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Tissue Eng. Author manuscript; available in PMC 2010 February 12.

Published in final edited form as:
Tissue Eng. 2006 December ; 12(12): 3307. doi:10.1089/ten.2006.12.3307.

Engineering Complex Tissues

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Abstract

This article summarizes the views expressed at the third session of the workshop "Tissue Engineering —The Next Generation," which was devoted to the engineering of complex tissue structures. Antonios Mikos described the engineering of complex oral and craniofacial tissues as a "guided interplay" between biomaterial scaffolds, growth factors, and local cell populations toward the restoration of the original architecture and function of complex tissues. Susan Herring, reviewing osteogenesis and vasculogenesis, explained that the vascular arrangement precedes and dictates the architecture of the new bone, and proposed that engineering of osseous tissues might benefit from preconstruction of an appropriate vasculature. Jennifer Elisseeff explored the formation of complex tissue structures based on the example of stratified cartilage engineered using stem cells and hydrogels. Helen Lu discussed engineering of tissue interfaces, a problem critical for biological fixation of tendons and ligaments, and the development of a new generation of fixation devices. Rita Kandel discussed the challenges related to the re-creation of the cartilage-bone interface, in the context of tissue engineered joint repair. Frederick Schoen emphasized, in the context of heart valve engineering, the need for including the requirements derived from "adult biology" of tissue remodeling and establishing reliable early predictors of success or failure of tissue engineered implants. Mehmet Toner presented a review of biopreservation techniques and stressed that a new breakthrough in this field may be necessary to meet all the needs of tissue engineering. David Mooney described systems providing temporal and spatial regulation of growth factor availability, which may find utility in virtually all tissue engineering and regeneration applications, including directed *in vitro* and *in vivo* vascularization of tissues. Anthony Atala offered a clinician's perspective for functional tissue regeneration, and discussed new biomaterials that can be used to develop new regenerative technologies.

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INTRODUCTION

Engineering complex tissues is perhaps the most ambitious goal of all tissue engineers. Engineering of functional vascular networks, interfaces, structural hierarchy, and complex functional features is emerging as an unparalleled scientific and technical challenge for the next generation of tissue engineers. This translates into the need to establish compositional gradients or subcompartments, temporal changes, and the use of cells to drive tissue and organ morphogenesis. An additional level of complexity is imposed by the orientation toward a "custom designed," individualized approach to defect repair that takes into account multiple additional requirements. New generations of interactive biomaterial scaffolds and bioreactors are now being developed to emulate features found *in vivo* and to bring this ambitious goal closer to reality. The fields of "adult biology," associated with tissue remodeling; transplant surgery; and immunology are also essential for supporting the efforts of tissue engineers in this direction. To noninvasively monitor the maturation and remodeling of such grafts by molecular imaging techniques, new sets of biomarkers may need to be developed. Efforts are under way to develop new modalities and to improve the existing ones for preservation of component cells and engineered tissues, in order to make tissue grafts available to patients in a timely fashion.

ENGINEERING OF COMPLEX ORAL AND CRANIOFACIAL TISSUES

Antonios Mikos from Rice University discussed tissue engineering approaches to dental, oral, and craniofacial rehabilitation for millions of people, as well as significant challenges that need to be addressed before successful clinical applications can be realized. The current paradigm that recognizes the complex interplay between biomaterial scaffolds, growth factors, and local cell populations during healing has been consistently utilized to design constructs that attempt to promote these interactions to restore the original architecture and function of complex tissues. Nevertheless, issues related to each component of this paradigm reflect the fact that "ideal" tissue engineering techniques are yet to be developed for the regeneration of complex composite structures such as alveolar bone, periodontal ligament, and teeth.

Part of the difficulty in designing constructs exposed to the oral environment relates to a unique set of interactions involved in the regenerative process in contrast to other parts of the body (Table 1). Augmented healing of alveolar extraction sites, where bone regeneration materials implanted into a tooth extraction socket encounter an environment very different from that of healing long-bone fractures, provides an excellent example. Interactions between the implanted material and the oral flora occur within an essentially "contaminated" wound-healing site, and a progenitor cell population responsible for bone healing is believed to be derived from remnants of the periodontal ligament, rather than from periosteum or endosteum. Further, reconstitution of a healed extraction socket involves the coordinated regeneration of both bone and epithelium. Thus, tissue engineering approaches to augmented healing of alveolar sockets have attempted to recapitulate the temporal and spatial relationships of growth factors and cells with the healing environment of the oral cavity, and constructs should be designed with these considerations in mind.

Biomaterial scaffolds

Biomaterials and growth factor delivery systems used for alveolar bone engineering have several fundamental shortcomings. From an anatomical standpoint, an ideal scaffold for bone regeneration should be injectable so that it conforms to the irregularly shaped defects produced by tooth extraction. Next, it should undergo an increase in mechanical stiffness, to mimic the mechanical properties of bone, in order to sustain the physiological loading conditions imposed by mastication. The scaffold material should also degrade at a rate complementary to bone formation so as to preserve the morphology of the alveolar ridges—an important consideration

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for the prosthetic rehabilitation of the patient. If the material degrades too quickly, the resultant defect will compromise denture construction or dental implant placement. A slower rate of degradation will reduce tissue in-growth and regeneration.

Although moldable polymeric materials exist, which upon cross-linking exhibit compressive and tensile strengths close to that of bone, these materials do not degrade on a timescale suitable for optimal bone healing. In addition, these materials are hydrophobic, which renders them unsuitable for cell encapsulation. Conversely, numerous synthetic and biocompatible materials capable of forming hydrogels are available. These degrade quickly and promote tissue in-growth, but their weak mechanical properties require rigid fixation devices to stabilize mechanically unstable bone defects. Thus, the challenge is to create an injectable biomaterial that has mechanical properties similar to bone, yet degrades quickly enough to support tissue in-growth, and allows for cell encapsulation.

Aside from the mechanical properties of a scaffold, the interactions between cells and the biomaterial surface also need to be addressed. While design improvements to biomimetic scaffolds have allowed an increase in cell adhesion, the adhesion of specific cell populations by altering scaffold properties has yet to be perfected. Surface properties are especially important in periodontal ligament regeneration, where successful formation of the periodontium and surrounding alveolar bone also requires the prevention of epithelial down-growth into the periodontal defect site. An ideal periodontal regeneration material should promote the attachment and in-growth of both osteogenic cells and periodontal ligament fibroblasts, while excluding epithelial cells from the periodontal defect.

Growth factor delivery

Another important area in tissue engineering is growth factor delivery. Numerous methods exist for controlled delivery of bioactive growth factors in bone and periodontal regeneration applications. To ensure sufficient bioavailability to the regenerating tissues, growth factors need to be loaded into scaffolds in concentrations that far exceed those normally seen in the body. Gene therapy might be able to address these issues using a different approach. Successful transfection of cells within the healing environment would up-regulate growth factor production and raise the concentration to required levels. However, viral gene delivery vehicles run the risk of becoming pathogenic and are limited in the size of genetic material they can carry, while nonviral gene delivery vehicles tend to be inefficient at gene transfection and are cytotoxic at high enough concentrations. Thus, from a cost perspective, more efficient growth factor delivery systems are needed, especially if such constructs are to be used successfully in clinical practice.

An equally important challenge in growth factor delivery for the engineering of complex tissues is control over the kinetics of release. Much research has focused on the characterization of key growth factors within the healing cascade and their temporal and spatial expression within complex tissue defects. However, the ability to direct the release of numerous growth factors on multiple timescales will determine the success of a tissue engineering scaffold in recapitulating the complex wound-healing environment seen in oral and craniofacial tissues.

Scale-up

Issues of scale-up present additional challenges. Tissue engineering strategies that prove to be successful in small experimental defects may fail in larger human applications where nutrient diffusion into the center of an extensive cell-based construct may be limited during the initial stages of healing before any appreciable angiogenesis takes place. Investigators must also consider the possibility of differences in physiology between animal and human subjects, and

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whether or not potentially promising results obtained in animal models can be reproduced in the clinical setting.

Animal models—With this consideration in mind, improvements in tissue engineering strategies require the development of effective *in vivo* models for the assessment of implanted constructs. While both cranial cavitational defect models and long-bone segmental defect models are commonly employed to test bone regeneration materials in small rodents, they fail to re-create the complex wound-healing environment found in the oral cavity. A model needs to be developed that can create an easily reproducible critical-size defect, which communicates with the oral cavity. The animal chosen must be large enough to make surgery practical, and reasonably inexpensive to permit studies with sufficient statistical power. In addition, the ability to quantify both the angiogenic and osteogenic potential throughout the volume of the surgical defect is essential.

Future directions and potential for dental and craniofacial tissue engineering

While advances in tissue engineering have created a variety of approaches toward both alveolar bone and periodontal ligament regeneration, an ideal material has yet to be developed. Additional clinical challenges that must be addressed include the ability to control the morphology of bone formed by an implanted biomaterial. For instance, the alveolar bone present in the posterior maxilla tends to have a larger proportion of cancellous bone when compared to the anterior mandible, which has implications for the primary stability of titanium implants placed in that location. There currently exists no synthetic biomaterial capable of controlling the architecture of bones generated *de novo* within a bony defect. Another challenge is to create dental implant systems for tooth replacement that can regenerate a periodontal ligament attachment to the surrounding alveolar bone and form a tight gingival seal between the implant surface and the oral mucosa. Finally, one of the most difficult challenges is to regenerate an entire tooth. Although some attempts have been made in this direction, they have been rarely successful. The ability to regenerate a complete tooth and its periodontal ligament within an extraction socket would be an extraordinary achievement, and would surely revolutionize the practice of dentistry.

Dr. Mikos concluded that continuing developments in the field of tissue engineering would undoubtedly lead to improvements in biomaterial scaffolds for cell-based therapeutics and the delivery of bioactive molecules such as growth factors and DNA. He also emphasized that the success of tissue engineering strategies would rely on the ability of the implanted construct to control the proliferation and differentiation of specific cell populations within the wound-healing site, using materials that are both cost effective and easy to manipulate in the clinical setting. In this way, tissue regeneration can be optimized in the complex wound-healing environment of the oral cavity.

OSTEOGENESIS AND THE VASCULATURE: SHOULD THE SCAFFOLD COME FIRST?

