premarket notification and not to devices requiring PMA (i.e., Class III). A medical device is SE if, in comparison to a legally marketed predicate device, has the same intended use and has the same technological characteristics of the predicate device OR has the same intended use and has the same technological characteristics of the predicate device AND does not raise new questions of safety and effectiveness AND demonstrates that the device is as safe and effective as the predicate.

However, if the proposed Indication for Use statement of the new device is NOT shared with another legally marketed device (i.e., predicate device) that was or is currently on the U.S. market OR shares an Indication for Use statement with a product approved via a PMA, the new device will require a PMA submission before marketing.

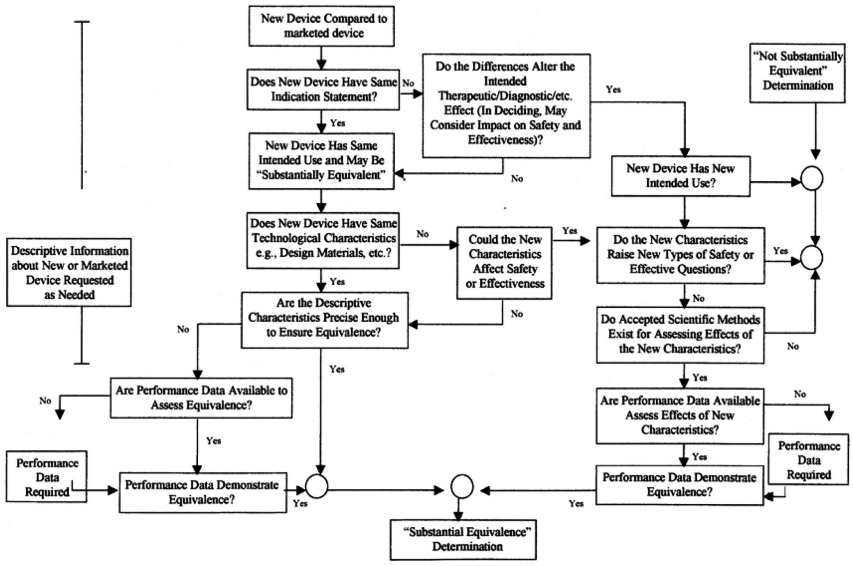
A medical device is SE to a legally marketed predicate device if the device

- Has the same intended use AND
- Has the same technological characteristics of the predicate device

  OR

- Has the same intended use as the predicate AND
- Has different technological characteristics of the predicate device AND
- Does not raise new questions of safety and effectiveness AND demonstrates that the device is as safe and effective as the predicate.

FIG. 11.1 Substantial equivalence decision-making process.



Substantial equivalence is established with respect to intended use, design, energy used or delivered, materials, performance, safety, effectiveness, labeling, biocompatibility, standards, and other applicable characteristics. A claim of substantial equivalence does not mean the devices must be identical. **Figure 11.1** presents a decision tree used to determine substantial equivalence.

If the FDA finds the device to be substantially equivalent, then the FDA will send the manufacturer a marketing clearance letter, referred to as an "SE letter," and the device may be marketed as described in the 510(k). If the FDA finds the device not to be substantially equivalent (NSE), then the FDA will send an NSE letter. In the latter instance, the firm may choose to resubmit another 510(k) with new information; may petition the FDA requesting the device be reclassified into Class I or II, as described in Section 513(f) of the act; or may submit a PMA.

PMA [4]

PMA is the FDA process to evaluate the safety and effectiveness of Class III devices. Class III devices are usually those that support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential, unreasonable risk of illness or injury. Because of the level of risk associated with Class III devices, the FDA has determined that general and special controls alone are insufficient to ensure the safety and effectiveness of Class III devices. Under Section 515 of the act, all devices placed into Class III are subject to PMA requirements. PMA by the FDA is the required process of scientific review to ensure the safety and effectiveness of

Class III devices. An approved PMA application is, in effect, a private license granted to the applicant for marketing a particular medical device.

The review of a PMA application is a four-step review process consisting of

1. Administrative and limited scientific review by the FDA staff to determine completeness (filing review);
2. In-depth scientific and regulatory review by the appropriate FDA scientific and compliance personnel (in-depth review);
3. Review and recommendation by the appropriate advisory committee (panel review); and
4. An FDA Good Manufacturing Practices (GMP) inspection.

During the administrative and limited scientific review, the FDA determines whether a PMA includes the type of information required by the FD&C act and the PMA procedural regulations (21 CFR, Part 814) and is suitable for filing. The filing of a PMA application means that the FDA has made a threshold determination that the application is sufficiently complete to permit a substantive review. If the information or data are not presented clearly or completely or are not capable of withstanding rigorous scientific review, then the FDA may consider the PMA incomplete and not file it. The 180-day review period provided by the FD&C act begins when the PMA is filed.

Any PMA accepted for filing may undergo an in-depth scientific review by the FDA personnel and may be presented to an advisory committee representing the appropriate medical field. The FDA notifies the PMA applicant of any deficiencies. Within the 180-day review period, the FDA will send the applicant an approval order under 21 CFR 814.44(d), an approvable letter under 21 CFR 814.44(e), a not approvable letter under 21 CFR 814.44(f), or an order denying approval under 21 CFR 814.45 [**5**].

The FDA will notify the applicant by letter of its decision to approve or deny, and in a *Federal Register* notice they will announce the decision and the availability of a summary of the safety and effectiveness data on which the decision is based. The notice also provides the applicant and other interested persons with an opportunity for administrative review of the FDA approval or denial action.

Product Development Protocol [6]

The 1976 Medical Device Amendments created a mechanism for the regulation of Class III medical devices that would allow a sponsor to come to early agreement with the FDA as to what would be done to demonstrate the safety and effectiveness of a new device. It was recognized that early interaction in the development cycle of a device could allow a sponsor to address the concerns of the FDA before expensive and time-consuming resources were expended. Many manufacturers already use the concept of concurrent engineering; that is, they involve manufacturing and service personnel early in the design process to identify and address potential concerns. The Product Development Protocol (PDP) extends this concept to regulatory requirements. It is an attempt at "front loading" the approval process by considering all regulatory areas as

well as product design and testing in the early concept and planning stages because this will most efficiently solve most problems. Thereby, the regulatory oversight during product development is limited to administrative and conformance assessment.

The PDP describes the agreed upon details of design and development activities, the outputs of these activities, and acceptance criteria for these outputs. It establishes reporting milestones that convey important information to the FDA as it is generated where they can be reviewed and responded to in a timely manner. The sponsor would be able to execute their protocol at their own pace, keeping the FDA informed of their progress with these milestone reports.

Because each device is unique, negotiations should be conducted to reach agreement as to what activities will be performed during the course of product development. This guidance document is designed to identify all potential areas for discussion of what the outputs of these activities will be and the acceptance criteria that can be used to assess these outputs. This "contract" establishes a predictable path to market with a potentially shorter review time frame when the device is ready for market. This document is intended to provide guidance throughout the life cycle of the device ("cradle-to-grave") on the engineering, preclinical, clinical, manufacturing, and postmarket content of the PDP contract.

The PDP process is initiated with an optional consultation with the FDA to determine if the device is appropriate for review as a PDP, PMA, or other regulatory pathway. This phase is referred to as the *proposal*. Its purpose is to establish that the FDA and the sponsor are willing to commit to activities, outputs, and acceptance criteria that would support regulatory approval of the described device. The sponsor would prepare a submission based on the guidance promulgated by the FDA. The FDA has 30 days to review this information.

After it has been determined that the appropriate regulatory mechanism for approval is the PDP, the sponsor may submit the Detailed Contents of the Protocol.

The protocol generally consists of a table of contents, device requirements and description, details of proposed verification and validation activities, clinical trial data, quality systems, and postmarket information. It may have details and timing of milestones and reporting requirements, notices, and special requirements for Notice of Initiation of Clinical Trials and the last progress report before the Notice of Completion. The FDA has 120 days to review the Detailed Contents, during which time it can request additional information. After this review, the FDA can either accept the PDP or deny for lack of content.

Humanitarian Device Exemption [7]

On June 26, 1996, the FDA issued a final rule to perform provisions of the Safe Medical Devices Act of 1990 regarding humanitarian use devices (HUDs). This regulation became effective on October 24, 1996. A HUD is a device that is intended to benefit patients by treating or diagnosing a disease or condition that affects fewer than 4000 individuals in the United States per year. A device manufacturer's research and development costs could exceed its market returns for diseases or conditions affecting small

patient populations. Therefore, the FDA developed and published this regulation to provide an incentive for the development of devices for use in the treatment or diagnosis of diseases affecting these populations.

The regulation provides for the submission of a humanitarian device exemption (HDE) application, which is similar in form and content to a PMA application, but it is exempt from the effectiveness requirements of a PMA. An HDE application is not required to contain the results of scientifically valid clinical investigations demonstrating that the device is effective for its intended purpose. However, the application must contain sufficient information for the FDA to determine that the device does not pose an unreasonable or significant risk of illness or injury and that the probable benefit to health outweighs the risk of injury or illness from its use, taking into account the probable risks and benefits of currently available devices or alternative forms of treatment. In addition, the applicant must show that no comparable devices are available to treat or diagnose the disease or condition and that they could not otherwise bring the device to market.

An approved HDE authorizes marketing of the HUD and is tantamount to a PMA approval. However, a HUD may only be used after institutional review board approval has been obtained for the use of the device for the FDA-approved indication. The labeling for a HUD must state that the device is a HUD and that, although the device is authorized by federal law, the effectiveness of the device for the specific indication has not been shown.

REGULATORY STATUS OF BONE GRAFT SUBSTITUTES

Synthetic Bone Graft Substitutes

At the time of the Medical Device Amendments of 1976, an FDA advisory panel was charged with the responsibility of recommending device classifications (Class I, Class II, or Class III) to the FDA for all devices known to have been sold in interstate commerce. Bone graft substitutes were not classified because, at that time, interstate commercialization of such devices was not known. Hence, new devices intended to be used as bone graft substitutes required PMA before marketing the product. Consequently, the first bone graft substitute devices submitted to the FDA were Class III devices requiring a PMA application. Two products were subsequently approved via PMA: ProOsteon™ 500 Porous Hydroxyapatite Bone Graft Substitute Blocks & Granules (Interpore Cross, Irvine, CA) and Collagraft™ (Collagen Corporation, Palo Alto, CA).

More recently, the existence of pre-amendment bone void filler for orthopedic use—U.S. Gypsum's calcium sulfate dihydrate bone void filler—was established. According to Section 510(k) of the FD&C act, post-amendment devices may be found SE to pre-amendment devices if both devices have the same intended use and the same technological characteristics. The establishment of a pre-amendment device

provided a new regulatory path (i.e., premarket notification) for products having the same intended use and technological characteristics as U.S. Gypsum's product. Wright Medical Technology was the first company to obtain 510(k) clearance for a bone graft substitute for orthopedic use—plaster of Paris pellets. The indication statement for the product reads

> Wright Plaster of Paris Pellets are intended to be gently packed into non-load-bearing long bone voids. These bone voids may be surgically created osseous defects or osseous defects created from traumatic injury to the bone. The Wright Plaster of Paris Pellets provide a bone filler that resorbs and is replaced with bone during the healing process.

Since Wright Medical Technology's 510(k) clearance, numerous companies have used this approach to market synthetic bone graft substitutes having similar indications for use and technological characteristics. A list of products cleared for this indication is shown in **Table 11.2**.

DBM

DBM has an interesting regulatory history and dynamic regulatory status. DBM is prepared by removing the mineral from human bone specimens by chelating the calcium phosphate mineral component of bone. The process of demineralization removes mineral and cellular components while preserving and exposing the endogenous proteins and growth factors of the bone specimen. The product can be formulated as a dry powder or with other additives to provide different physical and chemical characteristics.

DBM was originally regulated as a human tissue intended for transplant as defined by 21 CFR Part 1270:

> "any tissue derived from a human body, which:
>
> 1. Is intended for transplantation to another human for the diagnosis, cure, mitigation, treatment, or prevention of any condition or disease;
> 2. Is recovered, processed, stored, or distributed by methods that do not change tissue function or characteristics;
> 3. Is not currently regulated as a human drug, biological product, or medical device;
> 4. Excludes kidney, liver, heart, lung, pancreas, or any other vascularized human organ; and
> 5. Excludes semen or other reproductive tissue, human milk, and bone marrow."

In 1997, CBER issued a document entitled "Proposed Approach to the Regulation of Cellular- and Tissue-Based Products." The purpose of the document was to provide a unified approach to the regulation of traditional and new products, to specify

TABLE 11.2 Summary of Commercially Available Synthetic Bone Graft Substitutes

Company	Product	Composition	Available forms	FDA status
Biomet	BonePlast®	Calcium sulfate	Powder and accelerating saline solution	510(k) cleared ▪ Bone void filler
	PlatFORM™ CM	Type I collagen and carbonate apatite mineral	Block, strip, pad, and putty	510(k) cleared ▪ Bone void filler
	Pro Osteon® 200R	Hydroxyapatite and calcium carbonate	Granules	510(k) cleared ▪ Bone void filler
	Pro Osteon® 500R	A thin layer of hydroxyapatite over a calcium carbonate core	Granules or blocks	510(k) cleared ▪ Bone void filler
Depuy Synthes	Calceon® 6	Calcium sulfate	Pellets	510(k) cleared
	chronOS® Bone Graft Substitute	β-TCP	Granules and preformed shapes such as blocks and wedges	510(k) cleared ▪ Bone void filler
	Conduit® TCP Granules	β-TCP	Granules	510(k) cleared ▪ Bone void filler
	CRANIOS REINFORCED® Bone Cement	Calcium phosphate and sodium hyaluronate	Injectable paste or moldable putty	510(k) cleared ▪ Bone void filler
	HEALOS® Bone Graft Replacement	Hydroxyapatite-coated collagen	Strips	510(k) cleared ▪ Bone void filler
	Norian® SRS® Calcium Phosphate	Calcium phosphate	Injectable paste or moldable putty	510(k) cleared ▪ Bone void filler
	Norian® Drillable	Calcium phosphate	Injectable paste or moldable putty	510(k) cleared ▪ Bone void filler

(Continued)

TABLE 11.2 Summary of Commercially Available Synthetic Bone Graft Substitutes (*continued*)

Company	Product	Composition	Available forms	FDA status
Exactech	OpteMx®	60 % hydroxyapatite and 40 % β-TCP	Granules, sticks, rounded wedges, wedges, and cylinders	510(k) cleared ▪ Bone void filler
	Ossigen®	Mineral-collagen composite	Blocks	510(k) cleared ▪ Bone void filler: used with autogenous bone marrow
Globus Medical	CONDUCT® Matrix	Carbonate apatite mineral and type I collagen	Putty, sponge, and strip forms	510(k) cleared ▪ Bone void filler
	Kinex™	Bioglass, collagen, and hyaluronic acid	Putty, gel, and strip forms	510(k) cleared ▪ Bone void filler
	MicroFuse®	Poly(lactide-co-glycolide) or poly(lactic acid) with or without barium sulfate and calcium sulfate	Granules, sheets, preformed blocks, putty, and ST MIS implants	510(k) cleared ▪ Bone void filler
Integra LifeSciences	Integra Mozaik™	80 % highly purified β-TCP and 20 % highly purified type I collagen	Strip and putty	510(k) cleared ▪ Bone void filler
Medtronic	MasterGraft® Granules	Biphasic calcium phosphate (85 % β-TCP and 15 % hydroxyapatite	Granules	510(k) cleared ▪ Bone void filler
	MasterGraft® Matrix	Biphasic calcium phosphate and collagen	Compression resist block	510(k) cleared ▪ Bone void filler: used with autogenous bone marrow

Company	Product	Composition	Available forms	FDA status
	MasterGraft® Putty	Biphasic calcium phosphate and collagen	Moldable putty	510(k) cleared ▪ Bone graft extender: used with autograft ▪ Bone void filler: used with autogenous bone marrow or autograft or sterile water or blood, or any combination thereof
	MasterGraft® Strip	Biphasic calcium phosphate and collagen	Compression resistant strip	510(k) cleared ▪ Bone void filler: used with autogenous bone marrow
NovaBone	NovaBone®	Calcium phosphosilicate	Putty, particulate, morsels	510(k) cleared ▪ Bone void filler
Stryker	Vitoss®	100 % β-TCP, or 80 % β-TCP/20 % collagen, or 70 % β-TCP/20 % collagen/10 % bioactive glass	Putty, strip, morsels	510(k) cleared ▪ Bone void filler
Wright Medical Technology	MIIG® 115 MIIG® X3	Calcium sulfate	Injectable graft	510(k) cleared ▪ Bone void filler
	OSTEOSET®	Calcium sulfate	Beads	510(k) cleared ▪ Bone void filler
	PRO-DENSE®	75 % calcium sulfate and 25 % calcium phosphate	Injectable paste	510(k) cleared ▪ Bone void filler ▪ Benign bone cysts/lesions
Zimmer	CopiOs®	Calcium phosphate dibasic and type I collagen	Sponge and paste	510(k) cleared ▪ Bone void filler

TCP: β-tricalcium phosphate.

criteria for regulation, and to provide harmonized review of applications within different centers of the agency.

The proposal provided guidance in its regulatory approach regarding DBM:

> The FDA would consider demineralized bone (decalcified freeze dried bone allograft) to be an unclassified pre-Amendments device rather than a tissue under section 361 because the bone is more than minimally manipulated. The FDA would seek a classification recommendation from the Orthopedic/Dental Advisory Panels. The device to be classified would be defined as including allograft bone that is processed ONLY to demineralize and preserve the bone, and ONLY intended to be used as a bone filler in orthopedic and/or dental procedures.
>
> Based on current information, the FDA expects to propose that demineralized allograft bone be regulated as a Class I medical device exempted from pre-market notification. In addition, the FDA expects that it would also propose to exempt demineralized allograft bone from the cGMP requirements except for certain requirements consistent with those proposed for human tissues regulated under section 361.

Since 1997, no action was taken to formally classify DBM products; the FDA used enforcement discretion in its regulation of DBM. That is, the FDA has allowed DBM manufacturers to continue marketing DBM products without premarket notification or PMA. However, in March 2002, the FDA's Office of Compliance issued a letter to DBM manufacturers announcing the requirement of premarket notification for DBM products. In filing 510(k)s, CDRH has instructed sponsors to use resorbable calcium salt bone void filler devices as predicates for demonstrating substantial equivalence. **Table 11.3** shows a list of DBM products cleared by FDA for use as bone graft substitutes.

BMPs

BMPs, when provided with an osteoconductive scaffold, are a combination of a biologic and a device, respectively, and as such are regulated as combination products. Under Section 503(g) of the FD&C act, the FDA must designate a center within the FDA to have primary jurisdiction over the premarket review based on the primary mode of action of the combination product. Premarket review of combination products composed of BMPs and osteoconductive scaffolds is currently performed by CDRH. These products are Class III and require PMA.

Stryker Biotech was the first manufacturer to receive approval to market a BMP bone graft material—namely, OP-1 Implant. OP-1 Implant is an osteoinductive and osteoconductive bone graft material containing recombinant human osteogenic protein-1 (OP-1; also known as BMP-7) and bovine-derived collagen (3.5 mg OP-1:1 g collagen). This combination product device was approved as a humanitarian device for use as an alternative to autograft in recalcitrant long-bone nonunions in which use of

TABLE 11.3 Summary of Commercially Available DBM

Company	Product	Composition	Available forms	FDA status
AlloSource	AlloFuse®	DBM in an RPM	Gel, paste, and putty	510(k) cleared ▪ Bone void filler ▪ Bone graft extender
Biomet	InterGro®	DBM in lecithin, some versions contain calcium carbonate	Paste, putty	510(k) cleared ▪ Bone void filler ▪ Bone graft extender
DePuy Synthes	Optium DBM®	DBM in an inert glycerol carrier	Gel, putty	510(k) cleared ▪ Bone void filler
	DBX®	DBM in sodium hyaluronate carrier	Putty, mix, strips, and injectable putty	510(k) cleared ▪ Bone void filler ▪ Bone graft extender
	I/C Graft Chamber®	Allograft cancellous chips and DBM particles	Lyophilized and packaged within a delivery chamber	Regulated under CFR 1270 and 1271 as a human tissue and 510(k) cleared
Exactech	Optecure® and Optecure® + ccc	DBM in a hydrogel carrier	Dry mix kit delivered with buffered saline	510(k) cleared ▪ Bone void filler ▪ Bone graft extender
	Optefil®	DBM in gelatin carrier	Injectable bone paste/dry powder	510(k) cleared ▪ Bone void filler
	Opteform®	DBM and cortical cancellous chips in gelatin carrier	Putty, dry powder	510(k) cleared ▪ Bone void filler

(Continued)

TABLE 11.3 Summary of Commercially Available DBM (*continued*)

Company	Product	Composition	Available forms	FDA status
Globus Medical	XEMPLIFI™	DBM	Putty, gel	510(k) cleared ▪ Bone void filler
Integra LifeSciences	Accell Connexus® Accell Evo3®	DBM, Accell bone matrix, and a poloxamer RPM	Injectable putty	510(k) cleared ▪ Bone void filler, extremities, pelvis, spine ▪ Bone graft extender
	Accell TBM®	DBM, Accell bone matrix	Strip	510(k) cleared ▪ Bone void filler, extremities, pelvis, spine ▪ Bone graft extender
	DynaGraft® II	DBM, RPM	Injectable putty	510(k) cleared
	OrthoBlast® II	DBM, cancellous bone, RPM	Putty, paste	510(k) cleared
Medtronic	GRAFTON® DBM family	DBM	Putty, gel, sheets, strips, packable graft, moldable graft	510(k) cleared ▪ Bone void filler ▪ Bone graft extender ▪ Bone graft substitute
	Progenix® Putty Progenix® Plus	DBM in a carrier consisting of bovine collagen and sodium alginate	Putty with or without demineralized cortical bone chips	510(k) cleared ▪ Bone void filler ▪ Bone graft extender ▪ Bone graft substitute

Company	Product	Composition	Available forms	FDA status
RTI Surgical	BioReady™	DBM with a carrier derived from DBM	Putty with or without cortical cancellous bone chips	510(k) cleared ▪ Bone void filler
	BioSet®	DBM with gelatin as a carrier	Paste, putty, strips, and blocks with or without cortical cancellous bone chips	510(k) cleared ▪ Bone void filler
Smith & Nephew	VIAGRAF	DBM combined with glycerol	Putty, paste, gel, crunch, and flex	510(k) cleared ▪ Bone void filler
Wright Medical Technology	ALLOMATRIX®	DBM with or without cancellous bone chips and a carrier medium	Putty	510(k) cleared ▪ Bone void filler
	PRO-STIM®	DBM with calcium sulfate and calcium phosphate	Injectable graft	510(k) cleared ▪ Bone void filler
Zimmer	Puros®	DBM with or without cortico-cancellous allograft chips	Putty	▪ 100 % derived from allograft tissue ▪ Regulated under 21 CFR parts 1270 and 1271 as a human tissue

RPM: reverse phase medium.

autograft is unfeasible and alternative treatments have failed. OP-1 did not provide the agency with effectiveness data because this device was approved under the HDE regulation.

More recently, Medtronic Sofamor Danek received a recommendation for marketing approval, with conditions, by the Orthopedic and Rehabilitation Devices Panel of the FDA. The product, InFUSE Bone Graft, when used with the LT-CAGE Lumbar Tapered Fusion Device, is indicated to treat spinal degenerative disc disease, a common cause of low back pain. The FDA panel conditions for approval included three additional postapproval studies in the areas of antibody response during pregnancy, dosing, and tumorogenicity. In addition, the panel recommended the product only be used with tapered cages. The FDA-approved bone graft substitutes containing growth factors are summarized in **Table 11.4**.