Susan W. Herring from the University of Washington explained that the vascular arrangement of the overlying periosteum precedes and dictates the architecture of the new bone, and proposed that engineering of osseous tissue might benefit from preconstruction of an appropriate vasculature and scaffolds with pore size and surface architecture conducive to the growth of vessels.[1,2] She emphasized that bone is completely dependent on its vasculature. Vessels provide nutrients and minerals to osteogenic sites, provide access for osteoclasts and other cells, and, possibly through their accompanying pericytes, supply a source of mesenchymal osteoprogenitors. Beyond the requirements of homeostasis, however, there is a morphogenetic connection. Vascular cells have dramatic inductive osteogenic effects,[3] and, at

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the same time, processes that stimulate osteogenesis induce angiogenic factors.[4] The association between membranous ossification and the preexisting vascular pattern in the connective tissue matrix is so anatomically close that one early worker referred to the process as "angiogenic ossification"[5] (translated by Inoue *et al*.[6]) and another described the bone as "modeling itself along the course of the artery."[7] Arnold Caplan, a founding father of modern tissue engineering, described the vasculature as the "orientor of osteogenesis" *in vivo*,[8] and his illustrations make clear that it is the vessels of the periosteum that provide the (negative) model for newly apposed layers of bone.

If it is generally true that the normal developmental sequence is for mineralized matrix to be patterned by a pre-existing vasculature, then the present practice violates the precept that tissue engineering works best if it mimics ontogeny. Manufacturing the matrix first may not be the most efficacious way to produce a well-perfused graft. Instead, the vascular network should be prepared first, from which living, remodeled woven bone could be produced naturally.

To establish that normal skeletal growth proceeds from vasculature to bone, rather than the reverse, Herring and colleagues assessed the role played by the periosteal vasculature in organizing the newly deposited matrix of growing craniofacial bones in a pig model. Piglets, 2–6 weeks old ($n = 5$), were anesthetized, injected intravenously with calcein (12.5 mg/kg; Sigma Aldrich, St. Louis, MO), and then perfused with heparinized saline followed by a silicone rubber–based vascular fill (Microfil®; Flow Tech, Carver, MA). The head was frozen overnight, and the zygomatic arch was cut into blocks and dehydrated and infiltrated with plastic embedding media. Cured specimens were thick-sectioned (50–1000 μm) and examined under epifluorescent illumination to compare the calcein-labeled matrix (mineralized in the last 3 hours of life) to the periosteal vasculature. Additional material included periosteal whole mounts from animals injected with Microfil® and histological sections of decalcified arches from piglets that had received injections of bromodeoxyuridine 3 hours before sacrifice.[9]

Different portions of the periosteum of the zygomatic arch were strikingly different in osteogenic activity. The medial surface was almost devoid of calcein label. Histological observations confirmed that the medial surface was not appositional, and was indeed being actively resorbed. The lateral surface showed extensive deposition of calcein, but the pattern was different for the two bones that comprise the arch, reflecting their different structures.[10] The more anterior zygomatic bone (Fig. 1) showed a continuous surface layer of calcein, approximately 100 μm thick, whereas the more posterior temporal bone (Fig. 2) exhibited calcein extending the tips of bony spicules by approximately 250 μm.

As has been described for the periosteum of limb bones,[11] the nutrient vessels of craniofacial periosteum were different from those of the bone-marrow spaces. Relatively large-caliber vessels were found within the plane of the periosteum, parallel to the bone surface. Beyond this general relationship, vascular pattern varied with region.

Despite its resorptive character, the medial periosteum was well vascularized with very large vessels close to the bone surface. Histological sections suggested that these vessels were composites of fusing smaller vessels and that they were continuous with the marrow circulation in the resorbing medial bone. In contrast, the largest vessels of the appositional lateral periosteum were more peripheral in the fibrous layer at some distance from the bone surface. These large vessels, which stemmed from nearby muscles and ligaments, formed an anastomosis in the plane of the periosteum. Closer to the bone surface, small-caliber vessels formed another planar network, interconnected with the large, superficial one. Only very small vessels penetrated the bone surface, and these frequently showed signs of angiogenesis, such as sprouts and endothelial tubes.

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Both the zygomatic and the temporal bone showed an intimate association between newly mineralized matrix and vascular architecture. In thick sections, labeled matrix could even be visualized as tubes around blood vessels (Fig. 3). However, only for the zygomatic bone was the vascular architecture that of the periosteum. Here the accreting matrix surrounded the deep periosteal network, becoming the negative image of the vasculature (Fig. 1). On the temporal bone, the mineralizing, elongating spicules were nested between radiating vessels that were perpendicular to the periosteal plane. Rather than coming from the periosteum, these vessels were clearly continuations of the intraosseous vasculature (Fig. 2).

In growing craniofacial bones, the relationship between new matrix and vascular architecture was close enough to justify the idea that vascular pattern dictates where mineralized matrix is laid down. However, the significant vasculature is not necessarily that of the periosteum. In the slower-growing zygomatic bone, the deep periosteal network did appear to be engulfed by mineralizing matrix and thus patterned the woven bone into its negative image. But in the faster-growing temporal bone, the appearance was that of radially oriented intraosseous vessels co-opting and transforming the deep periosteal network.

Susan Herring concluded that from a tissue engineering point of view, the important feature is that, in both cases, bone formed around the vessels. Osteogenesis can be organized either by internal vessels, as in the temporal bone, or by external vessels, as in the zygomatic bone. Thus, it may not be necessary, or even advisable, to engineer three-dimensional porous scaffolds for bone; rather, a vasculature should be constructed. Further, the vascular network need not be three-dimensional if it is osteogenic. A two-dimensional periosteum creates a three-dimensional bone. A two-dimensional vascular graft might be especially suitable for the membrane bones of the skull, which lack cartilage predecessors and are entirely formed and shaped by their periostea.

ORGANIZATION AND INTEGRATION OF ENGINEERED CARTILAGE

Jennifer Elisseeff from Johns Hopkins University discussed stem cell research in the context of musculoskeletal tissues and in connection with novel engineering tools that are being developed to improve tissue engineering techniques and facilitate their eventual clinical translation. She suggested that stem cells and novel enabling technologies are critical for future tissue engineering research.

The discovery of stem cells and the development of novel engineering technologies have infused energy into the field of tissue engineering and stimulated new advances that will further understanding of the tissue development process and promote clinical translation. Stem cells address a major challenge in tissue engineering—procurement of adequate cell numbers to place on a scaffold.[13] The cell is the building block of the developing tissue, proliferating and producing the desired tissue-specific extracellular matrix (ECM). Cells isolated from tissue biopsies oftentimes have limited expansion capabilities, and instead readily differentiate and secrete the tissue-specific proteins. The problems arise from the fact that during expansion these differentiated cells often change phenotype and lose their capability to form tissue.[14] In contrast, stem cells have the capacity to proliferate in an undifferentiated state, providing a potentially large source of cells.[15] The subsequent challenge, though, is to differentiate the stem cells during the tissue engineering process. In the case of adult stem cells, in particular, bone marrow–derived mesenchymal stem cells (MSCs), many of the signals (e.g., growth factors) required for tissue-specific differentiation have been elucidated.[16] In the case of embryonic stem cells (ESCs), manipulating or controlling differentiation remains a significant challenge.

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Stem cells

Stem cells are characterized by their ability to proliferate significantly in an undifferentiated state and, under appropriate conditions, to differentiate along one or more lineages. The discovery of these cells has stimulated significant excitement in the field as large cell numbers are required to seed the three-dimensional scaffolds, particularly if the scaffolds are the size of clinically relevant defects.[17]

Adult stem cells can be isolated from a number of tissues, most notably, the bone marrow.[18] Researchers continue to identify other sources of adult stem cells, including fat, cardiac, neural, limbus, bone, and muscle tissues. While there is still some debate over identity and classification, adult stem cells can be characterized by surface markers and their functional differentiation. The ESCs can be isolated from the inner cell mass of the blastocyst and the primordial gonadal ridge of the fetus[19,20] These cells are cultured on fibroblast feeder layers to maintain them in an undifferentiated state, for potentially very long periods of time. New feeder-free stem-cell culture systems are under development by a number of groups.

The proliferative capacity of the ESCs is intriguing for researchers in regenerative medicine who continually battle the challenge of having enough cells to combine with biomaterials to build new tissues. Differentiation of ESCs is usually initiated by formation of embryoid bodies (EBs), clusters of cells differentiating into all three germ layer lineages.[21] A number of researchers are selecting specific cell types or groups of cells from EBs to apply to tissue repair.[22] While these proliferation and differentiation characteristics lead one to assume unlimited capacity for ESCs to make any tissue in the body, we are now just touching the surface on how to control these cells and the unique requirements that we must elucidate to induce tissue development. A number of problems remain to be solved before stem cell research can be translated into clinical practice.

Tissue engineering of cartilage provides one important venue to evaluate a number of cell types: fully differentiated chondrocytes from cartilage, adult stem cells, and ESCs.[23–28] Figure 4 pictures cartilage engineered from chondrocytes isolated from cartilage tissue, bone marrow–derived stem cells, and cells derived from EBs made from human and mouse ESCs. While the base hydrogel scaffold was similar in all cases, embryonic cells required unique conditions for differentiation in each case.

Technologies: Understanding biology and engineering organized tissues

Biomaterials, along with cells, are a critical component of tissue engineering. The biomaterial scaffold provides a three-dimensional environment for cells and the developing tissue. Researchers are aiming to design scaffolds that can promote a desired cell behavior, including proliferation and differentiation.[29] This aim is challenging, since many of the signals required for stem cells, particularly ESCs, are unknown. This has led engineers to work with biologists to help discover more about the biology of stem cells and how they behave in complex tissue engineering scaffold systems.

Like many other tissues in the body, cartilage is structurally organized and contains a superficial, a middle, and a deep layer. The tissues in each of these layers are distinct with respect to matrix composition and organization and subsequent mechanical properties. The superficial layer contains more collagen than the other layers, and the fibers are aligned parallel to the joint surface. The deep layer of cartilage contains more proteoglycans and the collagen fibers are aligned perpendicular to the cartilage joint surface. The cells in the deep layer are larger compared to the superficial cells, which are smaller and flattened. The combination of these two layers and the intermediate third layer in between is what provides cartilage its unique mechanical properties and overall functionality as a smooth, lubricating surface that absorbs

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and transmits mechanical forces. Therefore, from a tissue engineering perspective, rebuilding the structure of even a seemingly simple tissue such as cartilage is critical to building more functional tissues. The need for organized structures applies to practically all tissues in the body, and these technologies may be applied in a similar manner to other tissue types.