Human Cells, Tissues, and Cellular- and Tissue-Based Products

The FDA's 21 CFR Part 1271 has described a unified registration and listing system for establishments that manufacture human cells, tissues, and cellular- and tissue-based products (HCT/Ps) and established donor eligibility, current good tissue practice, and other procedures to prevent the introduction, transmission, and spread of communicable diseases by HCT/Ps. According to 21 CFR Section 1271.10, an HCT/P is regulated solely under Section 361 of the Public Health Service act and the regulations in this part if it meets all of the following criteria:

- The HCT/P is minimally manipulated;
- The HCT/P is intended for homologous use only, as reflected by the labeling, advertising, or other indications of the manufacturer's objective intent;

TABLE 11.4 Summary of Commercially Available Growth Factor-Containing Bone Graft Substitutes

Company	Product	Composition	Available forms	FDA status
Medtronic	INFUSE®	rhBMP-2 and absorbable collagen sponge	Multiple kit sizes	▪ PMA approved for fusion with spinal cage ▪ PMA approval for open tibia fractures with IM nail
Stryker	OP-1® Implant	rhBMP-7 with type I collagen	Lyophilized powder reconstituted with saline	▪ HDE approval for long bone non-unions
	OP-1® Putty	rhBMP-7 with type I collagen and carboxymethyl cellulose	Lyophilized powder reconstituted with saline	▪ HDE approval for revision posterolateral lumbar fusion

rhBMP-2: recombinant human BMP-2; rhBMP-7: recombinant human BMP-7; IM: intermedullary.

- The manufacture of the HCT/P does not involve the combination of the cells or tissues with another article, except for water, crystalloids, or a sterilizing, preserving, or storage agent, provided that the addition of water, crystalloids, or the sterilizing, preserving, or storage agent does not raise new clinical safety concerns with respect to the HCT/P; and
- The HCT/P does not have a systemic effect and is not dependent on the metabolic activity of living cells for its primary function; or if it has such an effect or is dependent on the metabolic activity of living cells for its primary function, it is intended for autologous use, for allogeneic use in a first-degree or second-degree blood relative, or for reproductive use.

FDA regulations further define "minimal manipulation" for structural tissue as "processing that does not alter the original relevant characteristics of the tissue relating to the tissue's utility for reconstruction, repair, or replacement." In addition, FDA has stated that cutting, grinding, shaping, soaking in antibiotic solution, sterilization by gamma irradiation, lyophilization, freezing, and demineralization of bone are all examples of minimal manipulation. For cells or nonstructural tissue, minimal manipulation is processing that does not alter the relevant biological characteristics of cells or tissues. FDA has stated that density-gradient separation, cell selection, centrifugation, and cryopreservation constitute minimal manipulation. In contrast, the agency has concluded that cell expansion in culture is an example of more than minimal manipulation.

The FDA defines the term *homologous use* as "the repair, reconstruction, replacement, or supplementation of a recipient's cells or tissues with an HCT/P that performs the same basic function or functions in the recipient as in the donor." The FDA recognizes that homologous use does not necessarily require that tissue be used in its native location or even an analogous location.

A product eligible for regulation as a 361 HCT/P solely under Part 1271 is not subject to 510(k) premarket clearance or PMA. The FDA's CBER regulates HCT/Ps intended for implantation, transplantation, infusion, or transfer into a human recipient, including hematopoietic stem cells, and has jurisdiction over 361 HCT/Ps. The FDA has published comprehensive requirements (current good tissue practice, donor screening, and donor testing requirements) to prevent the introduction, transmission, and spread of communicable disease. An HCT/P that is subject to Part 1271 but does not meet the criteria for regulation as a 361 HCT/P may be subject to an additional layer of regulation as a drug, medical device, or biological product. For example, an HCT/P device is regulated by the FDA's CDRH and the device is subject to premarket notification or PMA.

The FDA has stated that cell-based therapies are one of the most rapidly advancing approaches to repairing, replacing, restoring, or regenerating injured or diseased tissues and organs. There are currently various commercially available products containing viable allogeneic cells for bone grafting, including the following:

- *Osteocell Plus® (NuVasive):* An allogeneic bone matrix containing living bone cells including MSCs and osteoprogenitor cells.
- *Trinity® Evolution™ (Orthofix):* An allograft of cancellous bone that is processed and cryopreserved to maintain viable MSCs and osteoprogenitor cells
- *AlloStem® (AlloSource):* Partially demineralized allograft bone combined with adipose-derived MSCs
- *Cellentra™ VCBM (Biomet):* DBM combined with 250,000 cells/mL viable osteogenic cells, including MSCs, osteoprogenitor cells, and preosteoblasts

However, there are other products that contain DBM and are designed to be mixed with bone marrow aspirate. Some of the products that are currently available are

- *Fusion Flex™ (Wright Medical Technology):* A dehydrated, moldable DBM scaffold that will absorb autologous bone marrow aspirate
- *Ignite® (Wright Medical Technology):* An injectable graft with DBM that can be combined with autologous bone marrow aspirate

It is worth noting that no products using in vitro cultured/expanded MSCs have been approved by the FDA for bone grafting. In an untitled letter to Regenerative Sciences, Inc., in 2008, the FDA declared that the MSCs that were promoted and sold by the company for use in the Regenexx™ procedure would be considered drugs or biological products because the MSCs were not considered minimally manipulated and were intended for nonhomologous uses [8]. Therefore, a submission of a New Drug Application (NDA) or Biologics Licensing Application (BLA) to the FDA is required. To date, no NDA or BLA has been approved by the FDA for this product. As of 2013, this company's expanded stem-cell procedure is only offered in the Cayman Islands. In the United States, Regenerative Sciences, Inc., provides Regenexx-SD™ Same-Day Stem-Cell Procedure, Regenexx-AD™ (Adipose Derived) Stem-Cell Procedure, and Advanced Blood Platelet Procedures, which are considered 361 HCT/Ps and do not require FDA approval.

CONCLUSIONS

Bone graft substitutes encompass various formulations from synthetic calcium salts to demineralized human bone tissue to recombinant growth factor technology and MSCs. These products differ in their technological characteristics and in their indications for use. In general, synthetic bone graft substitutes are indicated for filling bony voids or gaps that are not intrinsic to the stability of the bony structure and require premarket notification before commercialization of the product. That is, synthetic bone graft substitutes serve as osteoconductive matrices, allowing bone to grow on or within the products. DBM, recombinant human BMP, and MSCs are indicated as bone graft replacements because they provide osteoconductive and osteoinductive factors. This combination provides a signal to the cells to generate new bone and offers a scaffold on which these cells may deposit the newly formed bone. Because the

regulatory status of the medical devices depends on the technological characteristics and indications for use, it follows that these products have different regulatory classifications and hence different levels of regulatory control.

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Chapter 12 | Nanoscale Technologies for Bone Grafting

Linlin Sun,[1] Siyu Ni,[2] and Thomas J. Webster[3]

INTRODUCTION

According to a report by the American Academy of Orthopedic Surgeons, one in four people in the United States suffered from a musculoskeletal injury, with subsequent orthopedic implant operation and device costs totaling $20 billion (U.S.) in 2012 [**1**]. It is also clear that conventional bone implants have finite lifetimes and failure rates that increase with time. Currently, total hip and knee replacements have revision rates of approximately 6 % after 5 years and 12 % after 10 years; thus, significant additional costs will ensue because of implant failures alone. However, these statistics do not account for the additional overwhelming lack of return to a normal daily lifestyle after receiving such implants. Although patients do feel decreased pain in the long term after receiving such implants, those most physically active are not able to return to the active lifestyle they had before the event that led to the need to receive an implant. The related complications mainly result from implant loosening, infection, inflammation, and unmatched mechanical properties between the implant and juxtaposed bone leading to stress and strain imbalances [**2**].

Once implanted into the injury site, the direct connection between the implant surface and bone tissue (defined as osseointegration) without any interposed soft tissue layer is considered a significant success [**3,4**]. However, it usually takes several weeks to months for implants to osseointegrate with surrounding tissues. During that time, several issues can cause implant failure, such as fibrous capsulation, overloading, and infection [**5,6**]. For hip or knee implants, wear particles released from the implant surface may cause osteolysis, which is triggered by the immune response to such wear particles [**7**]. Osteolysis can induce the removal or loss of calcium from the juxtaposed

[1] Bioengineering Program and Department of Chemical Engineering, College of Engineering, Northeastern University, Boston, MA

[2] College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai, People's Republic of China

[3] Bioengineering Program and Department of Chemical Engineering, College of Engineering, Northeastern University, Boston, MA

bone, weaken the bone matrix, and cause implant failure [7]. Therefore, it is necessary to develop bone materials that promote bioactive bonding to bone, sustain long-term bonding to juxtaposed bone, have good biocompatibility, and possess high durability for long-term success—criteria that we do not have today with titanium or other currently implanted orthopedic materials.

It is clear that natural bones (and all tissues in the body) are nanostructured; consequently, their surfaces are nanorough [8]. The hierarchical structure of bone is mainly formed by collagen molecules (e.g., a type I collagen molecule is ~300 nm in length and 0.5 nm in width) and carbonated apatite (e.g., a hydroxyapatite [HA] nanoparticle is ~50 nm in length and 5 nm in diameter) [8,9]. Not only are bones composed of nanomaterials, but muscles, tendons, ligaments, joints, and connective tissues in the musculoskeletal system are also nanostructured [10].

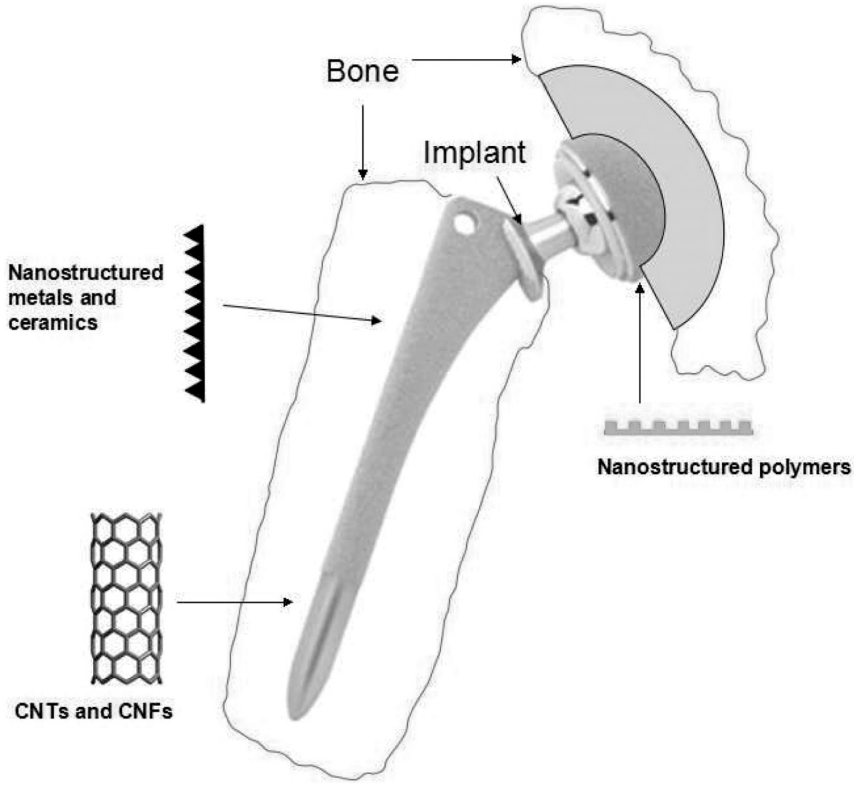
Therefore, learning from nature, once one changes the size of conventional materials from the macroscale down to the nanometre scale (<100 nm), numerous unique properties ensue, including unique optical, catalytic, energy, and electronic properties [11–13]. These novel properties of nanomaterials are all due to their significantly greater surface area and, thus, increased exposure of their chemistries [11–13]. For example, if developing nanoscale versions of hydrophilic conventional (or micron) chemistries, one can significantly improve the net surface energy. Likewise, if one creates nanoscale versions of hydrophobic conventional chemistries, one can significantly decrease net surface energy. Because the initial protein adsorption and subsequent cellular adhesion depend on implant surface energy, the ability to tailor net surface energy without altering chemistry can significantly help researchers pave the way to design better orthopedic implants [14,15].

Generally speaking, through this mechanism of controlling surface energy, nanotechnology facilitates the design of optimal bone implants in terms of increasing tissue growth, reducing an inflammatory response, and decreasing bacterial infection (**Fig. 12.1**) [14–16]. Because of the healing response, nanomaterials are able to enhance specific protein interactions (including adsorption and bioactivity), selectively increase bone cell (e.g., osteoblast, chondrocyte) growth, inhibit fibrous (e.g., fibroblast) formation, reduce immune cell responses (e.g., macrophages, neutrophils), decrease biofilm formation, promote bone regeneration, and control the biodegradability of implants for numerous applications [14–17].

NANOSTRUCTURED METALS

Since the 1950s, titanium and its alloys have been used for numerous orthopedic implant applications because of their corrosion resistance and suitable mechanical properties to support physiological loading conditions [18]. However, conventional (or micron-structured) metallic implants suffer significant drawbacks including weak osseointegration over the long term, osteolysis, infection (which is on the rise), and implant wear [19–21]. The interfacial movement of implants during use can cause the release of wear particles,

FIG. 12.1 Nanotechology has been able to facilitate an improved long-term performance of bone implants by modifying surface properties (such as energy) through the use of a diverse set of nanostructured materials, including nanostructured ceramics, nanostructured polymers, carbon nanotubes (CNTs), and carbon nanofibers (CNFs).



triggering cellular toxicity, osteoclast (bone resorbing cells) activation, and eventually enough bone resorption to lead to implant loosening [**20,21**]. Compared with conventional metals for orthopedic applications (e.g., Ti, Ti6A14V, and CoCrMo), metallic nanostructured topographies can increase osteoblast adhesion, proliferation, and subsequent long-term functions, as well as inhibit fibroblast (fibrous-tissue forming cells) functions [**12,22–25**], to compensate for such implant loosening.

Various nanostructured metallic materials have been developed based on the metallic chemistries used today, including titanium alloys, cobalt chromium, aluminum alloys, and stainless steel. These metals have been created to possess various types of nanoroughness, nanoparticulate morphologies, nanotubular morphologies,

and so on [12,25]. There are several main methods to create nanostructured features on metals, including chemical etching, powder processing, ionic plasma deposition, and anodization [12,25]. Importantly, it has been found that no matter which technique is used to create such nanotopographies on metals, improved bone growth results, thus demonstrating the versatility of nanotechnology in orthopedics.

As an example, with titanium, the nanoroughness increases the amount of grain boundaries, which increase the surface energy to enhance the select adsorption of proteins (e.g., vitronectin) to promote osteoblast adhesion (**Fig. 12.2**) [26]. In addition, the same promotion of surface energy can result when using anodization (an electrochemical method) to create a nanoporous or nanotubular layer on the surface of metals [25]. Anodized titanium, Ti6A14V, aluminum, steel, and nitinol have all been studied for orthopedic applications and show promising results [25].

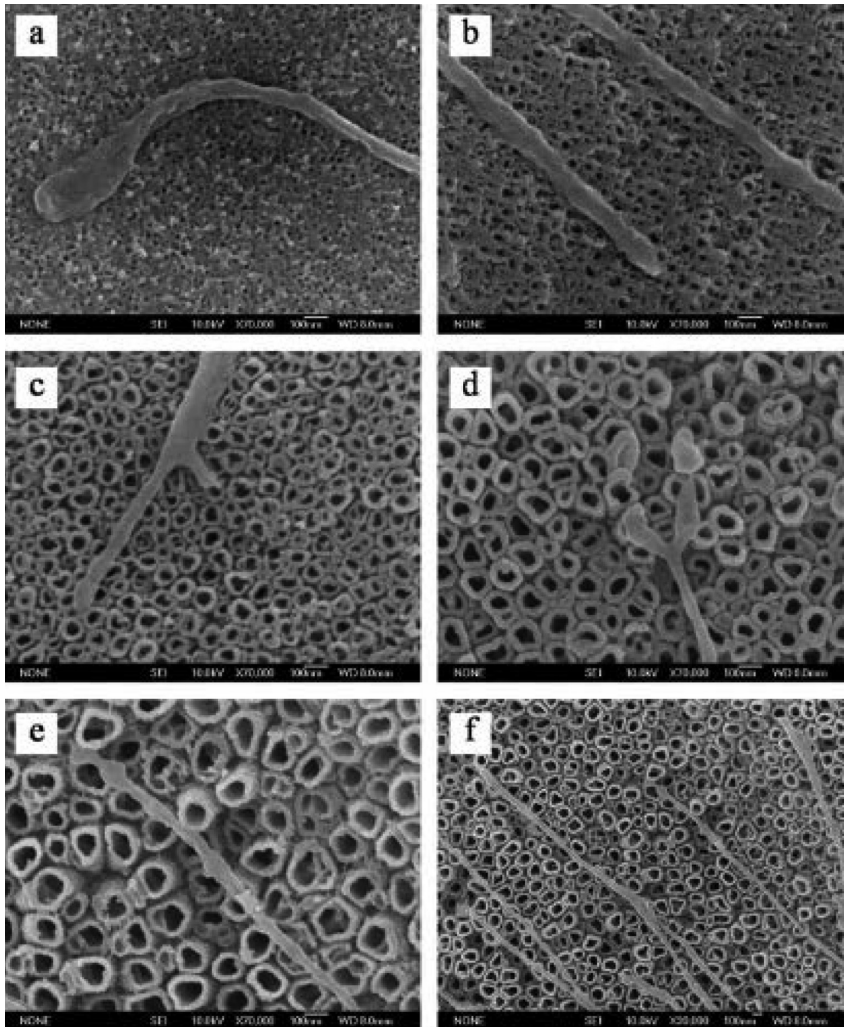
In vitro studies also showed that, once coated with nanophase alumina or titanium nanoparticles, the surface energy of conventional substrates was significantly enhanced and induced high-protein adsorption and bioactivity (specifically for vitronectin and fibronectin), which consequently increased osteoblast adhesion and long-term functions [27,28].

Some researchers have even demonstrated that titanium dioxide or zirconium dioxide nanostructures promoted mesenchymal stem cell differentiation into osteoblasts without the use of pharmaceutical agents or growth factors [27,29]. Moreover, titanium with leaf-shaped nanostructures promoted greater osteoblast gene expression from mesenchymal stem cells than nanotubular or nanoneedle structures. Although the mechanism remains to be elucidated, it is speculated that such enhanced stem cell differentiation to the osteoblastic lineage is due to the optimal initial protein adsorption on high surface energy nanostructured surfaces.

NANOSTRUCTURED CERAMICS

It is important to note that the above trend of using nanostructures to promote bone cell functions is not just restricted to metals, but it has also been used for ceramics, again demonstrating the versatility for the use of nanotechnology in medicine. Nowadays, there are main three types of bioceramics in orthopedics: bioinert ceramics (e.g., alumina, zirconia monoliths, carbon, etc.), bioactive ceramics (e.g., calcium phosphate, bioactive glass, etc.), and bioresorbable ceramics (e.g., HA) [28,30]. Among them, several commercial products of calcium phosphate (e.g., apatites, magnesium-substituted tricalcium phosphate, and biphasic calcium phosphates) are widely used to promote osseointegration and the performance of bone implants [31]. To mimic the composition and nanostructure of natural bone, HA has especially been formulated into various nanostructures and coated on metallic or polymeric materials to enhance osteoconduction [31,32]. To achieve enhanced bioactivity and a high mechanical strength, HA nanoparticles have been conjugated with many types of polymeric materials to form a matrix similar to the combination of collagen molecules and HA

FIG. 12.2 Scanning electron microscopy images of MC3T3-E1 preosteoblast cell filopodia extensions on different diameter titanium nanotube layers: (a) 20 nm, (b) 50 nm, (c) 70 nm, (d) 100 nm, (e) 120 nm (×70,000), and (f) 120 nm (×30,000) [**26**].



crystals in the natural bone structure [**33,34**]. Compared with the conventional materials, it has been shown that nanostructured ceramics trigger 30 % more bone growth than conventional versions. Moreover, once metals or polymers are combined with ceramic nanomaterials, their resulting nanofeatures induce positive biological

responses to promote the integration between bone and implants, reduce nosocomial infections, and even decrease bacteria infections (e.g., for *Staphylcoccus epidermidis*). It is important to note that this reduced infection is being accomplished without the use of antibiotics, for which bacteria develop a resistance toward anyway.

NANOSTRUCTURED POLYMERS AND COMPOSITES

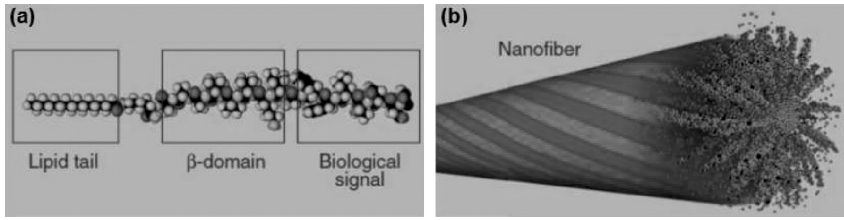
Again, it is important to note that the trends of improving bone cell functions (and inhibiting infection and reducing inflammation) using nanostructures have not been limited to metals and ceramics, but they have also been observed for polymers. Polymers are widely used in various forms for bone implants to provide tunable bone-matching mechanical properties and long-term reliability. For various polymers, they have been modified into nanopatterns, nanoparticles, nanopores, nanofibers, and so on. With these variations in nanostructures, the surface energy, area, hydrophilicity, and porosity of the materials can be tailored to improve orthopedic implant performance [**24**]. When combined with bioactive additives (e.g., HA or magnesium oxide nanoparticles), polymer composites have also provided coatings with low-stress, osseointegrative, and resorptive capacities to reduce implant loosening and improve long-term implant fixation.

SELF-ASSEMBLED MOLECULES

Compared with the conventional polymers that are composed of covalent bonds, natural biomolecules self-assemble via noncovalent forces; as such, these self-assembled molecules have recently become of great interest in orthopedics. This is because native bone has multiple levels of self-assembled hierarchical structures composed of organic and inorganic materials. Bones are roughly composed of calcium phosphate (mainly HA) at 69–80 wt%, collagen (mainly type I collagen) at 17–20 wt%, and other components such as water and proteins [**35**]. At the microscopic level, collagen fibrils form a tertiary structure with a 67-nm periodicity and 40-nm gaps where embedded HA nanocrystallites of matching size endow bone with rigidity. These collagen fibrils further form collagen fibers with diameters from 100 to 2000 nm [**36**]. Various noncollagen macromolecules classified as glycosaminoglycans and glycoproteins are also examples of self-assembled nanostructures.

Formed by noncovalent bonding and spontaneous organization, self-assembled molecules (e.g., polypeptides, oligopeptides, cyclic peptides, peptide amphiphiles [PAs], rosette nanotubes [RNTs], etc.) can mimic native biomolecules to have various nanostructures, including nanotubes, vesicles, nanofibers, helical ribbons, and β-sheets [**37–40**]. Much research has been undertaken to understand the essential factors required for self-assembling specific materials and to improve their biological properties for specific applications. Changes of temperature, pH, or ionic strength can affect the micro- and macrostructures of these materials [**37–40**]. Stupp and colleagues

FIG. 12.3 Schematic illustration of the PA nanofiber: (a) the structure of a PA monomer and (b) the PA self-assembles into a cylindrical supramolecular polymer [**41**].