Hydrogels can be used as scaffolds to suspend chondrocytes (and stem cells) in a three-dimensional environment for engineering cartilage.[30] Hydrogels are cross-linked polymer matrices capable of absorbing large amounts of water. Cells can be suspended in the liquid polymer solution before cross-linking. Light triggers the cross-linking process, and this enables a high degree of control over cross-linking—only when the solution is exposed to light does gelation occur. This technology was developed primarily to ease implantation, giving physicians control over gel scaffold formation *in vivo*.[31]

The light-based process is also useful for creating multilayered hydrogel structures. For example, a bilayered hydrogel can be created in a mold by placing one layer of partially gelled liquid polymer solution that forms a semi-solid, and then adding a second layer of liquid polymer solution to the first. The whole construct is then fully cross-linked to form a bilayered hydrogel. This technique can be extended to create more layers in the hydrogel. Different cell types can be encapsulated in each layer, without migration. Moreover, the interface of the hydrogel layers can be manipulated such that there is either a "clean" interface with no cell mixing, or a "fuzzy" interface with some degree of contact and mixing of the cells. Cells labeled with dyes encapsulated in a bilayered gel are pictured in Figure 5.

While the multilayered hydrogels are useful for creating tissues that resemble the normal architecture of tissue, they also provide a venue to study how cells "talk" to each other. Developmental biologists have for many years known the importance of cell organization, boundaries, and interfaces between cells in regulating tissue development. This concept can be applied to tissue engineering for enhancement of both tissue -formation rate and quality.

In both developmental biology and tissue engineering, little is known about how the cells in the different layers of cartilage "talk" to each other to regulate tissue composition. Biologists have determined a number of signals in limb development that may also be important in the multilayered hydrogel system. The application of multilayered, organized hydrogel scaffolds applies to the new realm of understanding how stem cells talk to other cells to regulate their own differentiation and also behavior of other cells. When chondrocytes isolated from the deep layer of cartilage were cocultured next to cells from the superficial layer, they decreased their proliferation rate and produced more ECM compared to when they were cultured alone. While we do not know all of the signals that were transmitted between the two cells, we can further investigate the mechanisms for the observed differences in cell function. Most important, it is clear that engineering biomaterial scaffolds to create organized tissue structures will be critical for functional tissue engineering and for understanding mechanisms of cell communication at the mm- and even the cm-length scales.

The hydrogel systems have also been used to study the behavior of mouse and human ESCs and human embryonic germ cells (Fig. 4). While we learn a great deal from working with fully differentiated cells and adult stem cells, the cell contact and matrix requirements for the above mentioned cells are all different. In all cases, the three-dimensional environment played an important role in differentiating the stem cells and even affected the cell response to growth factors.[25] Significant research still needs to be done to further understand the differences between these cell types.

There has been much interest in and debate over the role of stem cells in repair. It is clear that stem cells play a positive role in tissue repair, but questions arise as to whether these cells differentiate and help form the repair tissue or whether they simply secrete the appropriate

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cytokines that stimulate host cells to form new tissue and also scar formation. Tissue engineering systems can help in answering some of these questions. For example, MSCs were co-cultured in hydrogels adjacent to chondrocytes. Previous investigations by Gerstenfeld *et al*. have demonstrated that chondrocytes cultured in monolayers stimulate the differentiation of MSCs toward an osteogenic phenotype.[32] In similar coculture studies in a three-dimensional hydrogel environment, the MSCs stimulated the chondrocytes to proliferate and to produce more matrices. This effect was not observed with osteoblasts or fibroblasts, suggesting that MSCs promote new cartilage development. Coculture studies have thus been crucial in utilizing ESCs for cartilage tissue engineering applications.

Jennifer Elisseeff summarized that there are a number of exciting technologies in tissue engineering that currently, and will in the future, have a significant impact on the field of tissue engineering. Stem cells and enabling technologies will keep tissue engineers busy, as they elucidate the scaffold environments for more powerful cell types (stem cells) and design systems to enable translation of these technologies.

INTERFACE TISSUE ENGINEERING FOR SOFT TISSUE-TO-BONE INTEGRATION

Helen Lu from Columbia University discussed engineering of a functional soft tissue–bone interface, a problem critical for biological fixation of tendons and ligaments. She stressed that interface tissue engineering will likely be instrumental in the development of a new generation of fixation devices that can expedite the translation of orthopedic tissue engineering to the clinical setting. Using the anterior cruciate ligament (ACL)-to-bone insertion site as a model system, she explored the creation of the tissue interface by coculturing cells on a scaffold with a biomimetic gradient of structural and functional properties.

Application of tissue engineering methods[12,33] to musculoskeletal tissue regeneration has led to tremendous advances, whereby bone,[34–37] cartilage,[38–43] and ligament-like[44–48] tissues have been engineered *in vitro* and *in vivo*. Recently, the emphasis in the field has shifted from tissue formation to tissue function.[49] In addition to engineering tissues with physiological mechanical properties, a significant effort was invested into the biological integration of engineered grafts.

Musculoskeletal function requires soft tissue to integrate with subchondral bone and function in unison to facilitate joint motion. The insertion of ligaments or tendons into bone is usually achieved through fibrocartilage interface.[50–56] By design, the controlled matrix heterogeneity minimizes the formation of stress concentrations, effectively transferring complex loads between two distinct types of tissues.[51,57] Current soft-tissue reconstruction methods do not result in adequate graft integration, and the lack of interface can compromise graft function and long-term outcome. Lu proposed that the regeneration of the soft tissue-to-bone interface is a prerequisite for achieving biological fixation of soft-tissue grafts, and is essential for functional orthopedic tissue engineering. An important long-term goal is to devise tissue engineering strategies for regenerating the soft tissue-to-bone interface, and to apply these strategies to design integrative fixation devices capable of promoting the biological fixation. To this end, elucidating the structure-function relationships and the mechanisms of interface regeneration will be important for the design of integrative fixation devices.

Design considerations

The ACL is the primary knee-joint stabilizer and the most frequently injured knee ligament,[58] with over 100,000 ACL reconstruction procedures performed annually in the United States.[59] Autologous hamstring tendon-based grafts are increasingly utilized due to donor-site

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morbidity associated with bone-patellar tendon-bone grafts.[60] The soft tissue–based grafts can restore the physiological range of motion and joint function through mechanical fixation; however, biological fixation is not achieved as disorganized scar tissue forms within the bone tunnels and the native insertion site is lost due to surgery.

The ACL connects to bone through a characteristic fibrocartilage interface, with controlled spatial variation in cell type and matrix composition.[50,51,53–56,61] The ligament-to-bone interface consists of three distinct tissue regions: ligament, fibrocartilage, and bone (Fig. 6). The ligament proper is composed of fibroblasts embedded in type I and type III collagens. The fibrocartilage region is further divided into nonmineralized and mineralized zones. The nonmineralized fibrocartilage matrix consists of ovoid chondrocytes, and collagen types I and II within the proteoglycan-rich matrix. The mineralized fibrocartilage zone contains hypertrophic chondrocytes surrounded by a mineralized matrix.[54] Type X collagen, a marker for hypertrophic chondrocytes, is detected only within this region.[61] The last zone is the subchondral bone, within which osteoblasts, osteocytes, and osteoclasts are embedded in a type I collagen matrix.

The specific organization and controlled heterogeneity of the interface are important for minimizing stress concentrations and facilitating the transfer of complex loads between soft and hard tissues.[51,62] This multitissue insertion site is lost following ACL reconstruction surgery, and the graft is attached to bone via mechanical fixation using screws or pins. Without a biological interface, the graft-bone junction has limited mechanical stability, and the lack of integration is the primary cause of graft failure.[63–65] To date, functional integration of soft-tissue grafts with bone remains a problem, and advances are hindered by the lack of integrative graft solutions and sufficient understanding of mechanisms that govern interface regeneration.

Functional integration of soft tissue to bone may be achieved through the regeneration of the fibrocartilage interface, which in turn may require multiple types of cells, a multiphasic scaffold, and the development of controlled matrix heterogeneity mimicking that of the native insertion. Lu and colleagues have focused their efforts on three areas: (i) elucidating the mechanism of interface regeneration using models of homotypic and heterotypic cell-cell interactions, (ii) characterizing the structure-function relationship at the insertion site and identify interface-relevant design parameters, and (iii) designing biomimetic scaffolds capable of supporting multitissue regeneration.

Cell-cell interactions and mechanism of graft integration—It is well established that tendon-to-bone healing following ACL reconstruction does not lead to the reestablishment of the native insertion; rather, a fibrovascular or fibrocartilaginous tissue layer is formed within the bone tunnel.[66–69] This neo-tissue is located within the bone tunnel, whereas, physiologically, the insertion is situated outside of subchondral bone (Fig. 6). The formation of a fibrovascular tissue, instead of a mineralized matrix, within the bone tunnel results in a weak link at the graft-to-bone fixation site.[64] To promote biological fixation, it is thus physiologically more relevant to develop a fibrocartilaginous zone outside of the bone tunnel.

The formation of a fibrocartilage layer where the tendon graft directly contacts the bone is highly significant, and has led to the hypothesis that the interactions between cells derived from tendon (e.g., fibroblasts) and bone (e.g., osteoblasts) play a role in interface or fibrocartilage regeneration. Fujioka *et al*.[70] reported that by suturing the Achilles tendon to its original attachment site, cellular organization resembling that of the native insertion was observed over time. *In vivo* cell-tracking studies have revealed that the tendon graft is populated by host cells within 1 week of implantation.[71] While the source and nature of these host cells are not known, cell types other than osteoblasts and fibroblasts may be involved in fibrocartilage formation. Based on these observations, it is thought that osteoblast-fibroblast interactions mediate

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interface regeneration through two mechanisms: (i) phenotypic changes or transdifferentiation of osteoblasts, fibroblasts, or both, and (ii) the recruitment of progenitor stem cells or MSCs to the graft-bone interface, where they differentiate into fibrochondrocytes and form fibrocartilage.

One of the coculture systems focused on the interaction of fibroblasts and osteoblasts,[72] with the goal of emulating the *in vivo* condition where the tendon is in direct contact with bone tissue following ACL reconstruction. In this model, osteoblasts and fibroblasts were first seeded on opposite sides of a tissue-culture well separated by a hydrogel divider preformed in the center of the well (Fig. 7A). Once the cells reached confluence, the divider was removed, allowing the osteoblasts and fibroblasts to migrate and interact within the interface region. In another model system, an osteoblast monolayer was cultured atop a chondrocyte micromass[73] (Fig. 7B). It is likely that osteoblast-fibroblast interactions facilitate the recruitment and differentiation of stem cells or progenitor cells into chondrocytes or fibrochondrocytes. A triculture model was used to evaluate the effects of fibroblast-osteoblast interactions on chondrocytes.[74,75] These studies demonstrated the utility of *in vitro* coculture systems for investigating the mechanisms of interface regeneration.