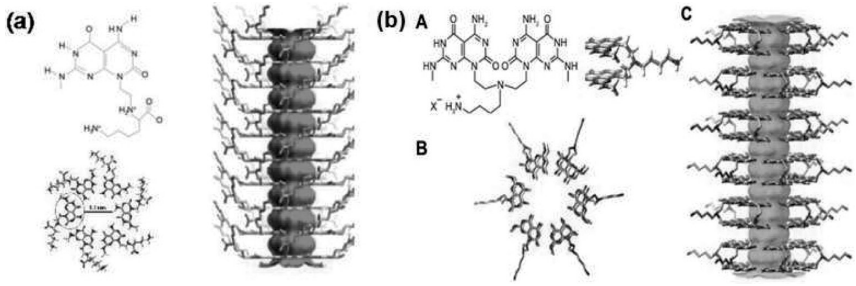


have reported that self-assembled PAs can form tunable networks by counter-ion screening, van der Waals forces, hydrophobic forces, ionic bridging, coordination bonding, and hydrogen bonding (**Fig. 12.3**) [**40,41**]. For example, in physiological conditions, self-assembled PAs form nanofibrous hydrogels. On the basis of the number of charges, PA is sensitive to the pH. Furthermore, the pKa of the peptides and their steric interference (e.g., supramolecular aggregation) affect self-assembly and gelation behavior. Responding to the pH value, the materials start to self-assemble in the acidic range and disassemble when the pH is neutral [**40,41**]. This tunable process can be used for drug delivery and prolonged, controllable release.

Peptide functionalized self-assembled materials also have a capability to guide cell behavior. For example, Hartgerink and colleagues used self-assembled PAs as fiber scaffolds to mineralize HA nanoparticles aligned along the long axis of the fiber, which resemble the natural composites of collagen fibrils and HA in bone [**42,43**]. For instance, Lee and colleagues conjugated bone morphogenetic protein-2 (BMP-2) with self-assembled peptides functionalized with a heparin-binding peptide domain to enhance bone regeneration in vivo. In their study, a hybrid scaffold (composed of collagen, heparin-binding PA, heparin, and BMP-2) prolonged BMP-2 retention to avoid burst release and increase drug efficiency, which reduced the therapeutic dose from 11 μg per treatment to 1 μg [**44**]. Therefore, reducing therapeutic doses is clearly beneficial to reduce potential systemic toxic effects.

Previous studies in our group showed that RNTs (**Fig. 12.4**) were able to promote osteoblast cell adhesion and proliferation [**45–49**]. Therefore, RNTs have been combined with titanium, poly(lactic-co-glycolic acid), and polyhydroxyethylmethacrylate to improve cell-binding capability and endow osseointergrative properties [**45–49**]. With similar dimensions as collagen molecules, RNTs may serve as a template and may also initiate and propagate mineralization. Self-assembling nanofibers are excellent candidates to serve as artificial structures to concentrate and retain biosignals in a high-density and organized geometry [**50**]. Moreover, because noncovalent bonds are

FIG. 12.4 Schematic illustration of the stepwise self-assembly process of RNTs: (a) helical RNTs and (b) twin-base-linkers. RNT molecules (A) self-assemble into a twin-rosette supramacrocycle (B), which self-organizes into RNTs (C) with 1.1/3.4 nm inner/outer diameters and up to several micrometres in length.



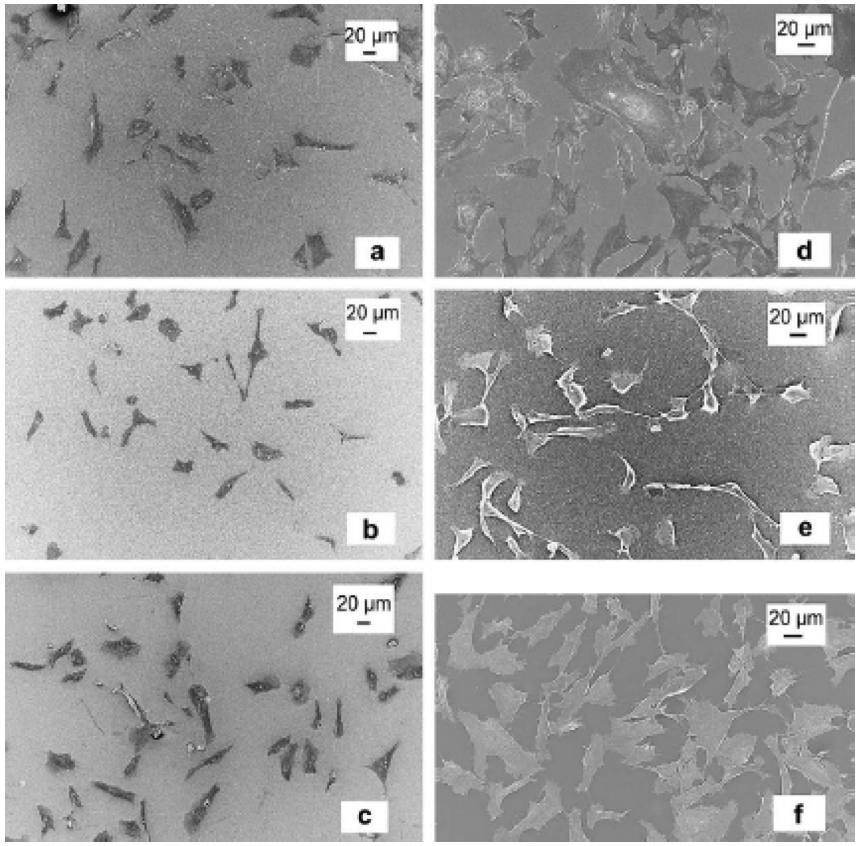
easy to break and reform, self-assembled supramolecules can adapt cell geometry more easily compared with conventional polymers formed by covalent chains [**41,51**].

The functional form of RNTs has been developed by covalently bonding peptides to a lysine chain on the RNT surface while hydrophobic drugs can be loaded inside of the nanotube (e.g., tamoxifen, dexamethasone, etc.) [**52**]. These functionalized RNTs have been designed to promote biological functions for heart, bone, cartilage, and skin applications [**53**]. For example, once RNTs were modified with the cell anchorage-dependent peptide RGD, they enhanced osteoblast adhesion and proliferation significantly more than the lysine functionalized RNTs, which promoted osteoblast functions significantly more than the numerous currently used orthopedic implants (e.g., titanium). Our current study showed that RNTs conjugated with BMP-7-derived short peptides selectively promoted osteoblast proliferation instead of fibroblast cells, which contribute to fibrous not bone formation [**47**].

CARBON NANOMATERIALS

Among the numerous nanomaterials now used for bone tissue engineering, carbon nanomaterials (e.g., nanodiamonds, graphene, carbon nanofibers [CNFs], and carbon nanotubes [CNTs]) have attracted much attention because of their exceptionally high mechanical properties, excellent conductivity, and unique thermal and optical properties [**54,55**]. For example, single CNTs have an estimated Young's modulus greater than 1 TPa and a tensile strength up to 63 GPa, and they possess a lower density than steel (and most metals) to make a light load-bearing bone graft [**56**]. Of course, it is important to note that an orthopedic implant material may be too strong compared with juxtaposed bone, which needs to be carefully considered. In addition, on the basis of the well-established carbon chemistry, it is convenient to modify the CNT or CNF surface

FIG. 12.5 Scanning electron microscope images showing osteoblast morphology on nanocrystalline diamond (a,d) and submicron crystalline diamond (c,f). Images a–c were taken after culturing osteoblasts for 24 h and images d–f were taken after 48 h. Osteoblast seeding density was 10,000 cells/cm^2 [**59**].



with various biomolecules or drugs for multifunctionality. Several studies have shown that CNTs promote osteoblast adhesion and extracellular matrix deposition whereas they inhibit fibroblast, smooth muscle, and chondrocyte cell functions [**15,53,57,58**].

Among the nanocarbon family, diamond has also attracted much attention in orthopedics. Specifically, compared with currently implanted titanium, studies have demonstrated that nanodiamond coatings on titanium (fabricated by microwave plasma chemical-vapor-deposition) enhanced osteoblast functions. Compared with micron-sized diamond, nanocrystalline diamond also promoted osteoblast adhesion and functions (**Fig. 12.5**) [**54,59**]. In vitro studies also indicated that nanodiamond titanium coatings were more biocompatible than carbon black, fullerenes, and CNTs [**60**].

CONCLUSIONS AND FUTURE DIRECTIONS

As highlighted in this chapter, nanotechnology clearly has great potential to solve many persistent issues related to conventional orthopedic implant failure, including controlling the structure and matching properties of natural bone, matching the mechanical strength of bone, further understanding the molecular mechanisms of tissue/implant interactions, reducing the inflammatory response, and decreasing bacterial infections (without using antibiotics). Nanotechnology is providing novel materials and methodologies to understand the cellular and molecular basis governing the interactions between implants and cells for numerous orthopedic applications. Of course, concerns still exist, such as toxicity and nanomaterial manufacturability, which are intensively being investigated to date. Toxicity is a concern because of wear debris that may result from the use of nanostructured materials in articulating surfaces. There is currently no consensus among the scientific community concerning the influence nanoparticulate wear debris will have on bone health. In addition, there are numerous questions concerning the scale-up of several nanomodification processes. For example, although anodization has been scaled-up for several industries, such as automotive and utensil polishing, scale-up of nanoparticulate manufacturing may be problematic. Nonetheless, the studies highlighted in this chapter have paved the way to use nanotechnologies for creating optimal bone implants.

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Chapter 13 | New Bone Grafting Technologies Using Stem Cells

Khadidiatou Guiro,[1] Sita M. Damaraju,[1] and Treena Livingston Arinzeh[1]

INTRODUCTION

Autogenous bone graft is considered the gold standard for a bone graft material because it contains the three essential bone formation elements, which are osteogenic activity and osteoinductive and osteoconductive properties. However, bone graft harvesting is associated with significant clinical morbidity in terms of pain, scarring, increased surgical time, prolonged hospitalization, delayed rehabilitation, increased blood loss, increased infection risk, and surgical complications (i.e., fracture, hematoma, neuroma, etc.). For these reasons, an impetus exists to develop alternative processes capable of replicating the performance of the autogenous graft while eliminating the associated morbidity and complications.

The use of stem cells in tissue engineering/regenerative medicine strategies has shown promise because of their ability to turn into various cell types and thus treat or repair a wide range of disease and damaged tissues. For bone repair, stem cells derived from various tissue sources have been combined with scaffolds/biomaterials to stimulate bone tissue formation. This approach moves closer to mimicking the three elements of autogenous bone grafts by providing stem cells as the osteogenic cell source and a scaffold that is osteoconductive but also may have osteoinductive properties. Implanted stem cells can have paracrine action in which they secrete trophic factors that can have a pronounced effect on recruiting endogenous cells to the defect site and promote their proliferation, differentiation, or both. This chapter will cover the use of the stem cells for bone grafting applications. The focus of this chapter will be on preclinical studies evaluating their use and current bone graft products containing stem cells.

PRECLINICAL STUDIES EVALUATING STEM CELLS FOR BONE GRAFTING

Bone tissue engineering strategies using stem cells have been investigated in small and large animal models to evaluate safety and efficacy. Critically sized bone defects

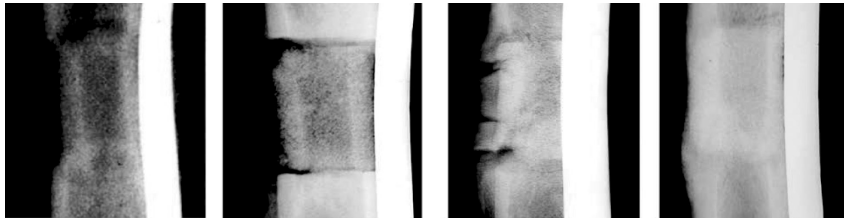
[1] Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, NJ

are routinely investigated because they are challenging to repair wherein they compromise the bone's own ability to spontaneously heal and restore normal function. Most preclinical studies for bone repair have been conducted using mesenchymal stem cells (MSCs) with the combination of a scaffold. MSCs are self-renewing, multipotent stem cells with the capability of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, myoblasts, and other cell types [1]. MSCs can be obtained from different sources such as bone marrow (BM-MSCs), adipose tissue (AD-MSCs), umbilical cord, periosteum, and dental pulp [2,3]. Of these, BM-MSCs are the most investigated [2,4].

BM-MSCs have been well characterized for their immunophenotype, multipotent capabilities, and proliferative capacity [1,5,6]. BM-MSC populations taken out to 15 passages and cryopreserved still have the capacity to differentiate and proliferate [7–9], suggesting that BM-MSCs may be valuable as a readily available and abundant source of cells in the tissue engineering and regenerative medicine fields. Furthermore, studies have demonstrated that the use of allogeneic BM-MSCs can successfully repair bone and other tissue types in various animal models without provoking an adverse immune response [10–12]. An allogeneic BM-MSC approach provides an off-the-shelf therapy in which allogeneic BM-MSCs are used as universal cells and, in turn, provide cells to a much larger clinical population. Allogeneic BM-MSCs are also currently in clinical trials for various disorders or conditions [13].

Bone repair has been investigated using BM-MSCs combined with ceramics or ceramic/composite scaffolds [4,14,15]. These scaffolds generally have proven osteoconductive properties [14]. Early studies demonstrated the repair of critical-sized 5-mm femoral diaphyseal defects in rats using BM-MSCs with ceramic scaffolds [16]. In canines, large, critical-sized femoral defects were treated with autologous or allogeneic BM-MSCs seeded on hydroxyapatite (HA)/β-tricalcium phosphate (TCP) porous ceramic scaffolds [11]. Both types of BM-MSCs supported new bone formation without an immunological response by 8 weeks postimplantation (**Fig. 13.1**). Similar findings were confirmed in a canine alveolar saddle defect for evaluating allogeneic BM-MSCs for craniofacial applications [17]. Critical-sized defects in caprine tibia and ovine femur underwent bone repair when autologous BM-MSCs were seeded on porous TCP or HA scaffolds. It is interesting to note that treated bone retained strength similar to control bones at 32 weeks for the caprine study and 8 weeks for the ovine study post-transplantation [18,19]. Likewise, natural coral exoskeleton scaffold was combined with BM-MSCs and implanted in large segmental defects in sheep. The results showed complete resorption of the scaffold along with recorticalization and the formation of a medullary canal with mature lamellar cortical bone in three of seven animals (**Fig. 13.2**) [20]. In addition, silicon and TCP scaffolds were combined with BM-MSCs and implanted in mid-diaphysis tibial fractures in sheep [21]. New bone formation coinciding with the scaffold resorption was observed 3 months after implantation. Although bioceramics are favorable materials for bone tissue engineering, they do exhibit brittle mechanical properties, which may be problematic for certain applications.

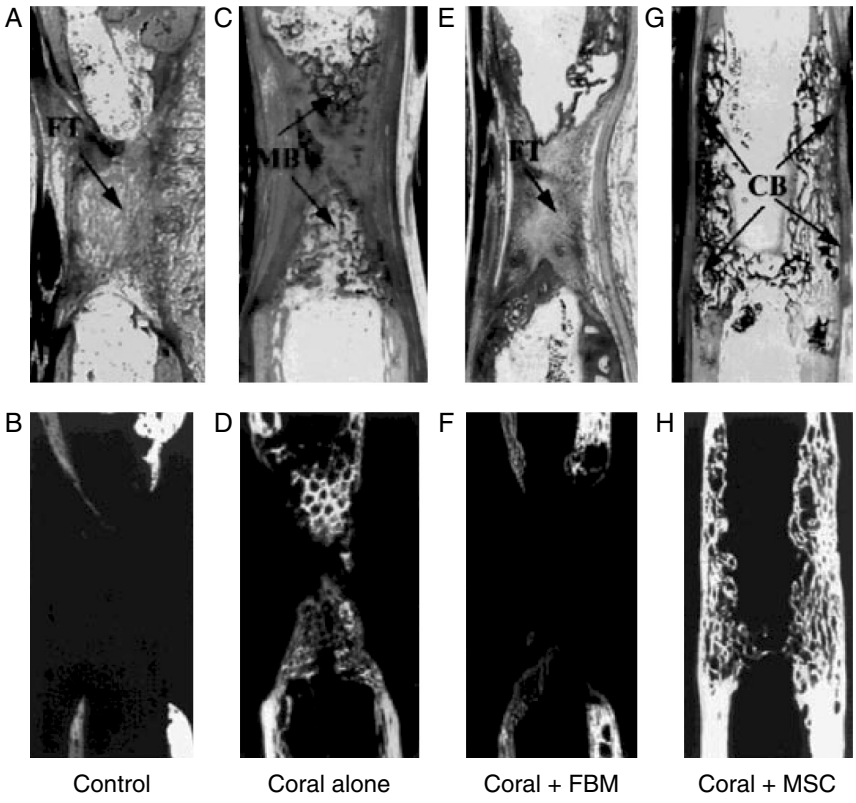
FIG. 13.1 Radiographs of segmental defects treated with allogeneic MSCs loaded onto an HA/TCP porous scaffold. Union at the host bone-implant interfaces was observed, and callus formed medially along the length of the implant by 8 weeks after implantation. The horizontal defects in the callus seen at 8 weeks were caused by the sutures holding the implant in place. At 16 weeks, the defect exhibited increase radiopacity and the medial callus was reduced [**11**].



Natural and synthetic polymers are also being explored as potential scaffolds for bone repair using BM-MSCs. Commonly used natural polymers for bone tissue engineering are silk, alginate, chitosan, and collagen [**22**]. These polymers have been shown to be biocompatible and biodegradable, and they have been shown to support cell attachment and proliferation in in vitro studies [**23**]. In animal studies, silk fibroin scaffolds in combination with BM-MSCs were implanted in critical-sized calvarial bone defects in nude mice for up to 5 weeks [**24**]. After 5 weeks, the scaffold with BM-MSCs was shown to integrate well with the surrounding tissue and stained positive for bone-specific proteins [**24**]. The scaffold alone or unfilled defect did not show similar repair. The implantation of chitosan-poly(butylene succinate) scaffolds with human BM-MSCs into critical-sized cranial defects in nude mice resulted in enhanced integration with the surrounding tissue and bone formation by 8 weeks [**25**].

Synthetic polymers such as polycaprolactone (PCL), polyglycolic acid (PGA), poly L-lactic acid (PLLA), and poly-lactic-glycolic acid (PLGA) are widely studied as scaffolds in combination with BM-MSCs. All of these polymers degrade where degradation products are removed by natural metabolic pathways in vivo. PLGA scaffolds fabricated to resemble trabecular bone morphology were preseeded with autologous BM-MSCs, which were later implanted in rabbit segmental bone defects. The defects were shown to undergo repair with new bone formation and scaffold integration with the surrounding tissue without the use of any supplementary growth factors [**26**]. Likewise, PCL scaffolds seeded with osteoblasts and BM-MSCs were implanted in critical-sized cranial defects of rabbits for up to 3 months. No significant differences in the rate and quality of bone repair were noted, and both of the tissue engineered scaffolds demonstrated bone formation and integrated well with the surrounding tissue [**27**].

FIG. 13.2 Micro-X rays and photomicrographs at 16 weeks. Histological sections of defects (A) left empty or filled with (C) coral (E) coral-FBM (fresh bone marrow), and (G) coral-MSCs. Note the invasion of the defect with fibrous tissue (FT) in A and E. In defects filled with coral alone (C), osteogenesis occurred within the medullary canal (MB). Defects filled with coral plus MSCs show cortical-like bone formation peripherally (CB). Cortical continuity was achieved between the edges of the defect. Micro-X rays confirmed the histological observations. There was no bone formation in defects (B) left empty or (F) filled with coral-FBM. Bone formation occurred in the medullary area in defects (D) filled with coral alone, but it was insufficient for bone union. In contrast, defects filled with (H) coral-MSC show osteogenesis chiefly at the periphery of the defect, leading to cortical bone union of the defect. Source: Reprinted with permission from Macmillan Publishers, Ltd. [**20**].



Composites consisting of natural or synthetic polymers with bioceramics have also been investigated because they combine the toughness and compressive strengths of polymers with the bioactivity of ceramics to achieve a more mechanically and physiologically similar structure to bone [**28**]. Moreover, to enhance osteogenic induction and bone formation, studies have used surface-modified bone scaffolds to improve

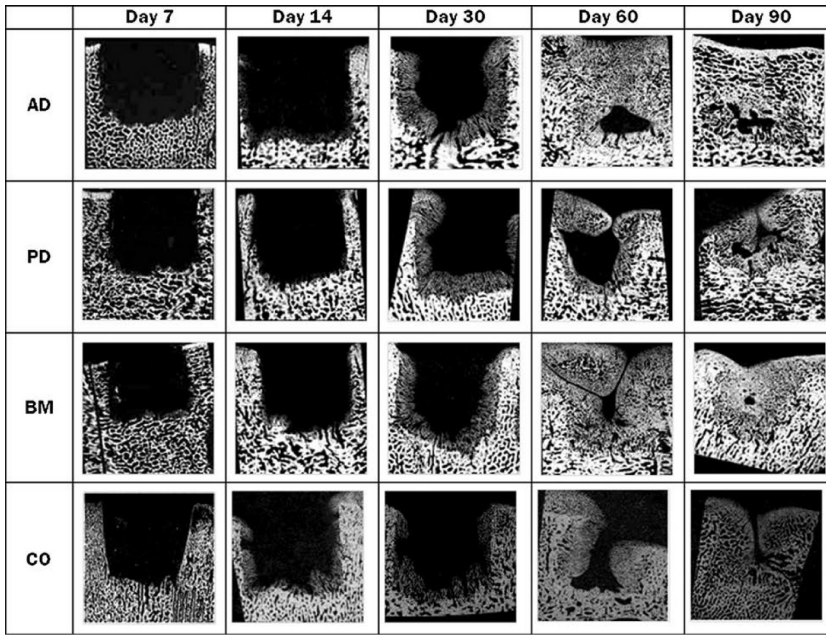
osseointegration and to deliver cytokines, growth factors, and specific genes [**29–33**]. BM-MSCs have been combined with bone allograft or demineralized bone, which provides additional osteoinduction, and they have demonstrated accelerated repair even in more challenging animal models such as diabetic rats [**34**]. These studies underscore the importance of the scaffold material in supporting stem-cell-induced bone repair.

Some studies compare the bone-forming capability of BM-MSCs with MSCs derived from other tissues. Findings demonstrate that BM-MSCs may have better osteogenic potential than AD-MSCs [**2**]. The influence of platelet-rich plasma (PRP) on the osteogenic capacity of AD-MSCs was evaluated and compared with BM-MSCs. The BM-MSC group had significantly higher amounts of new bone formation at 10 weeks when compared with the AD-MSC group and AD-MSCs with the PRP group [**35**]. Conversely, some studies demonstrate there are no differences in the osteogenic potential of BM-MSCs and AD-MSCs [**2**]. A study recently compared MSCs obtained from bone marrow, fat tissue, and periosteum [**3**]. These cells were seeded on collagen scaffolds for the repair of calvarial defects in pig. No differences in the osteogenic potential were observed (**Fig. 13.3**). In fact, all groups underwent complete bone repair by 90 days. Because culture expansion techniques (e.g., culture media used and number of passages before implantation) can differ from one study to the next, it is difficult to draw conclusions. Therefore, a need exists for direct in vivo comparisons of MSCs from different origins using consistent protocols for culture expansion and full characterization.