Structure-function relationships at the ACL-bone interface—The first steps in functional tissue engineering are the determination of the material properties of the tissue to be replaced, the measurement of *in vivo* strains, and the calculation of stresses found in the native tissue.[49] Therefore, in order to successfully engineer the soft tissue-to-bone interface, the structural and material properties of the insertion site must be characterized. Identification of these properties will be critical for biomimetic scaffold design, and will serve as benchmark criteria for judging the success of engineered interfaces.

Current knowledge of mechanical properties of the insertion site is based on theoretical analyses,[55,76] as direct measurement of these properties is difficult due to the complexity and the relatively small-length scale of the interface (100 μm to 1 mm).[50,56,62] Applying the novel functional imaging method of ultrasound elastography, the strain distribution at the ACL-to-bone interface could be determined.[77] Elastography analyses of bovine tibiofemoral joints loaded in tension revealed that the displacement across the insertion was the highest at the ACL and decreased toward the bone (Fig. 8A). These regional differences suggest an increase in tissue stiffness from ligament to bone. These results agree with finite-element analysis of the medial collateral ligament, predicting that the maximum principal compressive stress occur near the distal edge of the femoral insertion.[76]

Based on Wolff's Law,[78] a structure-function relationship likely exists at the insertion site, and the observed interface organization and matrix composition are directly related to the nature of the strain experienced in the region. Fibrocartilage is often found in regions subjected to compressive stresses.[79] Combining microscopic mechanical testing with optimized digital image correlation methods, the region-dependent changes in compressive mechanical properties of the fibrocartilage interface were quantified.[80] Displacement under applied load was imaged using epifluorescence microscopy, and digital image analysis was performed to determine the mechanical properties. The incremental displacement decreased gradually from the ligament, to the nonmineralized to the mineralized fibrocartilage, and then to bone, implying an increase in tissue stiffness across these regions.

Analysis of these individual regions demonstrated decreased strain and a significantly higher elastic modulus for the mineralized fibrocartilage compared to the nonmineralized fibrocartilage region.[80] Surface characterization of the insertion site (Fig. 8B) revealed a corresponding increase in calcium and phosphate content progressing from ligament to interface and then to bone.[81] Benjamin *et al*. suggested that the amount of calcified tissue at

the insertion correlates to the force transmitted across the calcified zone.[82] Thus, the increase in elastic modulus is likely due to the mineral phase of the fibrocartilage interface.

Scaffold design and testing—A biomimetic scaffold is essential for providing an optimal environment for fibrocartilage formation. In addition to supporting the growth and differentiation of relevant cell types, the scaffold for interface tissue engineering must direct heterotypic and homotypic cell-cell interactions to promote multitissue formation and the maintenance of controlled matrix heterogeneity. Consequently, the scaffold should exhibit a gradient of properties mimicking those of the native insertion zone. The interface scaffold must be degradable and must exhibit mechanical properties comparable to those of the ligament insertion site. It should also be adaptable to current ACL reconstruction methods or should be preincorporated into the design of replacement grafts in order to facilitate *in vivo* graft integration. A triphasic scaffold (Fig. 9A) with one region (Phase A) intended for soft tissue, Phase B intended for fibrocartilage region, and Phase C designed for bone formation is an example of a biomimetic scaffold for engineering a tissue interface. Through the interaction of osteoblasts, chondrocytes, and fibroblasts on this triphasic scaffold with a gradient of material properties, it is anticipated that a fibrocartilage-like interface may be formed in the intermediate region, Phase B, under appropriate physical and chemical stimulation.

The interactions of osteoblasts and fibroblasts on the triphasic scaffold and the feasibility of this scaffold for interface tissue engineering have been evaluated *in vitro*[83] and *in vivo*.[84] Specifically, fibroblasts and osteoblasts were seeded onto Phase A (polymer fiber mesh without Ca-P) and Phase C (polymer-ceramic composite with Ca-P[85]), respectively, whereas Phase B (polymer with lower amount of Ca-P than Phase C) was left unseeded. The migration of both cell types into Phase B was monitored over time, and it was observed that fibroblasts (Calcein AM, green) and osteoblasts (CM-DiI cell tracer, red) were localized primarily at the opposite ends of the scaffold after initial seeding (Fig. 9B-i), with very few cells found in Phase B. After 4 weeks of culture, the fibroblasts and osteoblasts proliferated within their respective phases and both cell types migrated into Phase B (Fig. 9B-ii). The controlled cell distribution resulted in elaboration of matrix specific to each cell type on the relevant phase of the scaffold. A mineralized matrix was detected in Phase C only, with extensive type I collagen matrix deposition observed on both Phase A and Phase B.

When the triphasic scaffold cultured with osteoblasts and fibroblasts was evaluated in a subcutaneous athymic rat model, abundant tissue formation was found (Fig. 9C). The production of ECM compensated for the decrease in mechanical properties of the biodegradable scaffold and, more important, controlled matrix heterogeneity *in vivo*. Optimization studies are currently under way with the ultimate goal of developing integrative fixation devices for use in soft-tissue reconstruction procedures.

Helen Lu summarized that the integrative graft solutions would be a significant component of functional tissue engineering.[86,87] Building upon the foundation of tissue engineering methodologies developed in the past two decades, innovative approaches have been created to address the challenges of biological fixation of soft-tissue grafts. The success of interface tissue engineering will likely depend on a thorough understanding of the structure-function relationships existing at the native insertion site, and the elucidation of the mechanisms governing interface regeneration. The successful regeneration of the soft tissue-to-bone interface will augment graft function and enhance translation potential of engineered grafts.

TISSUE ENGINEERING OF ARTICULAR JOINT SURFACE INTERFACES

Rita Kandel from the University of Toronto discussed the need for, and the potential and challenges related to, the recreation of the cartilage-bone interface, in the context of tissue

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engineered joint repair. Resurfacing the articulating surfaces of synovial joints with synthetic prostheses is still a treatment of choice for end-stage diseases. She argued that though primary joint replacements implanted since the late 1980s have shown reasonable success, failure rates of up to 40% after 10 years have been reported depending on the type of implant.[90–93] It has been speculated that all joint-replacement implants made of synthetic materials will need replacement if the patient lives long enough. Recent efforts have focused on developing new treatment methods that will result in biological repair and preclude the need for non-degradable synthetic materials. One of these approaches entails the use of tissue engineering methods to regenerate articular cartilage either *in vitro* or *in vivo*.[89,94]

General requirements

The key to successful bioengineering of articular joints will be to generate implants that will address the contrasting needs of the two different tissues, cartilage and bone, as well as the cartilage-bone interface. Bone comprises well-organized trabeculae, consisting of mineralized, mostly type I collagen matrices. In contrast, the ECM of articular cartilage is composed predominately of water and type II collagen fibers admixed with large aggregating proteoglycans. The deep aspect (adjacent to bone) of the cartilage is mineralized and this zone anchors the hyaline cartilage to the underlying subchondral bone. In addition to this interfacing function, the calcified zone is involved in the transmission of forces across the joint.[95,96] The stiffness of the calcified cartilage layer is greater than that of the nonmineralized hyaline cartilage but is an order of magnitude less than that of bone, most likely due to the arrangement of collagen fibers.[97] It has been hypothesized that this stepwise transition in stiffness is important for the proper functioning of cartilage and distribution of forces.[97] It is well known that repair of joint defects with fibrocartilage, which does not have the organization and composition of hyaline articular cartilage, will degrade over time as it does not have sufficient load-bearing capability.[88,98] As the goal of cartilage tissue engineering is to generate articular cartilage that will function similar to the native tissue, it is likely that regeneration of cartilage with both of these zones will be required.

Biphasic tissue constructs

A variety of methods have been proposed to repair osteochondral defects. One way to bioengineer these articulating surfaces is to generate biphasic constructs composed of cartilage tissue overlying and integrated with a substrate that serves as the bone interfacing component (osteochondral-type biphasic constructs).[99–114] Similar to scaffolds, substrates are porous and usually biodegradable.[115,116] However, in contrast to scaffolds, the cells or tissue are placed on the top surface and not seeded throughout the substrate. As a result, the regenerated cartilage is anchored to the intended articulation surface of the substrate. The substrate both supports cartilage formation and facilitates fixation after implantation by in-growth of bone into the pores. Important characteristics of this substrate, if it is used to support cartilage formation *in vitro* prior to implantation, are (i) it is fully porous, or chondrocytes will not form cartilage, and (ii) the size and organization of the pores allow fluid flow but will not permit (or severely restrict) cell infiltration into the full thickness of the substrate. Substrates utilized for these types of constructs have been made from a variety of natural and synthetic materials, but as the substrates are meant to be bone interfacing they must have sufficient strength for weight bearing.[99–114]

A porous ceramic substrate composed of calcium polyphosphate (CPP), which has mechanical properties approximating cancellous bone, has been developed.[117] To generate the biphasic construct, articular chondrocytes are placed on the articulation surface of the CPP substrate. As the cartilage forms *in vitro*, the developing tissue fills and thereby integrates with the top portion of the substrate (Fig. 10). The cartilage tissue contains type II collagen and large

proteoglycans.[118] The CPP is biodegradable, and as the biomaterial is made of calcium and phosphate the breakdown products do not incite an inflammatory reaction.[119]

Potential for cartilage repair

This type of biphasic construct, in which the cartilage tissue is formed prior to implantation, has the potential to repair focal cartilage defects. As the cartilage is already formed, lateral integration to the adjacent host cartilage is possible immediately upon implant insertion. As the substrate (CPP) is porous, bone can grow into the pores after implantation, resulting in secure implant fixation, and because it is biodegradable the CPP will ultimately be replaced by bone. This approach allows generation of cartilage with a calcified zone.[120–123] By using chondrocytes isolated specifically from the deep zone of articular cartilage, it is possible to generate cartilage with a mineralized zone adjacent to the bone substitute (Fig. 10). The mineral that forms under these conditions is hydroxyapatite, similar in composition and size to that present in the native calcified cartilage zone.[122] The presence of a mineralized interface improved the compressive properties of the tissue as the equilibrium modulus increased approximately eightfold and the equilibrium stress increased twofold compared to cartilage without a mineralized zone. The interfacial shear strength (measures strength of attachment of the *in vitro*-formed cartilage to the substrate) also increased as the energy to failure increased twofold (unpublished data). As most tissue engineering methods to date generate cartilage with only a fraction of the mechanical properties of the *in vivo* tissue, the above approach demonstrates the importance of bioengineering cartilage with an organization that mimics the native cartilage.[124]