Similar to BM-MSCs, AD-MSCs have been shown to undergo osteogenic differentiation and may be immune privileged and immunosuppressive [**36**]. AD-MSCs have been investigated in various animal models, have demonstrated successful bone repair, and may hold promise as a viable MSC source [**37,38**]. Ceramic and polymeric scaffolds such as HA/β-TCP, PLGA, collagen, and PCL have been combined with AD-MSCs as an approach for repairing calvaria and critical-sized femoral defects in rats and mice. These studies have demonstrated the potential of AD-MSCs as a cell source for bone repair for critical-sized defects [**38**].

Embryonic stem cells (ESCs) are also being investigated for bone repair because of their pluripotent capabilities [**39,40**]. Studies performed by Jukes and colleagues differentiated ESCs in vitro toward the chondrogenic lineage and seeded them on ceramic scaffolds. The cartilage tissue-engineered constructs formed bone in vivo [**41**]. However, safety concerns exist with the use of ESCs because of potential tumor formation. A recent study demonstrated bone formation using human ESCs grown in vitro on decellularized bone scaffolds in a bioreactor and then implanted in immune-deficient mice [**42**]. It is interesting to note that no tumor formation was observed as a result of predifferentiation of human ESCs in vitro toward the osteogenic lineage. Induced pluripotent stem cells (iPSCs) derived from adult differentiated cells may be promising for autologous therapies. iPSCs share many characteristics with ESCs, including morphology, proliferation, surface antigens, gene expression, epigenetic status, and pluripotency [**43,44**]. Dental pulp MSCs, human fetal MSCs, and amniotic

FIG. 13.3 Microradiography of representative specimens of the different groups at specific time points (magnification 2.5x). At the early stage of wound healing up to 30 days after implantation, there were only slight differences of bone regeneration visible among the three test defects (AD (adipose-derived), PD (periosteum-derived), BM (bone-marrow-derived), and the control defect (CO). At day 60 and day 90, the area of newly formed bone inside of the defect of the test defects showed differences more clearly compared with the control. Source: Reprinted with permission from Elsevier [**3**].



fluid stem cells are currently under investigation as a potential cell source for bone repair [**45–47**]. Results so far indicate that these cells have the potential for bone repair and may contribute to angiogenesis.

Several in vivo studies have shown rapid bone formation using stem cells genetically modified to express osteogenic genes, suggesting the potential of combining cell and gene therapy to enhance bone regeneration [**48,49**]. Genetically modifying stem cells in combination with scaffolds have shown promise. Several studies have shown bone repair in radial and femoral defects by using genetically modified BM-MSCs or AD-MSCs in rodent models [**50**]. Recently, rat AD-MSCs were modified with bone morphogenetic protein-2 (BMP-2), bone morphogenetic protein-7 (BMP-7), or both by lentivirus and later seeded on TCP scaffolds [**51**]. These scaffolds were implanted in rat femur defects for 6 weeks. Results indicated that co-transfection of *BMP-2* and *BMP-7* genes significantly enhanced new bone formation over *BMP-2*- and *BMP-7*-alone

groups. Depending on the severity of the bone defect, genetically modified cells can be cultured ex vivo and later implanted in vivo for bone defect repair. Preclinical and clinical studies establishing safety with the use of genetically modified stem cells will be needed.

BONE GRAFT PRODUCTS CONTAINING STEM CELLS

On the basis of promising preclinical data demonstrating that MSCs derived from the bone marrow can repair bone defects and can be used as an allogeneic cell source without the use of immunosuppressive therapy, bone grafts containing BM-MSCs have been developed and are used clinically. Currently, two commercially available allogeneic MSC bone grafts exist: OsteoCel and Osteocel-Plus (Nuvasive, San Diego, CA) and Trinity Evolution (Orthofix, Lewisville, TX). As reported in 2010 [**52**], these stem cell products accounted for 17 % of all sales for bone grafts and bone graft substitutes, having higher sales than allograft bone. The OsteoCel products accounted for more than 92 % of the sales for the stem cell products. OsteoCel was launched in 2005 by Osiris Therapeutics, Inc., and later sold to Nuvasive, Inc., in 2008. OsteoCel has been described as the first product containing viable allogenic adult stem cells developed for the repair, replacement, or reconstruction of bone defects [**53**]. OsteoCel is composed of adult MSCs and osteoprogenitors embedded in a matrix of cancellous bone obtained from cadaveric donors [**54,55**]. The cancellous bone is treated with proprietary processing for the selective removal of immunogenic cells while maintaining living MSCs [**56**]. The processed tissues are then combined with demineralized cortical bone from the same donor and stored at −80°C as granules in a cryopreservation agent. OsteoCel has a shelf life of 5 years, and adverse events have not been reported [**56**]. Recent studies have demonstrated that more than 90 % of the cells are viable upon thawing, and most of the population of cells demonstrates the capacity for the extensive self-renewal and multipotential differentiation characteristic of the MSCs [**54**].

Because OsteoCel is marketed as human tissue for transplantation, it did not require clinical trials by the U.S. Food and Drug Administration for approval for use in humans. Therefore, clinical data demonstrating efficacy are limited. Recent clinical studies have demonstrated its use in spinal fusions and hindfoot and ankle fusion procedures. Studies have been performed using OsteoCel in conjunction with lumbar interbody spinal fusion procedures [**57,58**]. The studies demonstrated safety and effectiveness by establishing that most patients achieved fusion. Recently, Hollawell and colleagues used OsteoCel Plus in hindfoot and ankle fusion. These authors obtained results showing 100 % fusion in 20 patients and high fusion rates [**55**]. However, one of the limitations of OsteoCel that has been reported is the inability to resist compressive forces [**59**]. Similar to OsteoCel, Trinity Evolution is an allograft of cancellous bone containing viable osteogenic cells and osteoprogenitor cells within the matrix and a demineralized bone component. The allograft bone is processed by the Musculoskeletal

Transplant Foundation, which is a nonprofit service organization that provides allograft tissue. Similar to OsteoCel, Trinity Evolution can be cryopreserved at –80°C and no significant adverse effect reported [**60**].

Stem-cell-based grafts currently in clinical trials also may show promise. A Phase II safety and efficacy clinical trial at the University Hospital, Basel, Switzerland, is evaluating adipose MSCs in composite grafts for the treatment of proximal humeral fractures. To be completed in 2013, a Phase I and II clinical trial sponsored by Mesoblast, Ltd., will evaluate NeoFuse (allogeneic mesenchymal precursor cells) in combination with MasterGraft Resorbable Ceramic Granules as a carrier for posterolateral lumbar fusion. An early-stage clinical trial at the Hospital Sirio-Libanes will evaluate the use of a bone tissue engineering strategy using MSCs from deciduous dental pulp in combination with collagen and HA to reconstruct the alveolar bone defect in cleft lip and palate patients. At Indonesia University, they will evaluate the combination of MSCs, bioceramic, and BMP-2 in treating critical-sized bone defects.

FUTURE DIRECTIONS

Significant progress has been achieved over the past decade in identifying potential sources of stem cells for bone tissue engineering. Much of the work has contributed to the in-depth understanding of MSCs and their role in bone repair. The development of novel biomimetic scaffolds that can be used to direct implanted MSCs or endogenous cells or both to differentiate and form new bone tissue will continue to be of interest. The challenge with stem cell therapy approaches is that many studies cannot be directly compared because of the differences in stem cell culturing protocols, animal models, bone defects, etc. Standardized protocols for stem cell isolation, culturing, and determining the quality of the stem cells (e.g., differentiation potential and passages) will be needed for translation of preclinical studies into a clinical setting.

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Chapter 14 | Strategies toward Engineering Vascularized Bone Graft Substitutes

Ángel E. Mercado-Pagán[1] and Yunzhi Peter Yang[1,2]

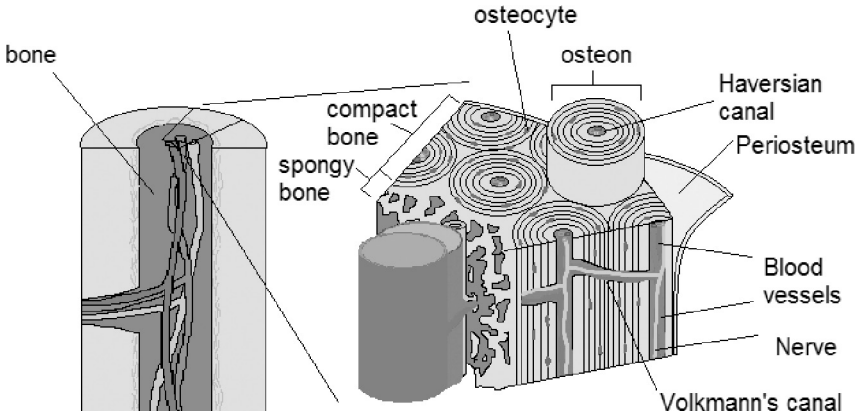
INTRODUCTION

For years, the main challenge for bone tissue engineering has been the vascularization of bone graft substitutes [**1,2**]. Although these grafts have had various degrees of success, revascularization of the affected areas remains a strategic issue, and restoration of damaged or injured bone tissue is hindered because of the limited ability of the body to restore complete functionality of the vascular system in and around the implanted grafts [**3**]. Compounding the problem, when bone tissue is damaged, not only vasculature is affected but also mineral matrix, cells, nerves, and microstructures. The problem to fix bone completely stems mostly from the complexity of bone, which is not easy to replicate through a biomaterial, or even a tissue engineering, standpoint. The complicated network of small vessels, the composition and array of the mineral matrix, the arrangement and behavior of cells, and the physical microarchitecture of bone are some of the topics that have opened new areas of research in bone tissue engineering.

The structure of bone is shown in **Fig. 14.1**. Bone is composed of inner cancellous (spongy) and outer cortical (compact) tissue. In addition to the highly mineralized matrix of these tissues, intricate vascular and neural networks sustain the rich cellular environment of bone [**4**]. The principal unit of compact bone is the osteon, a cylindrical layered microstructure containing cells, nerves, and vessels [**5**]. Osteons are interspersed through the compact stratum of bone, kept together by interstitial lamellae. The nerves and vessels of osteons are interconnected to each other through the bone structure by small perforating canals known as Volkmann's channels [**6**]. The periosteum is a strong connective tissue membrane rich in blood vessels, collagen, and fibroblasts, and it surrounds and connects to the compact tissue, providing an essential blood supply to the bone. Although weaker, more porous, and less dense than

[1] Department of Orthopedic Surgery, Stanford University, Stanford, CA
[2] Department of Materials Science and Engineering, Stanford University, Stanford, CA

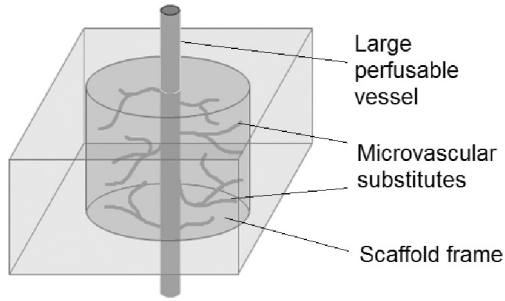
FIG. 14.1 Schematic of bone structure.



cortical bone, cancellous bone is highly vascularized and contains many bone marrow cells, which are responsible for red blood cell formation. Thus, it has been suggested that bone has a very important role in keeping vascular homeostasis [7] and, at the same time, depends on peripheral blood perfusion to remodel, regenerate, and sustain itself [8,9].

As illustrated by these descriptions, bone has a highly ordered vascular grid, which provides a thorough supply from its surroundings to its inner regions. However, this highly specialized, configured, functionalized environment presents an inherent problem when trying to restore it to its original condition when disrupted. When trauma occurs, this highly ordered structure is severed and perfusion of blood stops in injured areas, now disconnected from the vascular network. This leads to necrosis and tissue regression because cells do not receive enough nutrients and oxygen and waste elimination becomes impossible [10,11]. Advanced necrotic tissue needs to be surgically removed and replaced if the role of such tissue is essential for normal body function, but the success rate of replacement procedures has been varied [12,13]. This adds additional burden on the surgical techniques currently in use, such as induced membranes [14], distraction osteogenesis [15], and even the use of U.S. Food and Drug Administration (FDA)-approved vascular devices [16]. In a surgical and engineering approach, restoring the vascular network to its original spatial configuration, linking it to the points of disconnection, and fully reestablishing biological and structural function throughout the bone substitute is essential [17, 18], but it is a task that still has not been met with complete success because of the complexity of restoring the small vascular beds inside of bone with complete anastomosis with the host system. Moreover, the graded nature of mechanical properties of bone, from hard cortical bone to a less stiff cancellous bone—a difference of two orders of magnitude

FIG. 14.2 Levels of vascular repair in bone substitutes.

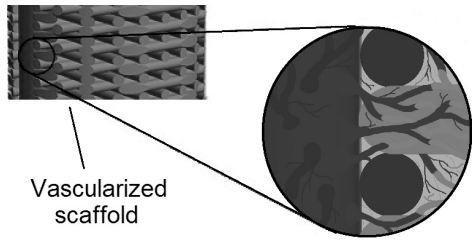


[19]—makes it essential to use a graded approach for developing mechanically sound structures that can resemble bone architecture.

The variability in the sizes and extent of bone injury has presented the need to repair vasculature at two levels: large perfusable vessels for blood drainage into and through the affected areas and microvascular substitutes for the irrigation of the internal structure of the grafts and surrounding tissues (**Fig. 14.2**). While the larger blood vessel substitutes provide the constant blood supply needed for the bone graft and restore the interrupted blood flow for adjacent sections of the host system, the smaller microvascular substitutes extend the coverage to the entire area of the bone graft, sustaining viability of the graft and promoting osteogenesis and osseointegration until bone function is restored. Numerous substitutes have been developed for the replacement of large vessels, from polymer-based constructs to biologically derived tissue-engineered vessels. Microvasculature approaches have been targeted by the use of cells, hydrogels, microfluidics, or combinations. The intended target for vascular tissue engineering in bone substitutes is to achieve their complete vascularization by integrating both approaches (**Fig. 14.3**).

Furthermore, for repair of bone using vascularized substitutes, two main routes have been adopted: premade vascular structures and vascular-inducing structures. For premade vascular structures, the body is provided with an engineered guide to conduct revascularization and bone repair. Because the vascular structure is designed before implantation, the osteogenic and vasculogenic processes can be tailored to a specific site, function, or application. However, the behavior of cells and materials used for premade vascular structures is yet to be optimized. For example, patency retention of the vasculature of the substitute is of critical importance [20]; if the structure collapses or closes up, then blood flow and vascularization are hindered. Another determination to be made with premade substitutes is the manner of integration with the existing host vasculature. Because the vasculature or guided channels are already in place on the substitute, the vascular section and its physical support must be already

FIG. 14.3 Concept of scaffold/graft integration. Large vessels or vessel grafts supported by the scaffold should enhance sprouting and anastomosis with preexisting or developing microvasculature throughout the scaffold for integration.



suitable for surgical implantation and anastomosis. Lastly, keeping the vascular implant in the substitute viable for extended periods of time can present a long-term issue in extreme trauma repair. On the other hand, vascular-inducing substitutes do not have a preset designed network, but they provide cues that trigger an angiogenic response from the host. In these cases, the body is conditioned for self-repair and vascular repair is not limited to predetermined dimensions of vessel or channeled constructs. Nevertheless, the requirement for this is reproducibility [**21**]. Given the different ways an organism can respond to a surgical implant, dosage of bioactive agents, and immunogenic factors, the outcomes are not necessarily even, and prognosis would be generally unpredictable or poor [**22**]. Even if the host system starts to respond in an angiogenic way, the actual promotion and formation of completely functional vessels, with the same properties before injury or tissue resection, remain a significant challenge for vascularization in tissue engineering [**23**].

Nonetheless, there have been recent encouraging results for the use of bone substitutes that promote vascularization at large and microsized scales in defects as we will see in this chapter. In the case of premade substitutes for vascular guidance, considerable research has been done on channeled solid matrices for vessel integration and imprinted polymer microfluidic devices, among others. Degradable hydrogels, highly interconnected porous scaffolds, and solid scaffolds with angiogenic factors are some of the devices created for induced vascularization. Results generally indicate (1) that the body can respond to physical, chemical, and biological cues, inducing angiogenic processes inside of and around the devices [**24–26**]; (2) that surgical procedures to graft the substitute to the host can restore vascular flow, albeit with material limitations [**27,28**]; and (3) that the success of substitutes is dependent on the extent of angiogenic invasion and vasculogenic generation in and around the scaffold, although defect or graft size so far has been limited to small sizes [**23**]. However, the results have also indicated that anastomotic integration, optimal geometric configuration [**29**], ideal

bioactive agent release profiles and distributions [**30–32**], cell behavior [**29**], and fully restored completely functional vascular beds [**33**] have not been attained yet. The research into overcoming these obstacles is ongoing, with novel approaches meeting a degree of success, as will be presented in this work.

Although considerable work is still needed to address the design of vascular constructs, the underlying basis for optimization has been established. However, the key now for future bone substitute design is on the combination of graded grafts that mimic the bone architecture with large vessels that can perfuse blood into the microvasculature inside of the graft. Hence, vascular graft technology needs to be developed hand-in-hand with osteogenic scaffolds to create a seamlessly integrated tissue replacement that can properly restore and improve function for bone tissues and vascular networks. Whether this involves the combination of strategies or further investigation of optimal parameters remains to be seen. We will now explore in more detail the current options for development of vascularized bone substitutes, the research into producing these devices, and future perspectives and prospects attained so far.

CURRENT ENGINEERING STRATEGIES FOR VASCULARIZING BONE GRAFTS SUBSTITUTES

Biomaterial Selection and Synthesis

The first step in designing a scaffold is selecting a proper starting material for a scaffold base. The selection of an adequate biomaterial is of importance for the behavior of cells and tissues in contact with the scaffold. Key characteristics of vascularized bone scaffolds should include guidance for neovessel formation, mechanical stability, chemical signaling, antifouling protection, tailored degradation profiles, and permeability for adequate nutrient and waste exchange [**34–36**]. A successful vascularized construct should seamlessly restore the flow of fluid through the affected area while maintaining the structural integrity and stable interface between synthetic grafts and host tissue. A substitute with a well-defined patent structure can assist in the guidance of fluid flow and prevent destabilization of the newly restored flow.

Thus far, bone scaffolds have been made with a variety of materials, including biologically based materials, ceramics, metals, and polymers [**37**]. Each of these materials has their advantages and disadvantages. Biologically based materials provide excellent biocompatibility. For example, collagen has been extensively used for the development of bone tissue engineering scaffolds because of the high content of this material in bones and cartilages [**38–42**]. Chitosan and alginate scaffolds have also been developed for use in bone tissue engineering [**43–47**]. However, engineering of biologically based constructs has so far been unable to match the hierarchical assembly of native tissues, which limits their mechanical strength. They are also limited by availability, costs, and immunological response. Metals provide high mechanical properties that can provide support in load-bearing applications, but the inherent difference

in magnitude between these properties and the ones of bone hinders osseointegration, mostly because of stress shielding [**48**]. However, the ability to process the surfaces of metals used for scaffold fabrication has also made them possible candidates for vascularized scaffold fabrication. For example, Dabrowski and coworkers developed new titanium bone scaffolds with high porosity, pore interconnectivity, and mechanical properties by a powder metallurgy technique [**49**]. Van der Stok fabricated titanium frames by selective laser melting, which showed a high degree of integration with surrounding bone in a critical-sized defect in rats after 4 weeks [**50**]. The high interconnectivity and porosity in these cases expressly increased tissue and cell infiltration, which eventually led to mineralization and vascularization inside of the graft for more efficient osseointegration. Calcium phosphate ceramics also provide high mechanical properties, but they also add another dimension—chemical similarity to the mineral matrix of bone [**51**]. This not only facilitates osseointegration, but it also enables the seeding of cells for bone differentiation and osteogenesis [**51,52**]. A major limitation of ceramic scaffolds is their inherent brittleness; the balance between porosity and mechanical strength is the key factor in the fabrication of ceramic scaffolds [**53**]. Highly interconnected porous scaffolds have been created with ceramic precursors, with the intention of promoting cell growth throughout the void volume. For example, Fielding fabricated printed scaffolds with calcium phosphate, doping them with silicon and zinc oxides to promote osteoinduction in vivo [**54**]. We have developed highly interconnected porous scaffolds using our template casting method for concurrent angiogenic and osteogenic differentiation of cells [**55,56**]. Gu used hydroxyapatite (HA) scaffolds doped with strontium salts to stimulate the release of angiogenic factors from cultured osteoblasts [**57**]. All of these results suggest that ceramic scaffolds could be very valuable in promoting osteogenic and prevascular formations for immediate functional restoration of the repair area.

Polymers have possibly provided the most versatile group in vascularized scaffolds for bone tissue engineering. The main advantage of polymers is their ease for customization according to application. Monomers can be selected to yield a relatively unlimited number of polymers with properties that can be used for graft fabrication, from the development of soft hydrogels to load-bearing frames. Grafts have been made with polyhydroxyacids [**58–62**], polycarbonates [**63,64**], polyanhydrides [**65,66**], polysphosphazenes [**67–69**], polypropylene fumarates [**70–72**], and polyurethanes [**73-75**], among others. Chief among these polymers, elastomers have shown the best properties for the development of load-bearing materials [**76**]. Elastomer scaffolds are biocompatible, degradable, and can withstand loads parallel and perpendicular to the main longitudinal axis [**77**]. Moreover, their biocompatibility and biodegradability make them suitable for seeding cells directly for vascular and osteogenic differentiation. Because of their elasticity, it is not surprising that these polymers have also been used extensively for the development of vascular grafts [**78–83**].

The limitations of individual types of materials are the main driving force to push for integration of more than one material. For example, metals have been modified

with ceramics to improve their integration to the bone surface [84–86]. Ceramics and polymers have been combined to improve on the overall properties of each individual component [87–89]. As seen previously, metal dopants in calcium phosphate scaffolds have been used successfully to promote angiogenesis [90]. Polymers have also been composited with new carbon nanotubes to improve on the mechanical properties of tissue-engineered constructs [70,91]. The possible combinations in recent years have been numerous. Integration of materials with different properties has shown to improve on the osteogenic and vasculogenic potential of vascularized bone substitutes.

Thus, the proper selection of biomaterials is essential in the development of devices for bone substitutes. However, the base material is just part of the overall design of the bone graft. To develop vascularized bone grafts, fabrication methods should facilitate the incorporation of features, cells, and biochemical signals that can replace or mimic the structure of bone and its internal vasculature. In the following sections, we discuss how this integration occurs.