Rita Kandel summarized that the most important challenge is identifying a cell source that will provide sufficient numbers of cells capable of forming hyaline cartilage large enough to repair clinically relevant defects, and still having the capability to form cartilage with an upper non-mineralized zone and a deep mineralized zone. Articular chondrocytes are phenotypically unstable when grown in monolayer culture, and the cells can dedifferentiate to fibrocartilage-type cells with even one passage.[125] So, if the approach is to expand the cell number of differentiated chondrocytes in culture, it will be necessary to identify cell culture conditions that will allow for cell proliferation while still maintaining chondrocyte phenotype.[126] Alternatively, one can design materials with appropriate characteristics so that when the passaged cells are placed on the material it would induce the cells to redifferentiate toward articular chondrocytes. Nanomaterials or “smart” materials may be more suitable for this approach.[127,128]

Another approach currently being explored is the use of mesenchymal progenitor cells (MPCs). Most of the work focusing on this cell type and its role in tissue engineering is preliminary, in part because of our limited understanding of the biology of these cells and because the markers used to separate out cells that can differentiate to chondrocytes from a mixed cell population have not been well defined. However, MPCs after expansion and seeding into a scaffold are able to differentiate toward chondrocytes under the appropriate culture conditions, but whether they can generate tissue with the two zones is not known.[129,130] The biphasic (osteochondral-type) constructs can be created in a shape individualized for the defect, the soft tissue–hard tissue interface can be re-created prior to implantation, and they should be relatively easy to implant if designed appropriately.

HEART VALVE TISSUE ENGINEERING AND REGENERATION RESEARCH

The immense need for cardiovascular tissue engineering and regeneration has generated considerable interest and investigation in this field.[131–135] Frederick Schoen from Harvard Medical School stressed that the engineering of tissue requires an understanding of the relationships of structure to function in normal and pathological tissues (including mechanisms

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of embryological development, and functional tissue biomechanics and other structure-function correlations) and the ability to control cell and tissue responses to injury, physical stimuli, and biomaterial surfaces.

Dr. Schoen described an approach to tissue engineering of heart valves based on concepts and data derived from valve anatomy, physiology, development, remodeling, response to injury, and substitution. He stressed that surgical repair or replacement of a diseased heart valve is a common procedure (over 85,000 cases per year in the United States and 285,000 worldwide), and the tissue of the heart valves cannot regenerate spontaneously. Although valve surgery generally leads to enhanced survival and quality of life, presently available valve substitutes provide imperfect functional restitution and have potential complications.[136,137] Moreover, in pediatric applications, where although physiologically corrective procedures can be successfully performed, repairs of congenital deformities require very small valve sizes, and subsequent (and repetitive) operations may be needed to accommodate growth of the patient, even in cases where typical prosthesis-associated complications have been prevented. The goal of heart valve tissue engineering is to overcome the limitations of contemporary valve substitution methods by creating or regenerating a living valve replacement that functions well hemodynamically, repairs ongoing tissue damage, and has long-term durability and growth potential similar to the natural heart valves.[138–141]

Functional structure of heart valves: Role of valvular ECM and cells

The aortic valve (the most extensively studied, most frequently diseased, and most widely transplanted valve) best illustrates the essential relationships of structure to function. The aortic valve cusps open to form an obstruction-free orifice during ventricular systole and close rapidly and completely in diastole. Although the pressure differential across the closed valve imposes a large load on the cusps, regurgitation and cuspal prolapse are prevented by substantial coaptation of the cusps, which is enabled by a microscopically inhomogeneous architecture with three well-defined cellular tissue layers.[142,143] Nearest to the outflow surface is the *fibrosa*, composed predominantly of circumferentially aligned, densely packed collagen fibers, largely arranged parallel to the cuspal free edge. The fibrosa provides strength and stiffness, and minimizes sagging of the cusp centers. The central layer of the aortic valve cusp, *spongiosa*, is composed of loosely arranged collagen and abundant glycosaminoglycans. This layer accommodates the dynamic shape changes of the cusp during the cardiac cycle, lubricates relative movement between ventricularis and fibrosa layers, and absorbs shock during closure. The thin layer near the inflow surface, *ventricularis*, is rich in radially aligned elastic fibers, and this enables the cusps to recoil and have minimal surface area when the valve is open, but stretch in response to back-pressure of blood in the closed phase. The valves are lined by a confluent layer of valvular endothelial cells (VEC); deep to the surface are valvular interstitial cells (VIC).

Studies of normal, pathological, and substitute valves have demonstrated that the principal determinant of valve durability is the valvular ECM, whose quantity and quality depend on viability and function of VIC.[144] The VIC comprise a population of resident cells of diverse and dynamic phenotypes, largely along a spectrum of fibroblast-like to myofibroblast-like.[145–148] The VIC synthesize the valvular ECM molecules and express matrix degrading enzymes such as matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) that mediate ECM remodeling and repair.[149]

In normal valves, VIC are fibroblast-like (i.e., immunoreactive to vimentin but not to smooth muscle actin [SMA, a marker of myofibroblast function[150,151]], SMemb, or MMP-13). Indeed, only 2–5% of VIC of normal intact valves express SMA.[148] In contrast, previous *in vitro* studies using isolated cells cultured from heart valves demonstrated that 56–78% of cells are SMA positive.[142] The higher percentage of myofibroblasts observed when VIC are removed from

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the intact valve (i.e., and placed in culture) suggests that removal from the normal tissue environment stimulates VIC growth. Moreover, treatment of isolated VIC with transforming growth factor (TGF) strongly activates VIC to the myofibroblast phenotype.[152]

Cardiac valve development, maturation in utero, and postnatal changes

During normal development of the heart, the valve cusps or leaflets originate from mesenchymal outgrowths known as endocardial cushions.[153,154] Some endothelial cells in the cushion-forming area that initially line the internal cardiac surface undergo endothelial to mesenchymal “trans-differentiation” and migrate from the luminal surface to the subendocardial ECM.[155,156] These cells remodel the cushions into leaflets and cusps, in a process also stimulated by TGF.[157]

The mechanisms of developmental maturation of heart valves were studied by analyzing human semilunar valves obtained from fetuses at 14–19 and 20–39 weeks of gestation and neonates 24 hours to 30 days old, and comparing these valves with those of normal adults.[158] Fetal VIC showed a high proliferative index, an activated/myofibroblast-like phenotype (indicated by SMA expression) and MMP-collagenases, indicating active matrix remodeling. In contrast, cell turnover and VIC activation were low in adult valves. A trilaminar architecture, evident by 36 weeks of gestation, was rudimentary compared to that of adult valves. Moreover, collagen content increased during valve maturation but not subsequently, although collagen fiber alignment was higher in adult than fetal valves.

Valve adaptation and repair

The VIC become activated and mediate functional biomechanical adaptation of valves when exposed to an altered mechanical environment.[148,159] Although the specific regulatory mechanisms are unknown, this activation is potentially reversible, and when equilibrium is achieved, the cells return to a quiescent state.[148] It is thought that VIC phenotype is regulated predominantly by the wall stresses in the valve leaflets, analogous to remodeling in cardiac hypertrophy and vascular walls.[160–162] Mechanical and chemical stimulation may be synergistic.[163]

A unique opportunity to study cellular phenotypic changes and ECM remodeling of valves in response to abruptly altered mechanical loading *in vivo* is provided by clinical replacement of a diseased aortic valve by transplantation of the patient’s own pulmonary valve into the aortic position (Ross procedure) done in some pediatric and young adult patients with aortic valve disease.[164] Analysis of the structure and interstitial cell phenotypes in clinical pulmonary-to-aortic valve transplants early (2–10 weeks) and late (3–6 years) postoperatively showed near-normal cuspal structure and collagen architecture, and viable VIC.[165] In early autograft cusp explants, VIC resembled myofibroblasts (19% had positive SMA) and had strong expression of MMP-13, indicative of active ECM remodeling. Subsequently, there was late normalization of VIC phenotypes toward those of normal aortic valve, with only 6% of VIC in late explants and 5% in normal aortic valves expressing SMA.

Physiological wound healing in mitral valves is also associated with phenotypic modulation of VIC from fibroblasts to myofibroblasts.[166] Moreover, VIC in mitral valve leaflets from patients with myxomatous mitral valve degeneration also express features of myofibroblasts, suggesting that this disease comprises a nonequilibrium state, consistent with the progressive course of this condition in many patients.[167]

The data summarized above on the dynamic VIC phenotypes that occur in several circumstances suggest the following general mechanistic paradigm by which cardiac valvular tissue is dynamically and reversibly responsive to environmental conditions, particularly

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mechanical loading: (i) Under equilibrium conditions, the majority of VIC are quiescent fibroblast-like cells, (ii) Altered mechanical loading (as in valve development, adaptation to changes in the local mechanical environment and in pathologic states) stimulates VIC to become activated and mediate connective tissue remodeling, and (iii) When equilibrium is restored, the cells return to the quiescent state.

Studies of contemporary bioprosthetic heart valve substitutes

Structural dysfunction is the major cause of failure of the most widely used bioprostheses (flexible-stent-mounted, glutaraldehyde-preserved porcine aortic valves and bovine pericardial valves). Within 15 years following implantation, 30–50% of porcine aortic valves implanted as either mitral or aortic valve replacements require replacement because of primary tissue failure. Cuspal mineralization and noncalcific damage to the cuspal structural matrix are the major responsible pathologic processes.[168,169] Calcification is markedly accelerated in younger patients. The mechanism of valve calcification involves the reaction of calcium derived from plasma with organic phosphorous residing in the cross-linked, nonviable cells of the preserved valve.[170,171] Alterations in tissue structure and chemistry of bioprosthetic valves that occur during valve fabrication and manufacture can contribute to the characteristic failure modes as follows: (i) The microstructure of the cusps is fixed in a static geometry characteristic of one phase of the cardiac cycle, inducing abnormal tissue stresses during function, (ii) Damage to endothelial coverage allows penetration of plasma and inflammatory cells into the cusps, (iii) Bioprosthetic valve VIC are nonviable due to the chemical pretreatments; without viable cells, synthesis of collagen or other ECM components cannot occur, and structural damage can accumulate.

Conceptual approach to heart valve tissue engineering

The rationale and design criteria for tissue engineered heart valves (TEHV) derive from five key concepts of functionally adaptive valvular remodeling or regeneration established over the past decade from studies in many laboratories of normal, developing, and diseased heart valves; bioprosthetic valves; and other tissue valve substitutes: (i) The highly specialized arrangement of collagen and other ECM components of heart valves (particularly elastin and proteoglycans) enable physiological function and extended durability, (ii) The quality of valvular ECM depends on the ability of VIC to modulate their phenotype and function and thereby mediate valvular adaptation to different environments, (iii) Structural deterioration of native and substitute valves is mediated by chemical and mechanical damage to collagen, (iv) Cell viability in nearly all current bioprosthetic tissue valve substitutes is compromised or completely eliminated during processing; thus, ECM damage that occurs following implantation of current bioprosthetic valves cannot be repaired, (v) The long-term success of a tissue engineered (living) valve replacement will therefore depend on the ability of its cellular components to assume normal function with the capacity to repair structural injury, remodel ECM, and potentially grow.

In the general paradigm of tissue engineering,[12] cells are seeded on a synthetic polymer or natural material that serves as a scaffold, and then a tissue is matured *in vitro* (bioreactor) until proliferating cells produce a sufficient amount and quality of ECM. The tissue thus formed is called a construct. In the second step, the construct is implanted in the appropriate anatomic location, where further remodeling *in vivo* may occur to recapitulate normal functional organ or tissue architecture. Alternatively, cardiovascular tissue engineering approaches can omit a step or steps in the general paradigm by taking advantage of specific properties of initial components (cell or scaffold) and endogenous cells. For example, in the cell-seeded scaffold model, a confluent endothelial coverage could be placed on a cardiovascular prosthesis at the time of implantation.[172,173] The cell transplant model used in experimental and early clinical trials of cell-based therapy for myocardial tissue repair uses cells injected at a specific tissue

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site, in the hope that these cells will differentiate and proliferate, and/or stimulate repair by functional tissue.[174] More recent approaches to valve and vascular graft engineering have utilized scaffolds designed to attract endogenous cells to repopulate and remodel a decellularized matrix.[175,176] Irrespective of the methodology, the key processes occurring during the *in vitro* and *in vivo* phases of tissue formation and maturation are (i) cell adhesion, proliferation, sorting and differentiation; (ii) ECM production and organization; (iii) degradation of the scaffold; and (iv) remodeling and potential growth of the tissue. Key targets for characterizing tissue engineered constructs include tissue composition, cellular gene expression and phenotype, ECM, key effectors of tissue remodeling, and tissue quality.

Engineered heart valves constructed from biodegradable polymer scaffolds

Composite trileaflet valve-wall constructs fabricated from poly-4-hydroxybutyrate-coated polyglycolic acid scaffolds seeded with autologous bovine endothelial and carotid artery medial cells were cultured *in vitro* up to 14 days in a bioreactor that mimicked valve cyclical motions by providing pulsed flow of cell culture medium at physiological pressure.[148,177] The ECM produced in this construct was predominantly proteoglycan, and collagen accumulation (detected by picrosirius red staining observed under polarized light) was limited to a few weak birefringence fibrils. Elastin was not detected histologically. Immunohistochemical staining revealed that cells in the construct were activated myofibroblasts, as determined by strong expression of SMA (microfilaments) and MMP-13 coexpression.

The constructs were implanted as pulmonary valves in lambs. Valves functioning for and explanted at 4–20 weeks demonstrated the dynamic changes of VIC phenotype and ECM in tissue engineered valve explants toward layered architecture and cellular configuration of native valves. Some cells in explants at 4–8 weeks showed weak staining for SMA, but all cells throughout the cusp stained strongly for MMP-13. Explants at 16–20 weeks had layered leaflets, with fibroblast-like cells, having undetectable SMA; some cells were still positive for MMP-13. Leaflets were partially covered with cells that stained positive for von Willebrand factor, which is characteristic of endothelial cells. Movat stain demonstrated collagen in the fibrous layer on the aortic (outflow) side, proteoglycans in the central loose layer, and elastin near the ventricular (inflow) side. The structure resembled that of native valve. These studies showed that an engineered tissue valve could be implanted into and function in the pulmonary artery, and that the remodeling of these valves recapitulated features of tissue development, response to injury and wound healing.

Harnessing the reparative potential of circulating endogenous cells

There is now accumulating evidence that circulating endogenous cells with the potential of differentiating into vascular cells can be recruited *in vivo* from the bone marrow to sites of biomaterials (and potentially unseeded scaffolds) or natural cardiovascular tissues. Endothelial progenitor cells (EPCs) promote endothelial regeneration in dog models by covering implanted Dacron grafts[178] and in human models by covering implanted ventricular assist devices.[179] Coating a nonseeded scaffold with appropriate cell-signaling molecules to encourage EPC adhesion is an exciting opportunity. Recent evidence suggests that bone marrow could be a source of progenitor cells contributing to smooth muscle-like cells in the vasculature, including heart valves.[180,181]

Inflammatory cells may also play a role in mediating remodeling of a scaffold implanted *in vivo* without prior cell seeding. In one experiment on pigs, each of the four pigs used had one pulmonary valve leaflet excised and replaced with a leaflet constructed from porcine small intestinal submucosa (SIS).[182] Histology indicated that the implanted matrix was progressively resorbed and replaced by fibrous connective tissue that had features of adult valve. Alternatively, a biodegradable graft containing collagen microsponge was fabricated and

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tested, with and without preseeding.[183] In both cases (SIS and collagen microsponge) there was no thrombus formation, the scaffold was absorbed, and there was endothelialization, parallel smooth-muscle cell alignment, elastin, and collagen fibers. These results suggested that the patch promoted *in situ* cellularization and regeneration of autologous tissue. However, an important limitation of this study was that the implanted patches were small; cellularization of a large patch may be less efficient.

Nevertheless, clinical applications of decellularized heart valves have been largely unsuccessful. Simon *et al*. used SynerGraft decellularized porcine heart valves as homograft valve replacements in the right ventricular outflow tract during Ross procedures in children.[184] Many valves failed, and postimplant examination of failed valves revealed incomplete decellularization, lack of cell repopulation, lack of endothelialization with severe inflammation, fibrous sheath formation inside and outside the graft, foreign body reaction, calcification, and severe degeneration of leaflets and wall. Histological examination of decellularized porcine Syner-Graft valves (Cryolife Kennesaw, GA) implanted without *in vitro* preseeding for 6 months in sheep suggested only minimal growth of host cells on intact leaflets and showed a lack of calcification.[185]

Challenges and future directions in clinical translation

Heart valve tissue engineering has exciting potential but many challenges and questions remain. Early studies focused on the design of individual valve leaflets.[186] As the field of tissue engineering evolves, attempts have been made to transition from the design of separate structures to the design of complete valved conduits.[177] Importantly, the remodeling of a single cusp attached to a native arterial wall may be different from those in a valved conduit.

There is a need to develop guidelines characterizing the safety, efficacy, and quality of tissue engineered products (be they heart valves, other cardiovascular implants, or prostheses in other clinical applications) before they can be implanted in humans. Challenges in tissue characterization for heart valve tissue engineering are summarized in Figure 11. The major research goals are to understand mechanisms, define animal models, develop biomarkers, develop assays/tools, and define surrogate and true end points. The major clinical goals are to characterize and assure control of quality of tissue constructs, accommodate patient-to-patient heterogeneity in tissue remodeling, and predict outcomes as early as possible.

Demonstration of long-term efficacy (implantability, functionality, long-term performance) and safety (biocompatibility, durability, modes of failure, ease of monitoring) of these valves in humans will be a particular challenge. Since current heart valve replacements serve their recipients predictably and moderately well in most patient populations (except children), acceptance of tissue engineering by the surgical community may be slow. Indeed, a leading cardiologist suggested that surgeons should consider the use of a tissue engineered valve in a patient (beyond appropriately controlled clinical research) only when the 15-year lifetime of conventional valve substitutes can be conclusively and predictably demonstrated.[187] This will be a substantial challenge since results from available animal models of bioengineered tissue may not necessarily translate directly to humans, and the most suitable animal model for testing TEHV has not yet been determined.

A key consideration is that *in vivo* behavior of TEHV may display considerable variability among recipients, owing to intrinsic variation in inflammation and remodeling potential among individuals.[188–191] Simply stated, some patients might inadequately remodel their tissue engineered valves, and the consequences could be disastrous. This issue is conceptually analogous to the emerging area of pharmacogenomics,[192] which shows that individual patient characteristics (for example, genetic mutations or polymorphisms) have a profound influence on drug metabolism outcome in some patients. Indeed, it has been shown that individuals with

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genetic defects in coagulation proteins may be unusually susceptible to thrombosis of prosthetic heart valves.[193]

To understand, monitor, and potentially control patient variability in wound healing and tissue remodeling *in vivo*, tissue biomarkers measurable by chemical or molecular assay or molecular imaging that predict success and failure of an engineered tissue must be identified. These biomarkers may be employed in preclinical *in vitro* and *in vivo* experiments and clinical studies using molecular tissue imaging,[194,195] to track the presence, migration, proliferation, and function of bone marrow–derived endogenous progenitor cells and cells used to preseed scaffolds. Imaging of magnetically labeled MSCs injected into porcine myocardium has been performed *in vivo*,[196] a technique that can potentially be expanded to study magnetically labeled EPCs and MPCs seeded on a scaffold and implanted into an animal or human model. *In vivo* molecular imaging has been used to demonstrate key enzymatic and cellular events in atherosclerosis and thrombosis.[197,198] Molecular imaging can probe polymorphisms of ECM gene expression *in vivo* in models of wound healing[199] and cardiovascular disease.[200,201] These studies can potentially be translated to perform real-time *in vivo* characterization of scaffold matrices (either preseeded or with the potential of attracting endogenous cells) implanted in animal models. Moreover, relevant biomarkers might be measured distant from the tissues in which they are generated; for example, researchers are currently working to identify serum-specific biomarkers of ECM remodeling in diseases, such as MMPs in acute coronary syndromes,[202] and urine-based biomarkers in early cancer progression.[203]

Frederick Schoen concluded that the assessment of bioactive and engineered implants will require a broadened scope of the concept of biocompatibility, and that the approaches employed in implant retrieval and evaluation will necessitate the identification of tissue characteristics (biomarkers) that will be predictive of (surrogates for) success and failure. Although considerable validation of these concepts and enabling technological advances will be necessary, a most exciting possibility is that such biomarkers may be used to noninvasively image or monitor the maturation or remodeling of engineered devices in individual patients through bioassay or molecular imaging technology.