Vascularization Strategy and Fabrication

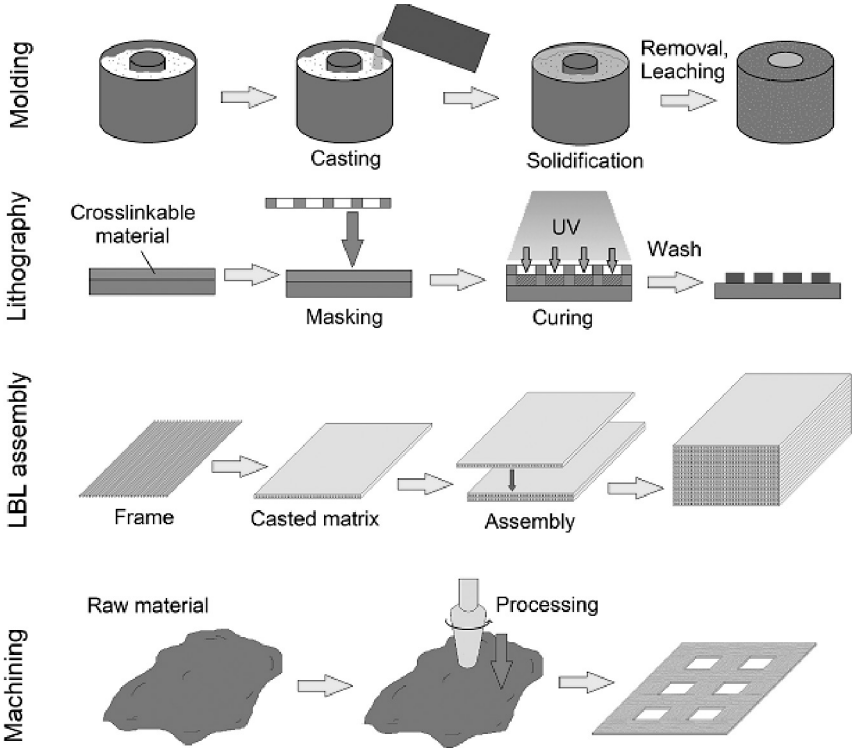
The need for vascular constructs also calls for novel methods for creating new bone grafts. A vast and increasing number of bone substitutes for simultaneous vascular and osteogenic repair are currently under research, each made through different methods. The most common remains the prefabricated scaffolds [92], with channels to facilitate the growth of bone or vascular tissues. Although not commonly considered true prevascularized structures, nonchanneled bone grafts with vascular and angiogenic cues have also been adapted for vascular restoration. In this section, we will briefly introduce some of the methods that have recently been developed, with focus on vascular bone repair.

Devices with Controlled Structures

Structures with controlled architectures comprise the most obvious structures tailored specifically for vascular growth. The general argument for the fabrication of this kind of device is that the preformed structures will facilitate vessel formation and scaffold integration to the preexisting host tissue. Many of these devices are scalable; therefore, they can be adapted for different defect sizes and shapes. Current methods for creation scaffolds capable of vascularization (**Fig. 14.4**) include molding [93,94], layer-by-layer assembly (LBL) [95], lithographic techniques [96], printing [97,98], and machining [99,100]. It has been proposed that successful scaffold vascularization and osteogenesis will be achieved with interconnected pores ranging from 100 to 300 μm [101-103], but both processes have been observed in a wider range of sizes, as we will discuss in the following sections. Overall, device dimensions ultimately depend on the intended function of the scaffold, the location of injury, and the flow conditions in the affected area.

Macrofabrication Techniques. The rationale to create substitutes with macroscale structures or channels (ranging, depending on author, from a couple of microns [104],

FIG. 14.4 Simplified illustration of some methods for scaffold fabrication.



to hundreds of microns [**105**], to a few millimetres [**100,106,107**]) is that these can allow for the introduction of large vascular grafts or that their size can allow for the infiltration of large-size preexisting vascular tissue. Because necrosis is a challenge to overcome for any large size replacement for bone injuries, the restoration of a major blood supply is needed. Molding presents a simple way of creating grafts with a set shape. Although a major disadvantage of molded grafts is the limitation to the dimensions of the mold, their ease of production and high-throughput designs make them attractive candidates for bone healing. LBL has been used for years to develop several scaffolds, but only in recent years has it gotten a push for the creation of vascular scaffolds. Machining permits shaping grafts to the needed geometry for implantation, either by hand or computer-aided design. It is to be noted that in macrochanneled tissue engineered substitutes such as those fabricated by Akita [**106**] and Haholu [**100**], relatively large host vessels were directly inserted into the channels to promote vascular formation and infiltration into the scaffold. Although a large fraction of these bone

substitutes, because of their size, are acellularized constructs, several recent approaches have considered the introduction of cells to line the surfaces of such materials. We will discuss these approaches in the following sections.

Microfabrication Techniques. Because large vessels are needed for high-volume perfusion of blood in and around the bone substitute, small vessels are essential to distribute this volume of blood into every section of the new matrix. Therefore, substitutes with microscale architectures, ranging from nanometre size [**108**] to several hundred microns [**109**], according to author interpretation, are a very significant area of research because they can provide a structure with very small defined pathways that will eventually serve as guidance paths for neovessels. The scale of the devices usually makes them ideal for cell seeding and guidance into defined shapes that will eventually form into functional structures; in turn, these functional vessels allow for complete perfusion throughout the scaffold. Rapid prototyping (RP) of HA scaffolds has been achieved with high reproducibility, with well-defined microchannels that have shown vascularization 4 weeks after implantation in nude mice [**110**]. Printing has been successfully used for developing channeled ceramic scaffolds for vascular bone tissue engineering [**111**]. Polycaprolactone (PCL) films made by LBL microfabrication techniques have been shown to support cell proliferation in calvarial defects in rats after 2 months [**112**].

Microfluidics deals with the perfusion of small fluid flows or fluid flow through very small diameter channels. Recent advances in microfluidics have been achieved by hydrogel and chip technology to create microchannels that will eventually be used for the formation of microvasculature [**113,114**] and the evaluation of concurrent angiogenesis and osteogenesis [**115,116**]. The use of soft materials for creating small diameter channels is justified because of the feasibility to integrate the patterned structure into bone defects by using external fixators such as surgical plates or the bone graft itself. Because many of these gels have also been used for encapsulation of cells for osteogenic differentiation, it is possible that introduction of microchannels can enhance the concurrent differentiation of cells into bone and vascular tissue or at least promote fluid flow inside of the polymer matrix whereas tissue in-growth promotes vascular formation. Moreover, integration of solid, mechanically resistant scaffolds with channeled hydrogels can enhance the formation of vascular networks (inside of channels in the hydrogel matrix) and mineralized tissue (inside of the hydrogel matrix and around the graft struts).

Noncontrolled Structures

Although made for osteogenic and vascular repair, many bone substitutes do not have defined structures to guide the formation of elongated luminal structures for vessel repair. However, these devices are fabricated in such a way that interconnected networks can form or infiltrate through matrices containing cells or polymers. Although most of these have no defined guided channel, these grafts facilitate network formation from cells either pre-seeded inside of or invading the graft. The increased

distribution of blood flow through the resulting networks regulates the exchange of nutrients and wastes, increases the viability of osteogenic cells undergoing differentiation into bone tissue, and decreases necrotic damage to the frame of the graft.

Interconnected Porous Scaffolds. The first group of substitutes made with the intention of forming vascular networks through their inner structure comprises scaffolds with interconnected pores. These scaffolds have been made from various ceramics [**117-119**], metals [**120,121**], polymers [**122,123**], and composites [**124–126**]. Formation of interconnected porous scaffolds can be achieved by various methods, including bubbling (foaming) [**127–129**], leaching [**94,130**], template casting [**131,132**], and molding [**133**]. Although these methods have been able to achieve high interconnectivity and porosities, thus potentially increasing the space for cell growth, the increase in void fraction in the graft decreases its ultimate mechanical properties and accelerates degradation. Thus, many of these scaffolds, particularly ceramic and polymer scaffolds, have required additional mechanical support when implanted in defect sites. The best outcomes for creating porous scaffolds then result from the balance between porosity and mechanical strength.

Degradable Matrices. To promote growth of vascular tissue inside of a scaffold, void space is not necessarily needed. If matrices can degrade to leave space for the newly forming vascular network, then there could be a possible control and regulation of its shape and rate of formation by cellular and enzymatic rates of degradation. Moreover, the matrix itself can be used to guide growth by the release of biological cues, which we will discuss in the next section, or by suspension of cells that can assist in angiogenic behavior of the invading tissue. By virtue of these properties, most of these degradable materials are soft hydrogels, which are synthesized mostly by gelation in preformed molds or after injection at predetermined sites [**134**]. Other methods of forming degradable matrices include direct printing, hydrogel layering, and polymer mixing. For example, patterned microsized alginate fibers encapsulating cells have been printed into three-dimensional constructs; results showed that cells can proliferate and undergo differentiation into osteogenic and vasculogenic lineages [**135**]. Hydrogel composites composed of polyethylene glycol fumarate and gelatin microparticles supported osteochondral tissue regeneration, with possible assistance in inducing vasculogenic responses [**136**]. Betz and coworkers created porous hydrogel trilayers of 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate and polyethylene glycol diacrylate, which showed enhanced differentiation of mesenchymal stem cells (MSCs) into osteogenic lineages [**137**]. However, synthetic polymers are not the only materials used for degradable matrix synthesis. Natural-based materials have provided inspiration for the fabrication of bone substitutes with a substantial degree of success in vivo and in vitro [**138**]. Several proteins, including extracellular matrix (ECM) components, have been used to create gels that can be enzymatically degraded. Because ECM is naturally found in every tissue, presents an environment rich in proteins and other degradable components, and has molecular signals that promote natural tissue homeostasis and regeneration [**138**], it presents a potentially

ideal material for fabrication of bone and cartilage substitutes. The outlook for its use recently took a novel step by the creation of a decellularized ECM graft [**139**]. Particularly intended for bone tissue engineering, Martino and coworkers used hydrogels modified with fibronectin derivatives to evaluate their osteogenic potentials toward MSCs, showing that osteoblastic differentiation strongly depended on the level of binding affinity of surface integrins with the fibronectin components [**140**]. He and Jabbari used matrix metalloproteinase degradable ECM-like hydrogels to encapsulate MSCs for tissue engineering applications [**141**]. Our group has shown that deposition of ECM onto ceramic scaffolds modulates growth factor release and promotes cell growth [**56**].

Biological Guidance

By themselves, it is possible that bone substitutes will not be able to incite vascularization all throughout the spaces intended for vascularization. This is particularly important when considering diffusion limitations and the physical design of the scaffold. Furthermore, without proper cues, tissue growth could occur randomly through the bone substitute, leading to the presence of areas without sufficient cell in-growth and vascularization. As such, strategic placement of cues along the bone substitute presents a necessary approach to guided growth and differentiation of cells through the scaffold. In this section, we will briefly go over some of the work done for biological guidance in vascularized bone substitutes.

Growth Factor Delivery

The most common way to promote growth, differentiation, and migration of cells is by the use of growth factors. Growth factors are biological macromolecules, usually proteins or hormones, which control these processes by acting directly with binding receptors on the surfaces of cells and conducting signaling between cells. The presentation of growth factors in a bone substitute is done primarily by two methods: sustained release of the chemotactic agent or application of a gradient to a surface or matrix. Among all growth factors used for bone tissue engineering, bone morphogenetic proteins (BMPs) have been applied as a powerful growth factor for the differentiation of progenitor cells into osteogenic lineages in a vast array of applications, including commercially available products. For example, ceramic scaffolds impregnated with BMP-2 have been used to promote differentiation of cells inside of highly porous, interconnected calcium phosphate scaffolds [**56,142**]. Loading of BMP in hydrogel-coated printed scaffolds also accelerated in vivo bone growth 2 months after ectopic implantation into the back of mice [**143**]. These results have been consistently observed on BMP-loaded bone scaffolds. BMP-2 gradients have also been developed for bone-cartilage repair [**144**]. Fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) are examples of other growth factors that have been extensively used for angiogenic differentiation and promotion of cells. These have been incorporated into the design of several tissue-engineered grafts to promote vascularization. For example, Qu developed nano-HA/polyamide 66 scaffolds seeded with

bone marrow stromal cells (BMSCs) transfected with FGF-2 to improve on concurrent angiogenesis and osteogenesis in a calvarial critical-sized defect model in rats [**145**]. Stereolithographic/RP techniques were used to create preformed calcium phosphate cement scaffolds, which were then combined with VEGFs for vascular repair in a bone defect in rats; results showed that the scaffold could promote tissue growth and the formation of new vessels after 2 and 4 weeks [**146**]. The combination of BMPs and VEGFs into composite polymer scaffolds has shown that sequential release of growth factors might enhance the bone formation process [**147,148**]. Lastly, directed growth is essential for scaffolds where complex geometries and diffusion limitations are present. Growth factors have been proposed as chemotactical agents (promoting directed migration of cells) for the development of specific pathways inside of the scaffold. For example, included in this group are platelet-derived growth factors (PDGFs) and CXC chemokines such as stromal-derived factor-1 (SDF-1). Phipps used PDGFs released from PCL/collagen/HA scaffolds to show their chemotactical properties toward MSC [**149**]. Leotot showed that platelet lysate improved MSC interaction with and migration into ceramic scaffolds [**150**]. SDF-1, used in combination with BMP-2 or transforming growth factor-β1 (TGF-β1), showed increased cell homing and differentiation for cells in implanted scaffolds in a rat model [**151**]. Release of SDF-1 from poly(lactide ethylene oxide) fumarate hydrogels showed increased migration of BMSCs [**152**]. Although it inhibits EC proliferation in vivo, TGF-β1 can stimulate angiogenesis by recruiting cells that secrete more promoting growth factors [**153**]. Growth-factor-derived peptides have also shown to induce osteogenic and vasculogenic responses from progenitor cells [**154,155**]. Notwithstanding these results, regulation of release, method of delivery, and appropriate dosages remain as parameters to be optimized [**156**]. However, the ongoing research has shown that biochemical signaling is still a very powerful technique for controlling the cell proliferation rate, osteogenic and vasculogenic differentiation, and directionality of vessel growth.

Other Forms of Cellular Signaling

Growth factors are not the only form of signaling available for directing the functions of cells. In some cases it is desirable to avoid the use of biochemical cues because of safety, costs, or feasibility. Physical cues have also shown effectiveness for guidance of new vessels and vessel sprouts when applied to the bone graft. One of such methods is the use of physical surface morphology. In this method, surface characteristics of the bone graft are modified to elicit different behavior from the cells, including potential vasculogenic, osteogenic, and neurogenic responses [**157,158**]. For other grafts, it has been demonstrated that stimuli such as electrical impulses [**159**], temperature [**160,161**], and mechanical stresses [**158,162–164**] can also direct cell growth, differentiation, and function. One major limitation of the latter methods is that these can usually only be used in the prefabrication of cellularized vascular networks and not in the active development of networks in the body. Lastly, pH-responsive materials have also been shown to provide a way of directing cell growth by controlling the structure of the scaffolding

material. Although implementation of these methods has yet to be demonstrated widely in vivo, the directed fabrication of functional cellularized structures into prevascularized tissue can assist in the integration of cells and scaffolds for direct implantation in bone repair.

Cellular Guidance

In some bone substitute materials, cells are included as precursors of the networks that eventually will form throughout its frame. Whether cells or not should be added into the scaffolds is still a matter of debate; this will be addressed formally in upcoming sections. However, if cells are to be included in the bone substitute design, then it is desirable that proper cell lines are used as precursors. For example, small precursor vessels, if they are to mimic small capillaries in the body, would be expected to be constituted only by endothelial cells (ECs) whereas larger vessels would include smooth muscle cells (SMCs) to provide the strength needed for sustaining increased pressure resulting from blood flow. Moreover, the components of the scaffold where cells are to be contained, whether encapsulated or on the surface, need to allow viability, growth, and proliferation of the selected cells. However, applicability of cellularized constructs has not gained considerable support for clinical implementation, mostly because of the feasibility, biocompatibility, and long-term sustainability of engineered tissue-construct systems. Current research has aimed at targeting these issues, and several important milestones toward solving these problems have been attained. Although cells have been extensively used for the purpose of inciting vasculogenesis and osteogenesis in scaffolds, in this section we will focus on some of the cells selected for vascular generation in grafts for bone tissue engineering.

ECs

ECs constitute one of the largest groups of cells studied for vascular tissue engineering. These cells interact closely with one another to form sheet-like structures that form walls of tissues, organs, and blood vessels. These cell layers allow permeation of wastes and nutrients between tissues; have mechanical, chemical, and physical stability; and serve as angiogenic surfaces for blood vessels. Unger showed the angiogenic potential of ECs seeded in silk fibroin [**165**]. Santos showed that ECs seeded on starch/PCL fibrous scaffolds can assemble into capillary-like structures [**166,167**]. Cell colonies seeded on bicalcium phosphate/BMP-2 ceramics were observed to undergo vasculogenesis and host vessel anastomosis in vivo 4 weeks after implantation in mice [**168**]. Their response has been shown to be highly regulated by chemical signaling and surface characteristics [**158**]. Thus, the ability to control their spatiotemporal characteristics inside of several surfaces and scaffolds has been consistently shown in vitro and in vivo.

SMCs

Although all capillaries and small vessels are formed almost exclusively out of ECs, larger blood vessels and complex organs require another layer of SMCs. This layer is

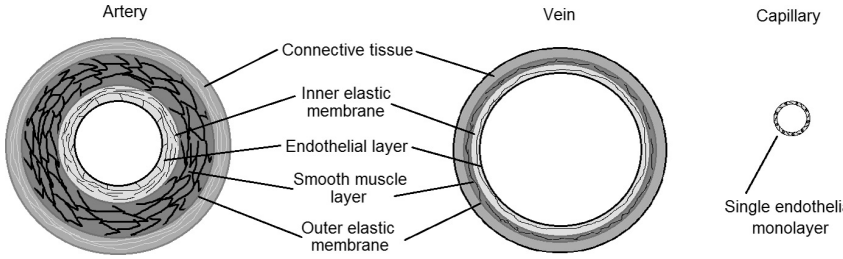
larger in arteries than in veins because the former have to carry blood away from the heart. Layers of SMCs provide the mechanical compliance needed for withstanding pulsating blood pressure. The cells in the SMC layer are elongated and can withstand longitudinal and radial stresses. In large blood vessels, as shown in **Fig. 14.5**, the endothelium lines the internal lumen whereas the thicker SMC layer surrounds it. Cellularized layers are connected by intermediate strata composed of fibrocollagenous tissue.

Thus, incorporation of SMCs into the vascular design in bone substitutes is hypothesized to provide new vessels a structure resembling those of natural large vessels in the body. If the substitute is intended to replace a large section of damaged bone rich in vascularized tissue, then it is expected that incorporation of large functional angiogenic vessels will be needed to completely irrigate the affected area with perfused blood, and formation of proper vessels can be facilitated by SMC interaction with ECs. The use of preexisting vessels in the surrounding muscular tissue to fill this role, although potentially effective, can have undesired results because the muscular blood supply is sacrificed for an increase in blood flow inside of the scaffold. With this objective, research efforts could include SMCs in their design, either by preseeding of cells or expected migration and invasion of SMCs. Elbjeirami and West showed that ECs exposed to VEGF-producing SMCs showed angiogenic potential [**169**]. Wang has shown that SMCs and ECs infiltrate porous vascular polyglycerol sebacate (PGS)/PCL constructs after interposition grafting in a rat abdominal aorta model, degrading the matrix and reforming into a neoartery [**28**]. SMCs have been an adequate alternative for cell seeding in vascularized bone substitutes.

Stem and Precursor Cells

When developing cellularized materials for bone regeneration, stem and precursor cells are the most used. The use of cells with different differentiation potentials permits controlling the rate of new tissue formation for more efficient repair. The varied differentiation behavior of these cells makes them versatile for the development of several kinds of tissues, including bone and vascular tissues. Their differentiation is triggered by signaling provided either by the host body or the graft. Careful selection of cell lineages can lead to specific differentiation patterns inside of the bone substitute, increasing mineral tissue content as a result of osteogenic differentiation, or interspersed vascularized structures formed through vasculogenic differentiation. For example, BMSCs can form a mineralized matrix whereas endothelial progenitor cells can be activated to produce sprouting into cords and luminal structures [**170**]. A ceramic scaffold with MSCs and femoral vessel insertion showed increased bone formation and capillary infiltration when removed after 4, 8, and 12 weeks implantation in rabbit femoral defects [**171**]. A gelatin scaffold containing MSCs showed increased angiogenesis and tissue repair when implanted into a rat spinal injury model [**172**]. Human embryonic stem cells have been used in decellularized bone grafts for fabricating new bone tissue, with conservation and improvement of the bone matrix when implanted

FIG. 14.5 Comparison between the structures of arteries, veins, and capillaries. Images are not to scale.



in immunodeficient mice for 8 weeks [**173**]. Even adipose-derived stem cells have been suggested as precursors for cellularized vascular bone grafts [**174,175**]. The versatility of multipotent cells is a powerful and convenient tool in developing cellularized constructs.

Co-Culture Systems

A vascularized bone substitute is expected to replace and repair two different kinds of damaged tissues. As such, it is hypothesized that including cell lineages of vasculogenic and osteogenic types in a co-culture can provide simultaneous repair of vasculature and mineralized bone tissue. However, spatial distribution of cells is relevant in co-culture because functional vasculature needs to expand throughout the matrix as homogeneously as possible for complete nutrient, oxygen, and waste exchange. For example, Fuchs and coworkers developed a PCL scaffold co-cultured with outgrowth EC and osteoblasts, showing an enhanced vascularization in vivo compared with EC monoculture [**176**]. Kang showed that vasculogenic and osteogenic differentiation is more pronounced in a co-culture of ECs and MSCs than their respective monocultures [**55**]. Several other studies have underscored the importance of co-culturing for concurrent angiogenesis and osteogenesis [**135,170,177–183**].

Concurrent Angiogenesis and Osteogenesis

It is relevant to mention that concurrent angiogenesis and osteogenesis can also occur in the body provided that the pertinent cues are present at the site of repair. For example, expression of VEGF by the host body has been observed in response to distraction osteogenesis [**184–186**]. Several growth factors have shown to have angiogenic and osteogenic potential in vivo [**187,188**]. Adipose cells loaded onto scaffolds have also shown to respond to angiogenic and osteogenic factors concurrently, leading to formation of two distinct cell lineages from one kind of progenitor cell in the same scaffold [**189**]. The plasticity of some circulating stem cells is responsible for their multiple differentiation potential, which can be used for concurrent osteogenic and angiogenic

processes. For example, adult human circulating $CD34^+$ cells have also been shown to induce neovascularization and osteogenesis in nonhealing fractures in nude rats [**190**]. Pluripotent embryonic cells can also be used for concurrent differentiation into different lineages [**191**]. Co-culture methods certainly provide the opportunity to investigate the effects of both processes occurring at the same time. Because integration is key in developing vascularized bone substitutes, it would be of importance to evaluate how these biological processes are related to each other to enhance this integration.

TRANSLATION TO CLINICAL SETTING

All of the aforementioned methods have shown potential for concurrently restoring function for bones and vessels, as shown from preliminary results in vitro and in vivo. Several bone substitutes have managed to gain commercialization after extensive development, but the vast majority is yet to be translated into a clinical environment. The ultimate goal of all research directed at restoring blood flow and bone tissue is developing a cost-effective substitute that can be easily implanted after injury, with minimal burden on the patient and the surgeons. However, the main challenge right now is identifying the optimal conditions for successfully and seamlessly integrating the vascular bone graft to the host body. A key set of questions remain while developing these materials. We will give a brief overview of some of the most relevant aspects in the following section.

Surgical and Biological Anastomosis Versus Autogenous Vessel Formation

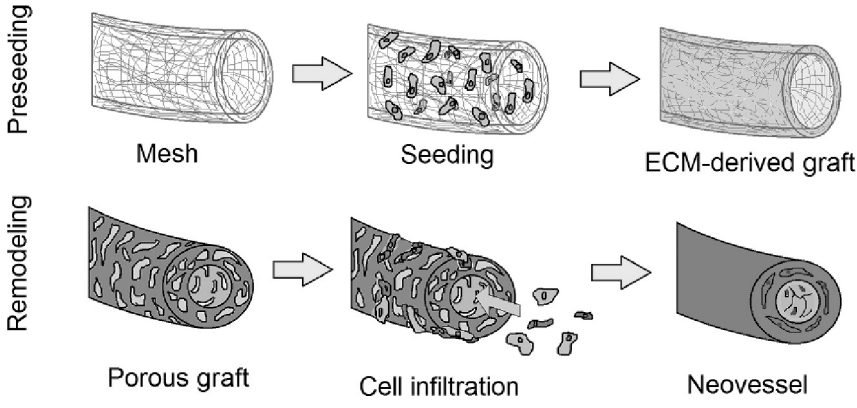
Vessel integration has been achieved in the clinic by surgically connecting vascular grafts to injured blood conduits in the body. However, so far, no engineered vascularized substitute has been developed to allow surgical approaches to connect bone and vascular tissue simultaneously in traumatic bone injury. The main focus of current synthetic graft research is to optimize how to allow the body to anastomose to these vascularized bone substitutes, cellularized or not. At present, no scaffold has an integrated vascular bed to readily anastomose with the host vasculature [**192**]. It has been proposed that having physical or cellular precursor vascular structures can be sufficient for facilitating the formation of anastomotic points to existing vessels [**168,193,194**], but this has yet to be widely and conclusively shown. On the other hand, increased porosity and interconnected pore sizes are likely sufficient for bone and vascular tissue to grow, but this remains to be proven satisfactorily [**192**]. In addition, this does not account for large bone scaffolds, which can have internal void fractions that are not properly infused with blood, increasing necrotic formations. Moreover, regardless of signaling, there is still no efficient control on vascular formation in these scaffolds. Even with some level of vascularization, full anastomosis has not been conclusively demonstrated in all vascularized bone grafts. So far, direct combination of vessels with vascular grafts and separate implementation of bone substitutes is the standard of clinical practice because of ease of implementation. Nonetheless, it is expected that the

development of new versatile scaffolds can allow for the direct connection of the scaffolds to the host vascular system, or in preexisting tissue approaches that reproducible luminal structures can be formed and directly anastomose to surrounding vessels.

Cells or No Cells?

Although cells have been used to show a degree of vascular formation in and around bone grafts, their use remains controversial. Having cells in the graft can raise the chance of forming precursor vascular structures, but the implanted cells could suffer from ischemia before reestablishment of blood perfusion, and cells could respond in unexpected ways inside of a host, especially if cell lineages are exogenous. Having no cells in the scaffold reduces biological immunoresponse and simplifies the design, but it also decreases the possibilities of rapidly endothelializing and mineralizing the scaffold, depending completely on body response. Examples of both approaches are illustrated in **Fig. 14.6**. For example, Yadong Wang and coworkers developed a heparin-coated porous PGS vascular graft with an outer PCL shell for added mechanical strength [**28**]. He showed that ECs could invade and slowly degrade the inner PGS layer while forming a functional endothelium whereas SMCs could invade and degrade the PCL layer. Seamless integration with the host vasculature was eventually achieved. On the other hand, L'Heureux [**195**] and Niklason [**196**] have developed tissue-engineered blood vessels from ECM deposited by cells, research that recently was successfully implemented by Duke University for the repair of blood vessels in an adult patient [**197**]. It has been suggested that developing new techniques for in vitro formation of functional blood vessels with perfusable lumens is indispensable for vascular tissue engineering. Although the debate continues, research up to this point has shown advantages for both approaches.

FIG. 14.6 Approaches for the design of degradable vascular grafts. In the preceeding approach, a mesh can provide temporary support for cells, which can deposit ECM. After decellularization, an ECM based tissue engineered graft is available for implantation. For the in vivo remodeling approach, a porous scaffold can serve as a template for cell invasion and subsequent neovessel formation.



Implementation on Current Surgical Procedures

There has been also increased interest in improving current surgical methods for the repair of bone by adding vascular approaches. A technique that has been used for vasculature enrichment in bone defects is the induced membrane. By surrounding a polymethyl-methacrylate (PMMA) cement spacer with soft tissue flaps in a bone defect, an induced tissue membrane rich in vasculature is created by the host body, which is then filled with autografts after the spacer is removed postoperatively [198,199]. Although this method has been successfully used in clinical repair, the method requires a secondary postoperative procedure for cement removal and placement of separately harvested autografts, which can lead to varied results, in particular lasting severe donor site pain. The technique can potentially be used with scaffolds and other grafts that can promote growth into their structure, and the synthetic membrane itself could be made with polymeric materials with cells and growth factors to induce vascularization in the surroundings of the implanted graft spacer. Distraction osteogenesis is another surgical procedure in which bone spacing in a defect is slowly increased to permit formation of new bone in the spacing created by using devices such as the Ilizarov apparatus [200,201] and the Albizzia nail [202,203]. This also increases the amount of surrounding soft tissue and vascularized beds surrounding the bone. Although the procedure is meant to provide the body with a slow gap opening for self-regeneration, the technique has potential for improvement by placement of bone substitutes and signaling factors that can accelerate the regeneration rate of the new hard and soft tissues [204,205]. Vascular grafting has allowed restoration of blood supply to distant parts in the host body, thus facilitating a rapid integration of bone substitutes after implantation. For example, in vascularized fibular grafting, the fibula is harvested from the leg, with its small attached blood vessels, and transferred to the hip, where microvascular anastomosis of vessels is performed. However, as with other autograft procedures, there is sacrifice of healthy tissue and possible unpredictable outcomes [206]. New research needs to look into actual combination of these new vascular grafts to either the affected site or the bone grafts to promote efficient bone repair.

Integration of Vascular Grafts and Scaffolds

As mentioned before, the surgical repair of blood vessels and bone is currently done mostly in separate procedures, but integration of vasculature and bone substitutes is starting to be developed. The key aspect in developing new grafts is integration of the current surgical methods with new breakthroughs in bone and vascular tissue engineering. For example, a bone substitute comprising a permeable suturable vascular graft integrated into a solid scaffold for mechanical support could provide an adequate one-time surgical implant that helps restore mineralized and vascular tissue by supplying blood immediately throughout the construct. In another example, if a luminal cellular structure with great anastomotic potential can be efficiently combined with a mechanically robust porous scaffold, then the same outcome could be expected. There are numerous device combinations that could be proposed and attempted. Thus,

translation into a clinical environment depends deeply on the combination of strategies into a single unit that can provide a frame for accelerated osteogenesis and an increased rate of incorporation into the surrounding vascular beds at the implant site.

FUTURE OUTLOOK AND CONCLUSIONS

The development of the optimal tissue engineering strategy is still ongoing, but recent research has provided more insight into the needs for vascularized grafts. The limitations of synthetic materials to osseointegrate to bone, the unpredictable outcome of internal graft vascularization, the difficulty of instant cellular anastomosis to preexisting host vascular systems, and reproducibility are just some of the challenges that have yet to be addressed. However, the fabrication of novel composite materials, controlled spatial addition of signaling cues, new methods for cell arrangement, and gradual improvement on cost-effective and less-burdening surgical techniques have slowly targeted these. It would be expected that future bone substitutes will have a solid porous biodegradable structure that will facilitate osteoconduction, osteoinduction, osteogenesis, and osseointegration. These substitutes would have an optimized architecture in which completely interconnected luminal networks will perfuse nutrients and remove wastes throughout the entire scaffold for efficient cell growth, differentiation, and reorganization into different tissues. Most importantly, the scaffold will easily and flawlessly integrate to the affected bone tissue and the surrounding vessels for eventual complete functional restoration of the affected areas. However, for a successful outcome of vascularized bone substitutes integration is essential. From the combination of synthetic and cell-based substrates and matrices, to the fusion of vascular and mineral grafts, to the combination of surgical approaches, integration is needed. Concurrent vascular anastomosis and osseointegration will only be possible by enhancing bone substitute design with vascular graft integration in a multifunctional approach.

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Chapter 15 | Regenerative Engineering: Fulfilling the Tissue Engineering Promise to Bone Regeneration

Tao Jiang,[1,2,3] Jennifer I. Rulka,[1,4] and Cato T. Laurencin[1,2,5,6,7]

INTRODUCTION

The principal calcified tissue of vertebrates is bone. Bone is a specialized form of connective tissue that, similar to other connective tissues, consists of cells and extracellular matrix (ECM). However, bone is the only connective tissue that forms mineralized ECM, conferring marked rigidity and strength to the skeleton. Bone is a complex, highly organized, constantly changing connective tissue in the body and its main functions are to (1) provide mechanical support and sites of muscle attachment for locomotion; (2) provide a protective shield for internal vital organs (brain, heart, lungs, etc.) and bone marrow; (3) generate red and white blood cells by the bone marrow for immunoprotection of other tissues and for oxygenation; and (4) provide storage of ions such as calcium and phosphate, as well as growth factors and fatty acids, for the maintenance of serum homeostasis and for normal body function [**1**].

On the basis of general shape, bone can be classified into short, flat, and long bone [**2**]. Short bones measure approximately the same in all directions and are trapezoidal, cuboidal, cuneiform, or irregular in shape. In contrast, flat and long bones have one dimension that is much shorter or longer than the other two. Bone tissue may also be classified into cortical (compact) and cancellous (trabecular) bone according to the

[1] Institute for Regenerative Engineering, University of Connecticut Health Center, School of Medicine, Farmington, CT
[2] The Raymond and Beverly Sackler Center for Biomedical, Biological, Physical and Engineering Sciences, University of Connecticut Health Center, School of Medicine, Farmington, CT
[3] Department of Medicine, Division of Endocrinology, University of Connecticut Health Center, School of Medicine, Farmington, CT
[4] Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA
[5] Department of Biomedical Engineering, University of Connecticut, School of Engineering, Storrs, CT
[6] Department of Orthopedic Surgery, University of Connecticut Health Center, School of Medicine, Farmington, CT
[7] Department of Chemical, Materials and Biomolecular Engineering, University of Connecticut, School of Engineering, Storrs, CT

morphology [2,3]. Cortical bone is almost solid (10 % porosity), with space only for osteocytes, canaliculi, and blood vessels. In cortical bone, densely packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes. Cancellous bone has less density and greater porosity (50–90 % porosity) than cortical bone. It has a loosely organized, porous matrix.

Bone repair and regeneration has become a common task for orthopedic surgeons. Although bone has an excellent ability to self-repair small defects; the healing capacity of bone has its limitations. Defects, especially large bone defects due to trauma and disease, sometimes may not heal by themselves and result in a nonunion [4]. Nowadays, more than 500,000 bone graft procedures are performed in the United States annually and approximately 2.2 million worldwide to repair bone defects in orthopedics, neurosurgery, and dentistry [5]. In this chapter, we first briefly introduce various commercially available bone grafting options including autografts, allografts, and bone graft substitutes. Then we provide a state-of-the-art review on the tissue engineering approach to bone regeneration using biomaterials, cells, and growth factors. Finally, we emphasize the emergence of the field regenerative engineering, aiming to overcome various challenges that researchers have faced in bone tissue engineering. The regenerative engineering approach to bone repair takes advantage of advances in materials science, stem cells, and developmental biology and in our opinion represents the next era in engineering bone tissue.

BONE GRAFT SUBSTITUTES

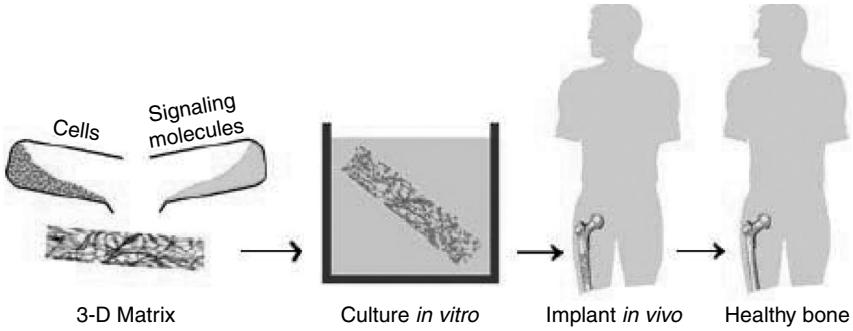
The first documented case of using bone grafts was in 1668 by a Dutch surgeon who filled a human bony defect with a xenograft from canine bone. In 1915, autologous bone grafting was described by Fred Albee, who used part of the tibia for spinal fusion [6]. Nowadays in the United States, musculoskeletal diseases, including bone loss and arthritis, are the leading category of reported chronic impairment. Bone grafting is a big business. During the 10-year period of 1998–2007, the sale of bone grafts and bone substitutes in the United States alone increased from approximately $150 million to more than $1.4 billion [7,8]. Autografts, allografts, and various types of bone graft substitutes have been used for bone repair. Osteoconductivity, osteoinductivity, and osteogenicity are three essential elements of bone grafts for successful bone regeneration. Osteoconductivity is the ability of a material to provide a three-dimensional (3D) structure for the in-growth of host capillaries, perivascular tissue, and osteoprogenitor cells. Osteoinductivity is defined as the ability of a material to stimulate primitive, undifferentiated, and pluripotent cells to develop into the bone-forming cell lineage with the capacity to form new bone. Osteogenecity implies that a bone grafting material has the intrinsic capacity to stimulate bone healing by the presence of mesenchymal stem cells (MSCs) or osteoprogenitors cells [9–11]. Autografts are cortical and cancellous bone tissues harvested from different donor sites of the host patient such as the fibula, rib, and iliac crest [12]. These grafts are currently considered as the gold standard for bone grafts because no immunogenicity problems are

associated with them and they possess all of the aforementioned attributes necessary for new bone growth. Although autografts provide the best replacement tissue for a bone defect, they do encounter limitations, such as donor site morbidity and limited supply. On the other hand, allografts are tissues taken from donors or cadavers. In this case, supply is not a concern. However, the disadvantage of allografts is the potential risk of disease transmission despite treatments to minimize this issue [**8,13**]. In addition, because allografts must undergo a series of processing, preservation, and sterilization steps to reduce the potential immune response of the recipient to the donor and the potential disease transmission, these grafts are acellular; thus, they are less successful than autografts because of the absence of viable cells. Apart from autografts and allografts, various bone graft substitutes exist classified as allograft-based, factor-based, cell-based, ceramic-based, and polymer-based bone graft substitutes [**8**]. Although these bone graft substitutes have their own advantages and have improved the quality of countless lives, they remain imperfect solutions and different concerns still remain. Allograft-based bone graft substitutes are human derived, and there is potential immunogenicity and risk of disease transmission. Factor- and cell-based bone graft substitutes usually require the addition of an osteoconductive graft for structural support. Ceramic- and polymer-based bone graft substitutes are generally osteoconductive but lack osteoinductivity. Other concerns may include unsuitable resorption rates and inferior mechanical properties [**13**]. Aiming to overcome the limitations of bone graft substitutes, new technologies and concepts have been continuously emerging and evolving, with tissue engineering being the most popular approach in the past 2 decades.

BONE TISSUE ENGINEERING

Tissue engineering was proposed for the first time in 1987 by Y.C. Fung and attracted great interest since the milestone paper by Langer and Vacanti [**14**]. It is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. Laurencin et al. further defined tissue engineering as the application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissues by using biomaterials, cells, and factors alone or in combination [**15**]. A popular and well-studied approach for bone tissue engineering is depicted in **Fig. 15.1**, which involves seeding cells together with certain signaling molecules on a 3D porous biodegradable matrix, culturing them in vitro, and implanting them into defects. In the tissue engineering approach to regenerating tissues or organs, biomaterials, usually in the form of 3D porous scaffolds, play multiple significant roles to provide structural maintenance of the shape of a defect and void volume for vascularization, and they serve as an initial temporary ECM for cell adhesion, proliferation, differentiation, and maturation. In addition, scaffolds can act as a delivery vehicle for growth factors and cells to the defect site for tissue morphogenesis and defect healing [**16–18**]. Ideally, tissue engineering scaffolds should degrade in a

FIG. 15.1 Schematics of scaffold-based bone tissue engineering. In this approach, cells, such as osteoprogenitor cells or stem cells, together with signaling molecules, are seeded on a 3D porous biodegradable scaffold and cultured in vitro before the constructs are implanted in vivo to repair a bone defect.



controllable manner so as to match the natural ECM formation, gradually transfer the structural and functional roles to the newly formed tissue, and finally get resorbed and metabolized. Cells, often tissue-specific cell types, progenitors, or stem cells that are capable of differentiating into multiple phenotypes, are critical for a tissue-engineered system to perform, succeed, and in many cases to compensate for a deficiency of the normally functional cell population [**17,19,20**]. Growth factors, typically acting as signaling molecules via binding to specific transmembrane receptors on the target cells, regulate several cell behaviors such as cell adhesion, proliferation, migration, and differentiation [**21,22**]. They play an essential role in tissue morphogenesis and new tissue formation in tissue engineering and regenerative medicine. Thus, growth factors are important components in tissue engineering systems to stimulate cells and alter cellular behaviors. In addition to the types of growth factors, the spatial distribution, concentration gradient of the growth factors on scaffolds, and the release kinetics are crucial for them to exert their biological effects on the target cells [**23,24**]. Although it is not indispensable that all three components are present in bone tissue engineering products, it is undoubted that a combinational approach may augment the therapeutic effect.

Scaffolds

In bone tissue engineering, scaffolds serve as temporary matrices for bone growth. Several criteria must be met for successful bone tissue engineering scaffolds. The biomaterials used must be biocompatible so that the scaffolds are able to perform with appropriate host responses when implanted in vivo. In addition, scaffolds should be biodegradable so that the materials can be broken down and eventually mediated by the biological system [**25**]. Furthermore, the 3D scaffolds for tissue engineering must

have an interconnected porous structure with appropriate pore size and pore volume to facilitate cell and tissue in-growth, vascularization, nutrient diffusion into the matrices, and metabolic waste removal. In addition to the 3D porous architecture, it is important to match the mechanical properties of the scaffolds to their in vivo microenvironment. Evidence suggests that implants are more biocompatible when the material properties approximate those of the local tissues [**26**].

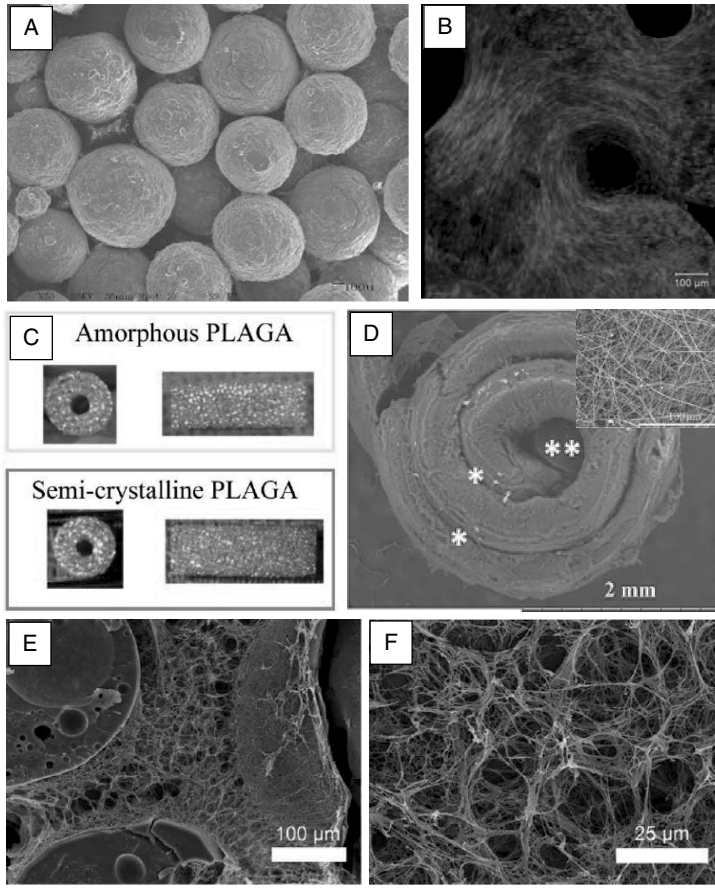
Several biomaterials including polymers and ceramics have been investigated for bone tissue engineering applications. Poly(α-hydroxy acids) such as poly(glycolic acid) (PGA) [**27**], poly(lactic acid) (PLA) [**28,29**], and poly(lactic-co-glycolic acid) (PLGA) [**30--33**] are the most extensively studied synthetic polymers for regenerating bone tissue. These materials are biodegradable via hydrolysis, biocompatible, and have already gained U.S. Food and Drug Administration (FDA) approval for use in several applications. One of the drawbacks associated with poly(α-hydroxy acids) is the acidic degradation products, which may cause adverse effects when scaffolds are implanted in vivo. Other synthetic polymers used in bone tissue engineering include poly(caprolactone) (PCL) [**34,35**]), polyanhydrides [**36,37**], polyphosphazene [**38,39**], and poly(propylene fumarate) [**40,41**]. Natural polymers originated from different sources have also been investigated for bone repair. Examples include collagen, chitin/chitosan, starch, hyaluronic acid, and bacterial-derived polyhydroxyalkanoates (PHAs) [**42**]. Natural polymers vary dramatically in physical, chemical, and biological properties. Some of the main advantages of these biomaterials include chemical versatility due to abundant functional groups and low immunogenic potential. Apart from polymeric materials, various inorganic materials have also been widely used in bone tissue engineering. Among them, calcium phosphate-based ceramics have been extensively studied because of their chemical similarity to hydroxyapatite, the major component of the inorganic matrix of bone [**43**]. The main advantages of calcium phosphate-based ceramics are the well-proven osteoconductivity and, in some cases, osteoinductivity. Nevertheless, these ceramics are brittle in nature and lack the mechanical properties suitable for bone tissue engineering. Therefore, composite materials consisting of polymers and ceramics have frequently been developed to fabricate bone tissue engineering scaffolds, combining the advantages of both components while offsetting the disadvantages of each material [**44**].

Scaffolds for bone tissue engineering can be fabricated by various techniques [**45**]. Several of the early developed technologies focused on fabricating highly porous scaffolds with high interconnectivity between pores, aiming for uniform cell seeding, nutrient transport, as well as tissue ingrowth and neo-tissue formation. For example, in the solvent casting and particulate leaching method, a specific-sized porogen such as salt or sugar particulates is dispersed in a polymer solution. The ultimate dissolution of the porogen produces polymer scaffolds with porosity usually greater than 90 %. This technique benefits from its simplicity and versatility with the additional advantage of efficient control over pore size and geometry. However, it is limited by the lack of control over interpore connectivity. Residual porogen particulates are potentially

associated with the structures, especially in thick scaffolds. Thermally induced phase separation and freeze drying is another popular technique to make porous scaffolds. Such a technique can be used to fabricate polymer and polymer/ceramic composite scaffolds with tunable porosity and interconnectivity. By varying the freezing conditions, mean pore diameters of 1–250 μm and porosity of up to 95 % can be obtained. The scaffolds produced by the aforementioned methods, although sufficiently porous to support cell attachment and tissue ingrowth, suffer from low mechanical properties, which significantly limit their applications. More recently, rapid prototyping (RP) integrated with computer-aided design (CAD) has been used to develop scaffolds with precisely controlled internal architectures [**46,47**]. For example, in 3D printing, scaffolds are fabricated by laying down successive powder layers of materials and selectively bonding the particles via the deposition of a binder solution, enabling precise control of internal porous structures. Using the RP technique, polymer or polymer/ceramic scaffolds with porosity from 30 % to 80 % and with a compressive modulus typically in the low-mid range of human trabecular bone have been produced [**34,48,49**].