BIOPRESERVATION FOR TISSUE ENGINEERING

Mehmet Toner from Harvard Medical School addressed the critical dependence of tissue engineering on the availability of large numbers of cells for growth and culture, and the related need for cryopreservation. As the field expands, and the need for cells continues to grow, preservation and storage of living cells for transport or later use will become increasingly important. He reminded the audience that the field of cryopreservation has been active for more than 50 years,[204] with the primary goal to move toward the preservation of whole organs. This goal has proven extremely difficult to achieve and failure to make significant progress has led to disappointment, but, recently, with the emergence of tissue engineering and cell-based therapies, cryopreservation research has been reinvigorated.

Cryopreservation and tissue engineering

From a tissue engineering perspective, the role of cryopreservation is not necessarily to preserve whole organs, but to preserve the cells necessary to create tissue engineered products as well as engineered tissues (Fig. 12). Cryopreservation research has led to the successful preservation of a large number of cell types. Successful freezing protocols for most cells rely on a combination of controlled freezing rates and the addition of cryoprotectant chemicals. Somewhat surprisingly, however, there are many cells types that have not been successfully preserved despite many years of effort. Illustratively, red blood cells have been successfully preserved and used clinically for 50 years, while there are still no clinically viable techniques for the long-term preservation of platelets and granulocytes. Cryopreservation techniques that

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have been successful for one cell type have proven to be difficult to adapt to other cells types. These problems have been compounded as the field has attempted to move into the preservation of tissues or whole organs. In order to support the growth of tissue engineering, the cryopreservation field, or the broader field of biopreservation, must create methods for the long-term preservation of any cell type, and ultimately of complex tissues.

Cryoprotectants—The accepted paradigm for successful cryopreservation is to avoid ice formation inside the cell during cooling. This is most easily accomplished using slow-freezing techniques. In slow-freezing protocols the cells are cooled at a controlled rate and ice is usually formed, or nucleated, outside the cells at a specific temperature. The formation of ice causes an increase in the osmolality of the extracellular solution because ice rejects solutes as it forms, and the cells shrink. As the cooling continues the cells continue to shrink, and because there is very little water inside the cells, ice does not form within the cellular interior. The intracellular solution eventually either forms a eutectic solid or enters into a glass phase. Unfortunately, this massive cellular shrinkage and the increase in extracellular electrolyte concentration may lead to cell death.[205–207] To prevent this, cryoprotectants, usually glycerol or dimethyl sulfoxide (DMSO), are added. These chemicals enter the cell, thereby reducing cellular shrinkage, and somewhat dilute the electrolytes. In general, however, if cells are frozen slowly they die, even with the addition of cryoprotectants.

In order to prevent cell death, the cells must be allowed to dehydrate, thereby reducing the amount of intracellular water, and then they must be frozen quickly to avoid excess shrinkage and damage from high electrolyte concentrations. Most successful cryopreservation protocols use a period of slow cooling and the introduction of cryoprotectants to induce some cellular shrinkage, followed by fairly rapid cooling to liquid nitrogen temperatures. If the cooling is too fast, so that the cells do not shrink, then ice forms inside the cells and they die. If the cooling is too slow, then the cells shrink too much and die from dehydration and electrolyte build-up. If it is just right, the cells may survive.[208] The three sources of cellular damage—dehydration, intracellular ice formation, and cryoprotectant toxicity—are all independent. There is no guarantee that for any particular cell type a cooling rate exists that will lead to high levels of viability. A cell that is very sensitive to dehydration effects may have low viability at cooling rates that extend right up to the point where ice starts to form inside the cells, which can kill them. Some cells are very sensitive to cryoprotectants and cannot tolerate even low concentrations of glycerol or DMSO.

Cooling rates—The rate at which cells must be cooled in order to induce some, but not too much, shrinkage is highly dependent on cell type.[209] For highly permeable cells such as erythrocytes, the optimal cooling rate is more than 1000°C/min, but for relatively impermeable stem cells the optimum is about 1°C/min. Determination of optimum cooling rates has been a highly fruitful area of research, both empirically and theoretically.[210] The original concept for defining an optimum was proposed by Mazur[211] as the fastest cooling rate possible that would not promote intracellular ice formation. He proposed that intracellular ice would not form if the cell had less than 10% of its initial water when it entered the ice nucleation zone of temperature. These values were refined to 5% of the initial water content when the cell reached −30°C.[212] Based on this definition and the water transport equations originally developed, a theory for determining optimum cooling rates for cell survival was developed.[211,212] This enabled prediction of optimal cooling rates that agreed very well with experimental values for a wide variety of cells, based solely on knowledge of the cell membrane permeability and the cell's initial surface area to volume ratio. However, the fact that the optimum value is cell-type dependent means that there is no one preservation protocol that will work for all cells. Also, as we noted earlier, for many cells there is no protocol of this type that will work at all.[209]

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Vitrification—Another method for avoiding ice formation inside the cell is to vitrify the entire sample, that is, have the entire sample enter the glass phase with no ice inside or outside the cells. There are two methods for achieving vitrification. The first method is to freeze the sample very fast. If water is frozen fast enough, 1,000,000°C/s, it will form a glass phase even without the addition of cryoprotectants.[213] For single cells and small samples this has been done successfully,[209,214] but scaling techniques of this type for large samples or even large numbers of small samples may be impossible. The second, more widely used, technique involves the use of high concentrations of cryoprotectant chemicals so that ice will not form no matter how slowly the sample is cooled. For instance, solutions containing 41–50% of propanediol will form a glass phase when cooled at any rate without forming ice.[215] Since ice is not formed anywhere in the sample, the cells do not shrink when they are cooled. They may shrink if cryoprotectants that do not enter the cell are used, and they usually are, but this shrinkage is controlled by the amount of chemicals added. Vitrification protocols, therefore, do not depend on cooling rate—which eliminates two of the three sources of damage in traditional freezing protocols. This also enables preservation of large tissue samples, at least in theory. Cooling large tissues at a uniform rate is impossible unless very slow cooling rates are used, but for vitrification protocols, very slow cooling rates can be used and uniformity of cooling is not so important. For these reasons, vitrification protocols are regarded by many as most promising for the successful preservation of whole organs.[216]

The problem with vitrification protocols is that they require very high concentrations of cryoprotectants, much higher than the concentrations used in freezing protocols. The mere addition and removal of the chemicals can kill the cells due to osmotic stress, and the chemicals are toxic.[215,217,218] It seems clear that many cell types will never be successfully vitrified unless new, nontoxic cryoprotectants can be found, and it is not clear that this will even happen. Vitrification may ultimately be effective for the storage of certain organs whose cells can tolerate the required chemicals, but it is unlikely to be the best method for preserving the many different cell types the field of tissue engineering will require.

Challenges ahead

Research continues along the traditional lines of vitrification and freezing protocols that attempt to prevent intracellular ice formation, but new research is moving in slightly different directions. Recent work has shown that ice formation is strongly enhanced by cell-cell interactions.[219,220] This suggests that efforts to prevent intracellular ice in complex tissues may be even more difficult than expected, since ice formation in even one cell of the tissue could trigger intracellular ice formation in surrounding cells or throughout the system. On the other hand, work by Acker and coworkers has shown that ice that forms inside a cell due to interaction with a neighboring cell is not lethal.[221,222] This complements some earlier studies by Rall,[223] which showed that the formation of intracellular ice is not always a lethal event. These studies suggest that future cryopreservation research might aim at controlling the damage caused by the formation of intracellular ice rather than its prevention. If this damage is prevented then faster cooling rates could be achieved with lower concentrations of cryoprotectants.

Other research is moving away from cryopreservation. Within the last 5 years there has been significant research on the various forms of anhydrobiotic preservation—preserving cells in a dried state. In 1992 Goodrich *et al*. reported the successful recovery of functional erythrocytes after freeze drying.[224] Using trehalose and sucrose to stabilize membranes and to promote glass formation, researchers have been able to preserve a variety of cells using air drying and freeze drying techniques.[225–227] There are two potentially significant advantages of drying techniques as a form of biopreservation for tissue engineering. The first is ease of storage and transport. Samples preserved in a dry state can be stored at or near room temperature. The

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convenience of off-the-shelf availability for the cells used in tissue engineering and for tissue engineered products is significant. In contrast, cryopreserved cells must be maintained at liquid nitrogen temperatures, which makes storage expensive and transport difficult. The second advantage of drying techniques is that the major additives used to promote survival of dried cells are sugars, usually trehalose or sucrose, which are nontoxic. This means that many cell types tolerate the addition of the sugars and that the sugars do not need to be removed before cell culture and clinical use. Drying techniques, therefore, may be applicable to cell types that do not tolerate traditional cryoprotectants.

Mehmet Toner concluded that the field of tissue engineering has allowed the biopreservation field to refocus. The long-held goal of whole-organ preservation remains important, but of new importance is the need to preserve cells of all types (including stem cells), even in small numbers, and engineered tissues. New techniques such as dried preservation, largely untested for most cell types, may not be applicable to whole organs, but may provide substantial benefits to tissue engineering. Techniques that are widely applicable to many important cell types do not yet exist, and it is not even clear which class of techniques will likely provide answers in the future. The traditional techniques of controlled freezing and vitrification have not yet yielded the desired results. A new breakthrough in biopreservation may be necessary to meet the full needs of the tissue engineering field.

TEMPORALLY AND SPATIALLY REGULATING GROWTH FACTOR AVAILABILITY

David Mooney from Harvard University discussed the process of angiogenesis as a universally relevant and useful model to study the relation of growth factor delivery to tissue regeneration, and stressed the need for control over the spatial and temporal availability of individual or combinations of factors. He reviewed the process of angiogenesis and what has been learned from past efforts to promote angiogenesis with growth factor delivery, polymeric sustained delivery systems, and certain key issues to address in future studies, as well as the potentially broad importance of this topic for tissue engineering.