In our laboratory, we have initially developed a microsphere sintering method to create 3D porous scaffolds to regenerate bone tissue using different types of polymer materials such as poly(lactide-co-glycolide) (PLAGA), polyphosphazenes, chitosan, and their blends and composites [**50,51**]. Orderly packing and heating the individual microspheres in a predefined mold led to the development of 3D scaffolds with mechanical properties in the mid-range of trabecular bone suitable for load-bearing bone tissue engineering. In addition, these scaffolds have approximately 30 % pore volume with median pore sizes ranging from 100 to 300 μm, allowing bone cells and tissue in-growth (**Fig. 15.2, A and B**). Furthermore, to more closely mimic the bone marrow cavity in native bone and promote transport flux through 3D scaffolds, Kofron and Laurencin et al. developed tubular scaffolds by sintering either PLAGA or PLAGA/hydroxyapatite microspheres [**52,53**] (**Fig. 15.2C**). Such tubular scaffold design allowed bone marrow stromal cells at the defect site to penetrate into the interior of the scaffold. Our findings indicated that amorphous PLAGA tubular scaffolds were completed degraded 24 weeks after implantation into rabbit ulnar defects and were replaced by mineralized tissue and osteoid [**53**]. Inspired by the hierarchical bone structures, Deng and Laurencin et al. recently developed a mechanically competent scaffold mimicking both the bone marrow cavity and the lamellar structure of bone (**Fig. 15.2D**). Electrospun polyphosphazene/PLGA blend nanofibers were oriented in a concentric manner with an open central cavity, creating a biomimetic nanofibrous scaffold [**54**]. The potential of using this unique spiral nanofibrous scaffold for regenerating bone tissue was evaluated in vitro by monitoring cellular activity and mechanical performance. Results demonstrated that the biomimetic scaffolds promoted osteoblast proliferation and differentiation throughout the scaffold architecture, leading to a similar cell-matrix organization to that of native bone [**54**]. Taking sintered microspheres

FIG. 15.2 Examples of 3D scaffolds used for bone regeneration. (A) A porous PLAGA/chitosan sintered microsphere scaffold. (B) The PLAGA/chitosan sintered microsphere scaffold supported osteoblastic cell proliferation on the scaffold surface and migration into the pore structure. Blue: cell nuclei stained with DAPI. Red: cytoskeletal protein F-actin stained with TRITC-conjugated phalloidin. (C) A novel tubular scaffold by sintering amorphous or semicrystalline PLAGA microspheres. Such tubular scaffold was designed to mimic the macroscopic structure of bone and allow bone marrow stromal cells at the defect site to penetrate into the interior of the scaffold. (D) A 3D biomimetic PLAGA/polyphosphazene nanofibrous scaffold formed by a rolling nanofiber mat of ~250μm in thickness. (*) indicates interlamellar space whereas (**) indicates central cavity. (E) A composite scaffold consisting of sintered polyphosphazene microspheres and PLLA nanofibers in the pores among the microspheres. (F) An enlarged scanning electron micrograph showing the PLLA nanofiber network within the polyphosphazene sintered microsphere scaffold in panel E. Source: Figures reproduced with permission from [**53–55,151**].



and nanofibrous structure together, Brown and Laurecin et al. further developed a novel scaffold that combines the excellent mechanical properties of the sintered microsphere structure with a highly nanofibrous network to mimic the mechanical properties of bone while also promoting the phenotype expression of osteoblast progenitor cells [**55**]. As shown in **Fig. 15.2. E and 2F**, a 3D poly(L-lactic acid) (PLLA) nanofiber mesh was successfully incorporated into the void space between sintered polyphosphazene microspheres via thermally induced phase separation [**55**].

Cells

Cells are a critical component of bone tissue engineering, in which cell-induced osteogenesis is highly dependent on the scaffold carrier. When combined with cells, a degradable scaffold provides a temporary environment for cells to attach and grow. Cells gradually migrate into the scaffold and differentiate to form new bone. These cellular components can be recruited from the local environment (endogenous) or can be introduced from an external cell source (exogenous). Endogenous cell recruitment occurs naturally when a bone defect is created. After the initial inflammatory process at the defect site, an influx of various cells is initiated because of chemotaxis. These osteoblastic and osteoprogenitor cells come from the bone, the periosteum of adjacent bone tissue, the bone marrow, and the surrounding soft tissue such as the muscle, as well as from the distant sites. In other words, the success of a tissue engineering strategy based on scaffold alone or scaffold with growth factors largely relies on the efficient recruitment of local cells. In some cases, an endogenous cell source may not be sufficient to regenerate bone. For example, the activity of the local cells may have been severely impaired because of the patient's medical conditions such as diabetes. Alternatively, the local cell source may be limited in the number of osteogenic progenitor cells because of aging and osteoporosis. However, in other cases, the bone defect may be too large for the body to repair and may result in nonunion if no exogenous cells are introduced. In such cases, several cell types may be chosen to be delivered directly to the defect site for regeneration.

Encouraged by the success of bone autografts in bone reconstructive surgery, autologous bone marrow, which remains in bone autografts and is believed to contribute to bone regeneration, has been investigated for bone tissue engineering because it contains osteoblast precursors. Various preclinical investigations [**56–59**] and several clinical studies [**60,61**] have shown that the implantation of fresh bone marrow could result in effective bone repair and regeneration. The effectiveness of bone regeneration by bone marrow transplantation greatly depends on the number of healthy osteogenic progenitors. However, in the best case scenario, these osteoprogenitors represent only 0.001 % of the nucleated cells in healthy adult marrow [**62,63**]. Therefore, strategies to select and expand the osteogenic progenitors ex vivo and implant those cells in vivo are of clinical importance. For example, Quarto et al. [**64**] reported the use of the culture-expanded osteoprogenitor cells grown on macroporous hydroxyapatite scaffolds to treat three patients with large bone defects. In all three patients, abundant callus

formation along the implants and good integration at the interfaces with the host bones were observed 2 months after implantation.

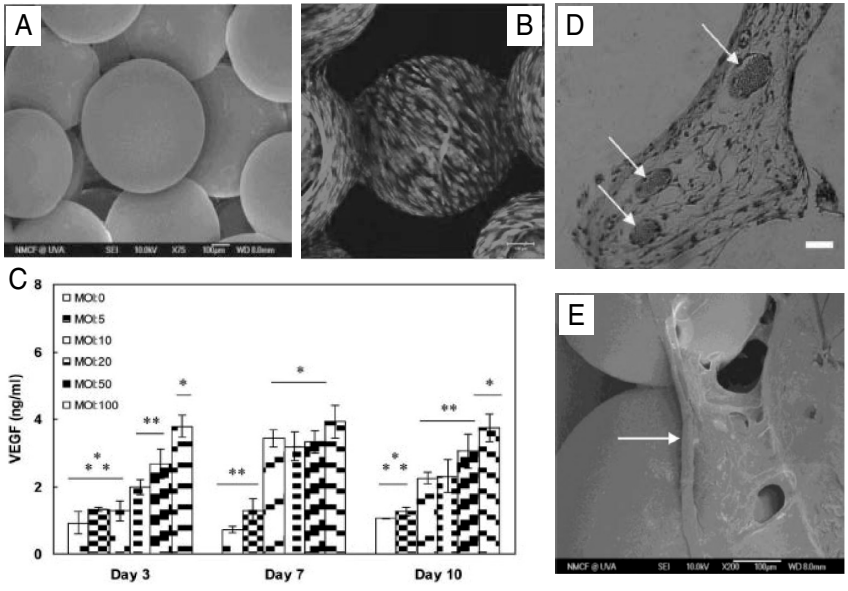
Different from fresh bone marrow that contains a mixture of various types of cells, stem cells expressing a characteristic panel of cell surface markers can be isolated from an early-stage embryo and various adult tissues and used to regenerate bone. A more detailed description of these cells and their applications can be found in the "Stem Cell Science" section below. In an attempt to accelerate or enhance bone regeneration, researchers have explored the use of predifferentiated osteoblasts in bone tissue engineering. For example, stem cells can be harvested from different tissues such as bone marrow and adipose tissue and predifferentiated into the osteoblast lineage using an induction culture medium containing dexamethasone, ascorbic acid, and β-glycerophosphate [**65,66**]. Yoon et al. [**66**] cultured human adipose-derived stem cells (ADSCs) on porous PLAGA scaffolds and differentiated these cells into osteoblasts in vitro for 14 days before being implanted into critical-sized calvarial defects in rats. It was found that scaffolds with differentiated ADSCs had noticeably more maximal and robust bone tissue regeneration than scaffolds with undifferentiated ADSCs. Because autologous osteoblasts are more difficult to isolate than stem cells and the expansion rate of osteoblasts are relatively low, generating mature osteoblasts from culture-expanded and predifferentiated stem cells have become an attractive strategy in bone tissue engineering.

Another important type of cell used in tissue engineering is genetically modified cells. Using a specific DNA encoding a functional, therapeutic gene to replace a mutated gene, researchers are able to transfect cells to express target proteins to enhance bone formation. Over the past decade, studies have continued to examine the delivery of the osteogenic and angiogenic proteins achieved by gene therapies for bone tissue engineering. Genetically modified cells expressing bone morphogenetic proteins 2, 4 and 7 (BMP-2, -4, -7), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) have been combined with scaffolds for bone tissue engineering [**67–71**]. For instance, Jabbarzadeh and Laurencin et al. isolated adipose-derived stromal cells and transfected the cells with adenovirus encoding the cDNA of VEGF. Results showed that the genetically modified adipose-derived stromal cells were able to produce an abundant amount of VEGF on the surface of the sintered PLAGA microsphere scaffolds. (**Fig. 15.3, A and B**) [**71**]. The dramatic advantage of gene therapy over the use of growth factors directly is the delivery of physiological levels of therapeutic protein using natural cellular mechanisms, avoiding the need of a high dose of proteins. However, gene therapy has potential risks such as toxicity associated with viral viruses and immune response.

Growth Factors

Bone is a rich source of several growth factors that serve as signaling molecules for cells. In skeletal development and bone fracture healing, these growth factors coordinate timing events between bone-forming and bone-resorbing cells and function to

FIG. 15.3 (A) Scanning electron micrograph showing the porous structure of a sintered PLAGA microsphere scaffold. (B) Immunofluorescent staining of VEGF produced by genetically modified ADSCs. An abundant amount of VEGF as evidenced by green fluorescent staining was produced by the transfected cells. (C) Quantitative VEGF production as a function of time and multiplicity of infection (MOI). VEGF was continuously produced by the cells during the 10-day experimental period. (D) Hemotoxylin and eosin staining showing the presence of blood vessel formation as evidenced by the luminal structure containing red blood cells (white arrows). (E) Scanning electron micrograph showing the growth of blood vessel (white arrow) after 21 days of subcutaneous implantation in SCID mice. Source: Figures reproduced with permission from [**71**].



influence critical processes such as cell proliferation, division, differentiation, and matrix synthesis. When growth factors are secreted by cells, they exert their biological effects on target cells via three possible signaling pathways [**72**]. Autocrine signaling refers to a mode of growth factor action in which a growth factor binds to receptors on and affects the function of the cell type that produced it. Paracrine signaling is the second form of cell-cell communication in which a cell produces a growth factor, secretes it into the immediate ECM, and induces changes in adjacent or neighboring cells. As opposed to paracrine signaling in which growth factors are only able to travel a short distance, endocrine signaling is a signaling pathway in which growth factors act on cells located at a distance from where the factors were synthesized via the circulatory system. Several growth factors have been shown to be expressed in distinct

spatial patterns during bone fracture healing. Among these growth factors are TGF-β, BMPs, fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), and VEGF.

The TGF-β family belongs to the transforming growth factor-β superfamily. TGF-βs are multifunctional peptides found in many tissues as at least three homologous isoforms, TGF-β1 through TGF-β3. In bone, TGF-β1, 2, and 3 are detected in bone cells and tissues such as the osteoblasts, the perichondrium, and sites of intramembranous bone formation. In addition, both chondrocytes and osteoblasts are enriched in receptors for TGF-β. These evidences suggest an important regulatory role of TGF-βs in osteoblast function, skeletal development, and bone fracture healing. Nevertheless, it is still debatable whether TGF-β is efficacious in bone healing, and the osteoinductive potential of TGF-β seems limited. Furthermore, there remains a concern for TGF-β to be used in the clinic for bone healing because TGF-β lacks specificity and enhances cellular proliferation of various cell types, potentially leading to unforeseen side effects [**72,73**].

BMP was originally discovered by Urist as an inducer of bone formation in ectopic tissue. BMPs are secreted polypeptides that exert pleiotropic effects on a variety lineage of cells via interacting with BMP receptors on the cell surface. Many of the BMPs belong to the TGF-β superfamily. Among all BMPs, BMP-2, BMP-4 (formerly BMP-2b), and BMP-7 (or osteogenic protein-1, OP-1) are more well known to play important roles in bone healing by means of their ability to stimulate differentiation of mesenchymal cells to osteoblast lineage. Numerous studies have evaluated the efficacy of recombinant human BMPs (rhBMPs) in the healing of critical-sized bone defects in different animal models [**74–76**] and in spinal fusion [**77,78**]. In our laboratory, we applied rhBMP-2 to a heparinized chitosan-PLAGA sintered microsphere scaffold and used the scaffold to repair a critical-sized bone defect in a rabbit ulnar defect model [**79**]. Results showed that the incorporation of rhBMP-2 on the heparinized scaffold promoted early bone formation as evidenced by complete bridging of the defect along the radius. In another study, Borden et al. [**56**] investigated the efficacy of BMP-7-loaded PLAGA sintered microsphere scaffolds for bone regeneration using a rabbit ulnar nonunion segmental defect model. It was found that BMP-7 induced penetration of new bone throughout the entire structure of the implant. Moreover, the addition of bone marrow and BMP-7 to the scaffolds resulted in the formation of mature cancellous bone within a short period of 6 weeks. rhBMP-2 and rhBMP-7 are currently approved by the FDA for several clinical applications, such as for treating long-bone nonunions and for anterior lumbar interbody fusions.

VEGF is a potent angiogenic factor and plays a critical role as a central regulator of vasculogenesis and angiogenesis. Some of the important biological functions of VEGF are to induce endothelial cell proliferation, promote cell migration, and inhibit apoptosis. VEGF has also been shown to exert its effects on osteoblasts by either directly acting on the cells [**80**] or paracrine signaling [**81**]. Endogenous VEGF also plays a key role in bone repair and remodeling through its effects on osteoblasts and osteoclasts. VEGF is expressed in a similar temporal and spatial pattern in the fracture

callus as occurs during long-bone development. Given the importance of VEGF in normal bone repair, treatment with exogenous VEGF might promote angiogenesis and bone formation and has attracted much research interests. In the study by Street et al. [**82**], exogenous VEGF was shown to enhance blood vessel formation, ossification, and new bone maturation in mouse femur fracture, and it promoted bony bridging of a rabbit radius segmental defect at a dosage of 250 μg per rabbit. Considering that neovascularization assists in providing critical nutrient support to the MSC and osteoblasts necessary for bone repair, enhancing angiogenesis within 3D scaffolds becomes crucial in bone tissue engineering. Thus, Jabbarzadeh and Laurencin et al. [**71**] used a combined gene therapy and cell transplantation strategy to induce vascular tissue growth into tissue-engineered PLAGA sintered microsphere scaffolds. The adenovirus-transfected ADSCs were shown to consistently produce VEGF during a period of 10 days, which, combined with endothelial cell transplantation, resulted in marked vascular growth within the 3D PLAGA scaffolds critical for bone regeneration (**Fig.15. 3, C–E**).

Other important growth factors have been expressed locally in the ECM or identified during the early stage of fracture healing and thus have been used in bone tissue engineering. FGFs comprise a family of structurally related polypeptides that are involved in the control of various processes. Specifically, FGF-1 and FGF-2 are the most abundant among FGFs in normal adult tissue. Numerous studies have found that FGF-1 and FGF-2 exert important effects on osteoblastic cell differentiation and bone matrix formation in vitro [**83,84**]. Via the interactions with FGF receptors, FGFs influence bone formation and play important roles in the control of fetal skeletal development in vivo [**85**]. IGFs are key players in skeletal development and physiology. The two major IGF peptides, IGF-I and IGF-II, are capable of stimulating osteoblast proliferation in vitro and promoting bone formation in vivo [**86,87**]. PDGF was originally identified as a product of human platelets; however, it was subsequently found to also be expressed by skeletal tissue. PDGF-BB is the most potent isoform of the growth factor among the PDGFs, the other two forms being PDGF-AA and PDGF-AB. As a potent mitogen for cells of the osteoblastic lineage, PDGF exerts important effects on bone formation, resorption, and remodeling [**88**]. More detailed review on the roles of applications of these growth factors in the repair of bone can be found elsewhere [**72**].

In addition to each individual type of growth factors, it has been gradually realized that a combined local delivery of multiple growth factors and, particularly, a well-controlled sequential growth factor delivery mimicking the orchestrated endogenous growth factor production during natural tissue morphogenesis, are critical to successful tissue engineering of bone. Therefore, several dual growth factor delivery systems have been developed, simultaneously or sequentially delivering two different growth factors such as BMP-2 and TGF-β [**58**], BMP-2 and BMP-7 [**89**], VEGF and PDGF-BB [**90**], or BMP-2 and IGF-I [**91**]. Because angiogenesis is crucial for bone regeneration and evidence has shown that angiogenesis precedes osteogenesis during endochondral

bone formation [92,93], the sequential delivery of VEGF and BMP-2 has attracted great interest. An early burst release of VEGF followed by a continuous release of BMP-2 from a composite biodegradable matrix has shown to enhance blood vessel formation and bone formation [94].

THE REGENERATIVE ENGINEERING APPROACH TO BONE REGENERATION

The field of tissue engineering has greatly advanced since its inception in the late 1980s. Simultaneously, the global demand of tissue-engineered products has increased dramatically largely because of the shortage of donor tissues. There clearly exists an imbalance between what are demanded and what tissue engineers could offer. Although we are fairly successful in engineering relatively simple tissues such as skin and cartilage and have made available several tissue-engineered products such as Epicel® for skin repair and Carticel® for articular cartilage regeneration, we face great challenges in regenerating complex human tissues and organs. These challenges lie in various aspects such as the need for advanced biomaterials, an appropriate and reliable cell source, and a thorough understanding of the biology of tissue morphogenesis [95]. Specifically, for regenerating bone tissue, although different bone void filler materials are available off-the-shelf, there are currently no FDA-approved devices that incorporate cells or other tissue-derived components. Our ability to regenerate large segmental bone defects is quite limited. Grand challenges such as mimicking the physical, chemical, and biological properties of native bone are yet to be overcome. In this regards, we believe that regenerative engineering represents the next valuable tool to tackle the problems. Regenerative engineering is defined by Laurencin and Khan as "the integration of tissue engineering with advanced material science, stem cell science, and areas of developmental biology" [96]. Although tissue engineering focuses on the ability to repair a specific tissue, regenerative engineering aims to regenerate or reconstruct complex tissues and biological systems such as the whole human limb by harnessing and expanding these newly developed tools.

Advanced Materials Science

Smart Materials

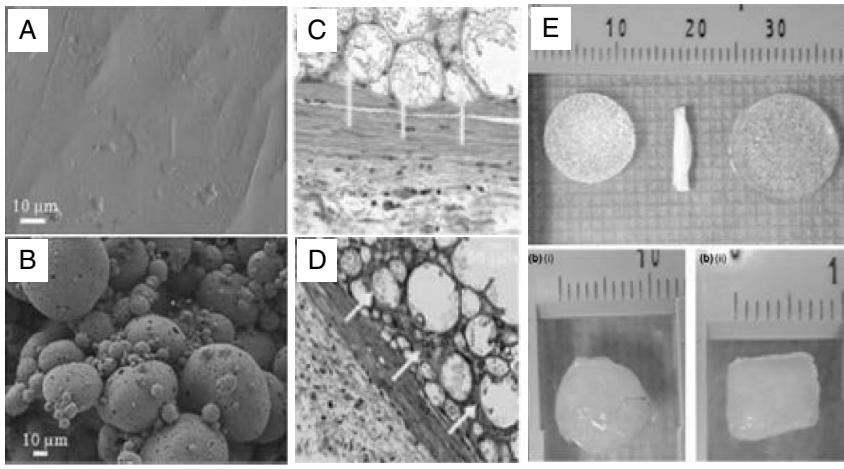
Nature presents us with intelligent materials possessing many inspiring properties such as sophistication, miniaturization, hierarchical organizations, adhesiveness, resistance, and adaptability. Chemists and materials scientists have fascinated us with various artificial materials closely mimicking the structure and function of the native tissues [97,98]. Moreover, the recent advancements in polymer science and novel fabrication techniques show promise in developing advanced biomaterials capable of performing in a complex in vivo microenvironment. For instance, polymer chemists are now able to synthesize highly versatile polymers such as polyphosphazenes to allow for desirable chemical modification [99,100]. Furthermore, bioengineers are capable of

evaluating many materials through biomaterials microarray and high-throughput screening [**101,102**]. On the other hand, advancements in micro- and nanofabrication have made it possible to create structures mimicking those of the natural tissues for regeneration purpose. These latest advancements in biomaterials and the milestones we have reached in tissue engineering are leading us to a new era of designing smart materials toward bone regeneration.

Smart materials differ from the traditional materials in a way that the former does not passively react to the microenvironment but perform their functions through molecular recognition or structural specificity or both. In other words, smart materials are bioresponsive to the microenvironment that they face. The design of smart materials for bone regeneration can be achieved in various ways. The most basic and commonly used approach to designing biomaterials exhibiting bioactivity and recognition is through immobilizing biologically active molecules on the surface of a biomaterial. For example, bioactivity has been introduced to different substrate materials by covalently coupling an RGD (Arg-Gly-Asp) oligopeptide sequence alone or in combination with other peptides, which binds to integrins in cell attachment proteins and leads to improved osteoblast adhesion, proliferation, and differentiation [**103–105**]. Growth factors such as BMP-2 could also be chemically immobilized onto polymer substrate to enhance bone regeneration [**106**].

The ability of certain molecules to self-assemble to form supramolecular architectures opened up great opportunities for biomedical engineers. Molecular self-assembly produces scaffolds with highly ordered structures that can be designed and built precisely for many tissue engineering applications [**107,108**]. Hartgerink et al. [**109**] fabricated a peptide-amphiphile nanostructured fibrous scaffold through molecule-by-molecule self-assembly or a bottom-up approach. The individual molecule included five distinct regions, namely a long alkyl tail for hydrophobicity, four consecutive cysteine residues to create disulfide bonds for polymerization, a linker region of three glycine residues to provide flexibility to the hydrophilic head group, and two functional regions containing phosphorylated serine residue and the cell adhesion ligand RGD. The molecules self-assembled into nanofibers via a pH-controlled and reversible mechanism. In addition, the phosphorylated serine residue induced nano-hydroxyapatite formation on the nanofibers. These mineralized nanofibers closely resemble the lowest level of hierarchical organization of bone. Using a top-down approach, Deng et al. [**110**] prepared completely miscible polymer blends of PLAGA-polyphosphazene attributing to strong intermolecular hydrogen bonding. When the materials were implanted in vivo, the polymers started to degrade, leading to the disruption of the intermolecular hydrogen bonding; intramolecular hydrogen bonding thus became dominant. As a result, the polymer chains self-assembled to form microspheres and in situ porous structure, facilitating tissue in-growth (**Fig. 15.4, A–D**). The in situ developed 3D structures showed interconnected pores with a porosity of 82–87 %. The findings are highly encouraging to develop 3D scaffolds for bone regeneration with initial higher mechanical properties that reduce over time as the

FIG. 15.4 Examples of advanced biomaterials. (A) Homogeneous polymer membrane consisting of 50 % poly[(glycine ethyl glycinato)1(phenylphenoxy) 1phosphazene] (PPHOS) and 50 % PLAGA. (B) In situ formation of polymer microspheres and porous structure after 12-week degradation in vitro. (C) Hematoxylin and eosin staining showing in situ microsphere formation (yellow arrows) after 12-week implantation in vivo. (D) Hemotoxylin and eosin staining showing robust collagen tissue infiltration within the matrix (yellow arrows) through the in situ formed pores after 12-week implantation. (E) Top panel: covalently cross-linked alginate scaffolds with an original shape of a circular disc (left) allows the lyophilized scaffolds to be mechanically compressed (center); the compressed scaffold reassumes its original shape and size upon rehydration (right). Bottom panel: Shape-memory alginate discs of circular and rectangular shape were used to regenerate cartilaginous tissues in the shape of the original scaffolds. Source: Figures reproduced with permission from [**110**].



polymer degrades while generating interconnected porous structures to allow tissue in-growth.

Smart materials may also be designed to readily sense the environmental changes, including temperature, pH, and light, and react to such changes to perform their functions. Many of these materials fall into the category of hydrogels that are useful in minimally invasive procedures in orthopedic applications. In a temperature-sensitive hydrogel system, some polymers undergo abrupt changes in solubility at a temperature known as the lower critical solution temperature (LCST). The phase transformation from soluble solution to hydrogel is generally viewed as a phenomenon governed by the balance of hydrophilic and hydrophobic moieties on the polymer chain and the free energy of mixing. Poly(*N*-isopropylacrylamide) (PNIPAAm) is a typical

thermo-sensitive material exhibiting a LCST of approximately 32°C in water [**111**]. Below the LCST, hydrogen bonding between the polymer and water dominates; above the LCST, intramolecular hydrogen bonding becomes favorable, leading to the hydrogel formation. Other polymers such as chitosan and its derivatives may be pH responsive. Chitosan-based polymers can be designed to possess a different acid dissociation constant (i.e., pKa value) so that the environmental pH may be used to induce hydrogel formation [**112,113**]. The phase transformation of these materials may be used to control surface mineralization. In several studies by Mano and colleagues [**111,114**], temperature-sensitive PNIPAAm and pH-sensitive chitosan hydrogels were used to modify composite membranes consisting of PLLA and Bioglass®. Results showed that the phase transition as a result of either temperature or pH change triggered significant mineral formation on the material surface.

There exist other types of smart materials that may react to the change of the microenvironment. Shape-memory polymers are a class of biomaterials that have attracted considerable research interests in the biomedical engineering field [**115,116**]. Specifically, shape-memory polymers are able to "remember" one or more shapes, each determined by network elasticity. These polymers are able to adapt temporary shapes by material immobilization and return to their original shapes once the external stimulus is removed. Thornton et al. [**117**] fabricated chemically crosslinked alginate scaffolds with shape-memory properties. The lyophilized scaffold could be delivered in a minimally invasive manner to an in vivo site and regain its original hydrated structure after implanted to regenerate cartilage tissue (**Fig. 15.4E**).

The surface texture or morphology of a material is another useful designing parameter to make functional scaffolds for bone regeneration. These artificial matrices, when introduced into carefully designed miniaturized surface textures, may become intelligent and instruct bone formation in an in vivo environment. Habibovic et al. [**118,119**] investigated the osteoinductive properties of biphasic calcium phosphate (BCP) ceramics consisting of a different ratio of hydroxyapatite and tricalcium phosphate. Different types of BCP materials used in the studies were identical in macroporosity but varied in microporosity. The high-microporosity materials exhibited a considerable amount of micropores in the micron and submicron range as compared with the low-microporosity materials. As a result, the BCP material with microporous texture induced bone formation whereas BCP with very low microporosity did not in a goat intramuscular model. Thus, it was concluded that the microporous surface texture rendered the BCP material osteoinductivity. Other evidence also suggested that appropriate macro- and microstructure could play an important role in the osteoinductivity of materials. In a study by Fujibayashi et al. [**120**], macroporous titanium scaffold was chemically treated with sodium hydroxide followed by a series of thermal treatments to induce microporous structure on the metal surface. The treated bioactive titanium was able to induce bone formation without the need of additional osteogenic cells or osteoinductive agents after implanted in the dorsal muscle of dogs for 12 months.

Nanotechnology

Nanotechnology as a field emerged in the 1980s after the publication of the book *Engine the Creation: The Coming Era of Nanotechnology* by Drexler and Minsky. Since then, nanotechnology has captured significant interests and has grown explosively. A general description was established by the National Nanotechnology Initiative, which defines nanotechnology as the manipulation of matter with at least one dimension sized 1–100 nanometres. In biomedical research, nanotechnology involves the visualization, manipulation, and fabrication of materials on the smallest scale, in dimensions of 1 nm up to 1000 nm [**121**].

Nanoscale materials can be in different forms, such as nanoparticles, nanofibers, nanospheres, nanotubes, nanogels, nanocapsules, and surfaces with nanotopography. These nanostructures can be prepared using various biofabrication methods such as electrospinning, spray drying, phase separation, molecule self-assembly, chemical vapor deposition, and nano-imprinting. Natural bone itself is a nanocomposite material composed mainly of hydroxyapatite nanocrystallites in an organic collagen-rich matrix. The individual collagen helical chains are 10 nm in length and self-assemble into oriented collagen fibers measuring approximately 500 nm in length. The mineral/inorganic component of bone, hydroxyapatite, exists as plate-like nanocrystals, measuring 20–80 nm in length. In addition, the triple helical structure of collagen provides bone with a structural framework, high tensile strength, and flexibility whereas nanocrystalline hydroxyapatite accounts for the stiffness and high compressive strength of bone [**121**]. In regenerative engineering, nanofabrication aims to recreate a nanoscale environment resembling the native ECM in which cells reside, grow, and make bone.

Because of their similarity to the natural ECM of bone tissue, polymeric nanofibers are of great interest for use as scaffolds to regenerate bone [**122**]. They are characterized by ultra-thin continuous fibers, high surface-to-volume ratio and porosity, and variable pore size distribution. Electrospinning is an attractive technique to fabricate nanofibers from various biodegradable polymers because of the ease of fabrication, efficient control over the process, and easy scale-up [**123**]. In electrospinning, polymeric nanofibers result from a jet of polymer solution under the influence of an applied electrical field between an ejecting needle and a collector [**124**]. A suitable polymer solution viscosity is critical to fabricate nanofibers without any beads or beads-on-a-string appearance. Laurencin and colleagues were the first to demonstrate that electrospun nanofibrous structure could serve as a novel scaffold to engineer tissues [**125**]. This pioneering work has been recently featured as 1 of the top 25 papers published by the *Journal of Biomedical Materials Research* over the past 50 years. Laurencin et al. have then investigated several electrospun biodegradable nanofibrous scaffolds using polyphosphazenes, PLAGA, and poly (epsilon-caprolactone) for wound healing [**126**], drug delivery [**127**], and regenerating soft [**128–134**] and hard tissues [**135**]. Furthermore, polyphosphazene-nanohydroxyapatite composite [**136**] and polyphosphazene-PLAGA blend [**137**] nanofiber scaffolds as well as composite scaffolds

comprising PLLA nanofibers and sintered microspheres [138] have been investigated for bone tissue engineering applications.

Stem Cell Science

Stem cells in bone tissue engineering have been one of the most promising areas of research that provide advances in clinical application to cure bone defects. Different types of stem cells including embryonic stem cells (ESCs), bone marrow-derived MSC (BM-MSCs), umbilical cord blood-derived MSC (UCB-MSCs), ADSCs, muscle-derived stem cells (MDSCs), and dental pulp stem cells (DPSCs) have been receiving attention in the bone tissue engineering field because of their distinct biological capability to differentiate into osteogenic lineage. Various experimental strategies are able to direct the differentiation of ESCs, BM-MSCs, UCB-MSCs, ADSCs, DPSCs, and MDSCs toward osteogenic lineage and their probable applications in regeneration of bone in the field of tissue engineering.

ESCs can be derived from the inner cell mass of the blastocyst. The cells have an unlimited activity of self-renewal and are able to give rise to any cell linage, which is one of the most useful characteristics of ESCs in tissue engineering and regenerative medicine. The specific lineage differentiation of ESCs can be done under specific culture conditions and by the manipulation of the microenvironment. Recently, there has been devoted attention to direct ESC differentiation into osteogenic lineage. In research, it has been demonstrated that osteogenic lineage cells with bone-forming capacity are derived from the somatic mesoderm or the ectomesenchymal cells of the neural crest. The differentiation has been characterized by distinct osteogenic gene expression profiles, mineralization activity, and animal studies [139]. These scientific findings have major implications for bone tissue engineering, in which ESCs can be used for fabrication of tissue-engineered bone in vitro.

BM-MSCs are the most frequently used cell source for bone tissue engineering. The bone marrow contains MSCs, which are able to contribute to the regeneration of mesenchymal tissues such as bone, cartilage, tendons, muscle ligaments, stroma, and adipose. The isolation process of MSCs from bone marrow had been based on mainly three methods: (1) acquiring only adherent cells on tissue culture plates after plating, (2) acquiring STRO-1- or CD105-positive cell populations, and (3) acquiring CD45- or Gly-A negative cell populations. BM-MSCs can be directed toward osteogenic differentiation if it is under the proper culture conditions containing certain exogenous factors. BM-MSCs are able to be harvested from the patient's own bone marrow, expanded in vitro, and then induced to differentiate toward osteogenic cell lineage, which is followed by the mineralized bone formation [140]. Borden and Laurencin et al. investigated the use of autologous bone marrow combined with an osteoconductive PLAGA sintered microsphere scaffold and osteoinductive BMP-7 to regenerate a segmental bone defect in a rabbit model. It was found that the addition of autogenous marrow increased the penetration of new bone into the central area of the scaffold

and increased the degree of revascularization. The combination of osteoinductive growth factor BMP-7 and bone marrow cells induced penetration of new woven bone throughout the entire structure of the implant and the formation of mature lamellar bone (**Fig. 15.5**) [**56**].

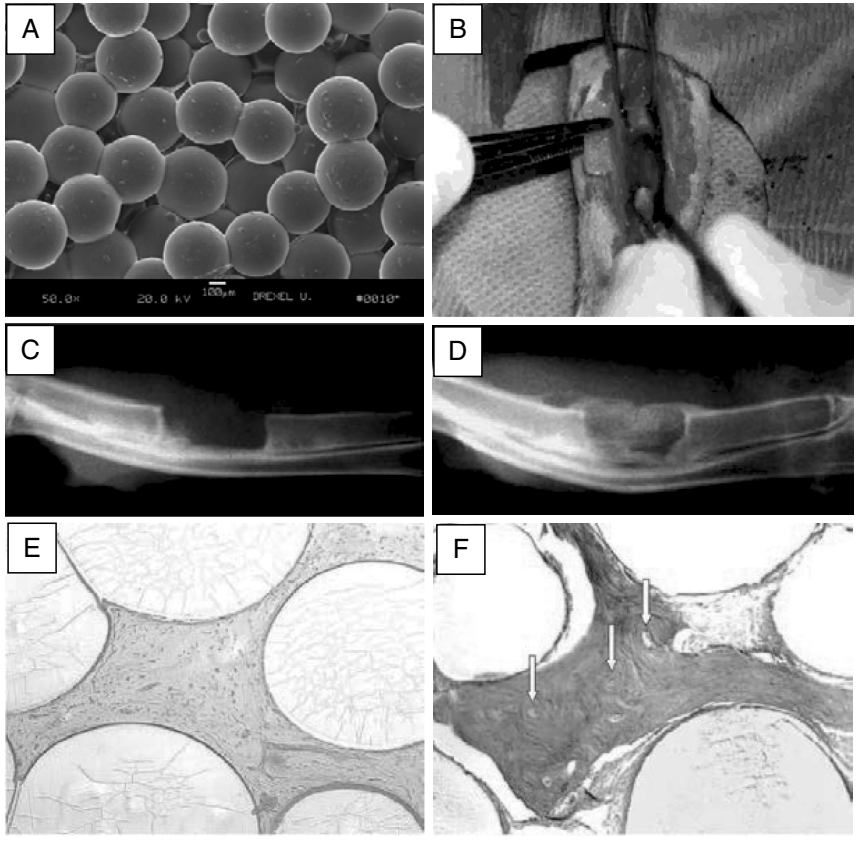
UCB-MSCs have been used as a source for hematopoietic stem cells. The process for UCB-MSC isolation collects umbilical cord blood from patients using heparinized tubes and proper salt-based solutions. The mononuclear cells are centrifuged to be able to collect the interface layer and diluted with phosphate buffered saline followed by further centrifugation. Finally, the cells are seeded into tissue culture flasks and cultured for a few days, after which only the adherent cells are purified. The isolated UCB-MSCs can be directed to differentiate into specific lineage cells. Many researchers have focused on the interaction of UCB-MSCs and biomaterials, and isolated UCB-MSCs loaded onto suitable biomaterials-based 3D scaffolds [**141**]. The combined constructs have been transplanted into immune-deficient animal models to evaluate their in vivo bone formation capacity. These findings have proposed the possibility of UCB-MSCs as a potent cell source for bone tissue engineering and the regeneration of bone tissue.

ADSCs come from adipose tissue, which is known to be a highly active metabolic and endocrine organ that produces various hormones and cytokines. When ADSCs are isolated, they can display multipotent differentiation capacity to differentiate into numerous cell types such as chondrocytes, osteoblasts, neural cells, adipocytes, cardiomyocytes, and endothelial cells depending on the specific culture conditions. Recently, ADSCs have been used by multiple researchers for bone tissue engineering applications to demonstrate that ADSCs show good adhesion, proliferation activity, and homogenous bone-like tissue formation on a range of biocompatible 3D scaffolds [**142**].

MDSCs have been reported to have multipotent differentiation capacity toward bone, skeletal muscle, and cartilage as well as neural, hematopoietic, and endothelial tissues. Isolated MDSCs from skeletal muscle tissues can be directed to differentiate into specific lineage cells when they are under the proper culture conditions. The isolated MDSCs were implanted with a combination of collagen membrane into a critical-sized cranial defect site of a normal rat, leading to bone tissue reconstruction [**143**]. These scientific findings propose a potent clinical use of MDSCs for bone tissue regeneration.

DPSCs are a recent finding as a potential cell source for bone tissue engineering. DPSCs are isolated from the digestion of pulp tissue, which allows the cells to display clonogenic and highly proliferative characteristics and illustrate the typical immunoreactivity profile as BM-MSCs. In developing teeth, dental mesenchyme was found by tracing genetically modified neural crest-derived cells that were in mouse models [**144**]. DPSCs are able to be isolated from digesting pulp tissue by a single colony selection and an immunomagnetic isolation method. DPSCs have been suggested to be a promising tool for bone regeneration [**145**].

FIG. 15.5 (A) A sintered PLAGA microsphere scaffold with porous structures allowing for infiltration of autologous bone marrow stromal cells and loading of osteoinductive growth factor BMP-7. (B) The bone marrow and BMP-7 loaded PLAGA scaffold was implanted into a 15-mm segmental bone defect in rabbit ulna. (C) A representative radiograph showing limited bone formation at the corner of the implant in the scaffold-only group at 8 weeks. (D) A representative radiograph showing robust bone formation throughout the scaffold in the scaffold plus bone marrow and BMP-2 group at 8 weeks. (E) Hematoxylin and eosin staining showing only fibrous tissue formation within the pore system in the scaffold-only group at 6 weeks. (F) Hematoxylin and eosin staining showing robust bone formation within the pores in the scaffold plus bone marrow and BMP-7 group. The woven bone found within the matrix showed signs of remodeling into mature lamellar bone (indicated by the white arrows). Source: Figures reproduced with permission from [**56**].



Bone Developmental Biology

The development, growth, maintenance, and healing of the skeleton require that bone be formed throughout life. The process of bone formation can first be recognized when undifferentiated MSCs or osteoprogenitor cells commit to the osteoblast lineage and begin to secrete a specialized ECM. This matrix mineralizes, and osteoblasts, surrounded by the mineralized matrix, become osteocytes. The appearance of osteoclasts begins the remodeling process that converts immature woven bone into mature lamellar bone. Bone development may be achieved through two distinct pathways: intramembranous bone formation and endochondral bone formation.

Intramembranous formation is initiated by the aggregation of undifferentiated MSCs into layers or membranes. These cells synthesize a loose organic matrix that contains blood vessels, fibroblasts, and osteoprogenitor cells. The osteoprogenitor cells differentiate into osteoblasts and deposit spicules and islands of organic bone matrix that then mineralize. Osteoblasts cover the surfaces of the spicules and islands and rapidly add new bone matrix. Osteoblasts caught within the bone matrix assume the form of osteocytes. Transcription factors Runx2 and Osterix with a zinc-finger motif are required for osteoblast differentiation.

Endochondral ossification that leads to the formation of embryonic long bones begins with the aggregation of undifferentiated cells that secret a cartilaginous matrix and differentiate into chondrocytes. After formation of the hyaline or hyaline-like cartilage, a periosteal covering appears around the diaphysis and begins to form a thin collar of bone. Some regions of the cartilage matrix mineralize, the chondrocytes enlarge, vascular buds invade the cartilage, and invading cells resorb the central portion of the cartilage, creating a marrow cavity. Osteoprogenitor cells accompanying the vascular buds then differentiate into osteoblasts and form a bone matrix on the mineralized cartilage. BMP signaling is likely required for endochondral ossification. Sox9 and its related molecules Sox5L and Sox6 play an important role in the commitment and maintenance of chondrocyte phenotype. The differentiation of early periarticular chondrocytes into flat, columnar proliferating chondrocytes and then into hypertrophic chondrocytes is likely to be regulated by various signaling molecules such as Indian hedgehog (Ihh), Wnts, and PTHrP. Osteoclasts, which develop from monocytic precursors of the hemopoietic lineage, then resorb the calcified cartilage and immature bone, and osteoblasts replace the mixture of calcified cartilage and immature bone with mature lamellar bone. In the differentiation of hematopoietic stem cells toward osteoclasts, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) are essential signaling molecules for osteoclastogenesis. In addition, microphthalmia-associated transcription factors (MITFs), nuclear factor κB (Nf-κB), Fos/Fra1, and nuclear factor of activated T cells-1 (NFATc1) are required for osteoclast differentiation [**146**].

A thorough understanding of the biology of bone development and bone fracture repair and the involved signaling molecules and signaling pathways will open new opportunities for investigators to further improve bone tissue engineering approaches.

Protein kinase A (PKA) plays an important role in mesenchymal cell fate decision. Specifically, the activated form of PKA leads to the phosphorylation of a large variety of downstream target proteins including various transcription factors that, in turn, ultimately regulate numerous cellular events. Siddappa et al. [**147**] exposed human MSCs to the PKA activator N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate (db-cAMP) to activate the PKA signaling pathway. It was found that the activation of PKA elicited an immediate response through induction of genes such as *ID2* and *FosB*, important for osteogenesis, followed by sustained secretion of bone-related cytokines such as BMP-2, IGF-1, and interleukin-11. As a result, PKA activation led to robust in vivo bone formation.

In one of the authors' own laboratories, a cAMP-dependent PKA-specific cAMP derivative, N^6-benzoyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP) was also shown to promote in vitro osteoblastic differentiation in osteoblast-like MC3T3-E1 cells and MSCs [**148**]. Thus, one of the strategies in bone tissue engineering moving forward is to identify functional molecules from developmental biology that, alone or in concert with others, promote bone tissue regeneration.

Convergence

Tissue engineering, advanced material science, and stem cell science have been the frontiers of biomedical research over the past 25 years, aiming particularly to repair or regenerate individual tissues and organs. Science and technology have greatly advanced since the term "tissue engineering" was coined in 1987, and they are reflected in the evolving strategies of tissue engineering and regenerative medicine. As we move forward, the importance of convergence research integrating the aforementioned three areas with seemingly disparate disciplines of physical sciences and developmental biology has been revealed.

The past several decades have seen the rapid progress of many disciplines in physical sciences, life sciences, and engineering, which have great impact on advancing medical therapies toward improving human health care. Leveraging the flexibility of organic synthesis, polymer scientists have been able to design and synthesize many polymer materials with varying chemical and biological properties based on a finite selection of monomers. Recent advances in the areas of stem cells and developmental biology have provided invaluable tools to biologists and practical therapies to clinical physicians and patients. In addition, borrowing engineering principles from other fields, biomedical engineers have initiated many important interdisciplinary areas such as nanomedicine, nanofabrication, biomechanics, bioimaging, biosensing, and bionics. The time has come for a convergence. In the field of biomedical engineering research, it is a convergence that integrates traditional tissue engineering with advanced materials science, stem cell science, and developmental biology—a new field that we term "regenerative engineering" [**149**]. We believe that the regenerative engineering approach will lead us to new solutions for regenerating complex human tissues such as long bones and limbs and will lead to personalized medicine in the future.

FUTURE PERSPECTIVES

The emergence of regenerative engineering empowers tissue engineers with invaluable tools to achieve bone regeneration using biomaterials, cells, and growth factors/signaling molecules. In the realm of regenerative engineering, we believe that future research should focus on advanced materials science, stem cells, developmental biology, and strategies to integrate these components into a functional biological system. In advanced materials science, technologies to synthesize a large array of biomaterials with varying chemical, physical, and biological properties and to test the biomaterials in a high-throughput manner have been advanced [**102**]. These technologies should apply to the bone tissue engineering field to aid us in designing and selecting candidates as functional scaffolding materials. In addition, to best mimic the unique structure and properties of natural bone tissue, a bioinspired approach to creating biomaterials with specific micro- and nanoscale topographical features, macroscale gradient structures, and biological domains to interact with target growth factors and cells is yet to be realized. In stem cell science, many great discoveries have been made recently. For example, pluripotent stem cells were induced from somatic cells by certain small-molecule compounds [**150**], virtually providing an unlimited stem cell resource for regenerative medicine. Because stem cell fate is precisely controlled by several factors such as the matrices, signaling molecules, and forces generated by the microenvironment and the cells themselves, interactions between stem cells and the artificial milieu that cells sense should remain to be an area of importance. Furthermore, bone development and bone fracture healing involve many signaling pathways with the participation of many growth factors and signaling molecules. Thus, pharmacological manipulation of the signaling pathways with small molecules represents an area of importance to enhance the regeneration of segmental bone defects.

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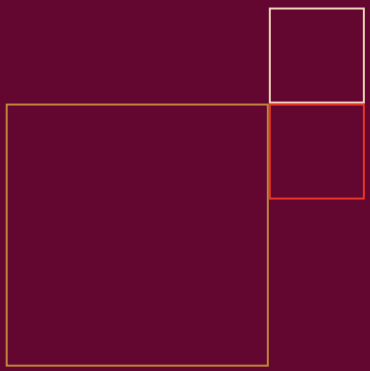

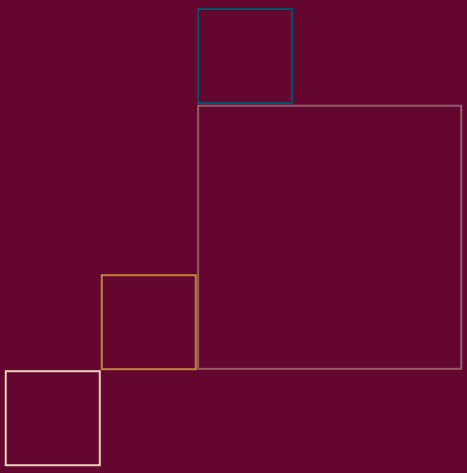

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