Polymeric systems allow one to regulate the tissue concentration, duration of signaling, and simultaneous or sequential availability of multiple factors. These levels of control enable the creation of networks of new blood vessels with controlled maturity and increased functionality in rodent models. However, the utility of these systems must be evaluated in larger animals and humans in the future, and, importantly, a quantitative understanding of growth factor signaling and its dependence on the host environment will likely be required to exploit these systems therapeutically. Systems that can provide desirable spatial and temporal signaling of growth factors could find utility in virtually all tissue engineering and regeneration applications.

Morphogens and growth factors regulate developmental, disease, healing, and regeneration processes. The spatial and temporal availability of these molecules is integral to their ability to regulate these processes and control the pattern of tissue formation. One type of tissue formation in which this interplay has been extensively investigated is new blood vessel formation (neovascularization). The cardiovascular system is the first organ system to develop and function in the embryo,[228] pointing to its central importance in development. While the role of the vascular system in the transport of nutrients and waste products has long been appreciated, its importance in regulating the availability of circulating growth factors and stem cells has become clear only recently.[229] Neovascularization results from the processes of vasculogenesis, angiogenesis, and arteriogenesis.[229,230] Angiogenesis is the major process by which vessels are formed in the adult, and serves as an excellent model for examining the importance of controlling growth factor availability in tissue engineering. The engineering of

any tissue of appreciable size (e.g., smallest dimension greater than a few hundred microns), with the exception of cartilage, will require angiogenesis to allow cell survival and tissue function.[231] Cell transplantation approaches to promoting neovascularization will not be specifically reviewed, and the interested reader is referred to a review of that topic.[232]

Angiogenesis and lessons from past studies

Angiogenesis occurs either via the sprouting of new vessels from the sides and ends of existing vessels or via the longitudinal division of a vessel.[233] Vascular endothelial growth factor (VEGF) initiates activation, migration, and proliferation of endothelial cells to form immature new vessels, and these immature vessels are ultimately stabilized through recruitment of mural cells (smooth muscle cells and pericytes).[229] Fibroblast growth factor (FGF) and angio-poietin-2 (Ang-2) act with VEGF to initiate angiogenesis, while platelet derived growth factor (PDGF), TGF-β, and angiopoietin-1 (Ang-1) act during the later stages to mediate maturation of the new vessels by recruiting and promoting interactions with mural cells.[234] The VEGF appears to be a particularly important factor, as a 50% reduction in its expression results in embryonic lethality.[235] While the VEGF family contains many related proteins and isoforms of these proteins, all references to VEGF in this review will refer to $VEGF_{165}$, the most commonly used isoform of VEGF-A.

The large number of patients suffering from ischemic diseases (e.g., coronary artery disease), in concert with the availability of recombinant angiogenic growth factors, has led to significant efforts to promote angiogenesis in a therapeutic context. In spite of promising early animal studies and small-scale clinical studies of therapeutic angiogenesis, large clinical trials with delivery of recombinant growth factors have not demonstrated as significant an effect.[236] In these approaches, single proteins (e.g., VEGF) have been delivered to drive angiogenesis as either a bolus injection(s) into the site of disease or via systemic administration. This strategy is limited due to the inherent instability of these proteins *in vivo* (e.g., the half life of VEGF is 90 min following intravenous injection,[237]) and the low targeting to the tissue of interest (e.g., single intravenous or intracoronary administration of growth factor leads to less than 0.1% retention in the tissue of interest after 24 hours[238]). The premature removal of VEGF likely leads to apoptosis of endothelial cells and regression of the newly formed vessels.[239] These issues have led investigators to deliver large, supraphysiological quantities of these factors in the clinical trials, but this approach may be constrained by possible side effects at distant sites (e.g., expansion of atherogenic plaques, growth of tumors)[236] and a still too rapid decrease in local factor concentration at the site of interest. Altogether, these results indicate that the duration of growth factor availability, and spatial segregation of signaling to the desired tissue will likely be crucial to the success of growth factor delivery to promote angiogenesis.

Another potential limitation of many efforts to promote angiogenesis may be a requirement to provide multiple growth factors, in an appropriate sequence, to promote the formation of a mature and stable vascular network. While VEGF is crucial in the initial stages, PDGF-BB promotes the maturation of blood vessels by recruiting smooth muscle progenitors to the endothelial lining of nascent vasculature. Arrival of these cells to the immature vessels is hypothesized to eliminate the requirement for VEGF.[234] The interaction of the endothelial cells with the pericytes/smooth muscle progenitors then leads to activation of TGF-β, which induces smooth muscle/pericyte differentiation and matrix deposition.[234] Angiopoietin-2 has been demonstrated to augment vascularization, presumably by destabilizing the existing vasculature and making it more responsive to initiators such as VEGF.[234] Angiopoietin-1, which binds to the same cell surface receptor (Tie-2) as Ang-2, appears to play an important role in recruiting and maintaining the endothelial support cells.[234]

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Polymeric systems to control temporal and spatial growth factor availability

Polymeric vehicles allow for sustained and localized delivery of growth factors and may provide an ideal means to regulate the local availability of single or multiple growth factors over desired time frames to promote therapeutic angiogenesis in the context of ischemic disease or as a component of a tissue engineering strategy. A variety of polymers and their varying physical forms have been developed to allow for localized and sustained delivery of various bioactive macromolecules.[240] Both injectable systems that are amenable to minimally invasive delivery,[241,242] and implantable, porous scaffolds[243–246] that may effectively serve as patches or vehicles for the combined delivery of angiogenic factors and cells required to engineer various tissues types (e.g., bone forming or neural cells) have been developed for angiogenic factors.

Providing a multiweek sustained release of VEGF from polymeric systems, in contrast to bolus delivery, leads to a significant increase in the density of blood vessels formed at the site of the polymer implantation,[244] enhances perfusion of ischemic limbs,[245] and prevents necrosis of these limbs (Chen and Mooney, unpublished data). Importantly, these effects can be achieved with total VEGF doses of orders of magnitude lower than what is typically used for bolus delivery, and the sustained VEGF presence in the tissue of interest is accompanied by little to no systemic exposure to the growth factor.[246]

An alternate approach to potentially sustaining the presentation of angiogenic molecules within a tissue of interest is to utilize localized gene therapy in order to promote a local production of the factor by the cells resident in the tissue.[230] The approaches to accomplish this goal to date, while they have led to very promising initial results, may ultimately be limited in their success due to the short-term expression of the plasmid DNA or adenoviral vectors used in these efforts. One approach to extend, in a controllable manner, the expression of the plasmid encoding the factor(s) of interest is to immobilize the plasmid in a polymer system that enables its sustained release. Incorporation of plasmid DNA into poly (lactide-*co*-glycolide) scaffolds can lead to a significant increase in the duration of expression,[247] and condensation of the plasmid prior to polymer encapsulation can significantly increase the level and duration of expression *in vivo*.[248]

Regardless of whether one promotes angiogenesis via direct delivery of a protein, or via indirect gene therapy approaches, it may be necessary to provide multiple factors in specific combinations or sequences to drive the formation of mature and fully functional vessel networks. A combined delivery of multiple factors important in the initiation of angiogenesis (e.g., VEGF and Ang-2) can increase the density of new blood vessels in an additive manner (Ennet and Mooney, unpublished data), but does not enhance the maturity of the vessel networks. A simultaneous delivery of factors involved in angiogenesis initiation and maturation decreases the formation of blood vessels,[249] likely due to the conflicting roles of the early versus later acting factors. However, providing sequential delivery of factors acting in the different stages of angiogenesis can maintain the vessel-forming activity of the early factors, while also promoting the subsequent maturation of these vessel networks.[249]

While the main focus in the development of polymeric systems for delivery of angiogenic molecules is the chemistry of the polymer, physical aspects of the system may also be critical to its performance. External mechanical stresses and strains imposed on the system may alter the kinetics of factor release,[250] and even the intrinsic mechanical properties of the polymer system can alter the responsiveness of the surrounding cells to the therapeutic molecule (e.g., plasmid DNA uptake and expression[251]).

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Future directions and potential for tissue engineering

Therapeutic angiogenesis clinical trials have, together with a large number of more basic studies, identified a number of key issues for the use of angiogenic molecules. These issues will likely be broadly applicable to the use of growth factors or morphogens in tissue engineering and regeneration. Little function typically results from providing supraphysiological concentrations of single factors for poorly controlled time frames and with little regulation over the distance of signaling, even if biological processes are significantly influenced. Instead, sophisticated delivery systems that provide the necessary concentrations, factor gradients, and sequential availability of multiple factors are more likely to initiate and drive the processes to a desired end point, and result in highly functional new tissues.

Polymeric systems offer great promise in enabling control over growth factor signaling, and considerable success with this approach has been achieved in small animal models. The function of these systems must, however, be tested in larger animals and humans to assess their utility. Critically, it will be necessary to understand the quantitative biology of the systems of interest, in addition to the qualitative understanding that is the norm, in order to appropriately design these systems for specific applications. For example, the specific duration of signaling, and the concentration and specific gradient of the factor(s) in the tissue of interest required to both activate and guide tissue formation are typically unknown, and are likely to vary with the age and disease state of the individual. A quantitative understanding of these issues will likely require the development of novel *in vitro* and *in vivo* model systems, and better mathematical models of the biological processes.

David Mooney concluded that polymeric systems that can drive therapeutic angiogenesis could find broad utility in tissue engineering and regeneration. In addition to the obvious utility in treating ischemic diseases,[236] VEGF signaling and angiogenesis are now more broadly appreciated as helpful in regulating many regenerative processes. Bone formation is dependent on VEGF signaling,[252] and the sustained delivery of VEGF from polymers can enhance bone regeneration.[242,253] Nerve and blood vessel wiring is dependent on common mechanisms,[254] and VEGF signaling may be beneficial to slow or reverse various neural diseases.[255] More broadly, angiogenesis is crucial to the survival of transplanted cells. Appropriate delivery of angiogenic molecules has been demonstrated to increase the engraftment of various cell types, including osteoprogenitors[256] and hepatocytes.[257] Stem cell populations may be dependent on vascular niches,[204] and systems that appropriately stimulate angiogenesis may reestablish niches crucial to regeneration and maintenance of tissues.

THE ROLE OF BIOMATERIALS IN DEVELOPING REGENERATIVE TECHNOLOGIES

Anthony Atala from Wake Forest University emphasized that the clinical application of tissue engineering has been elusive, owing to the difficulties in expanding cells outside the body, the challenge of providing adequate vascular support to growing constructs, and the nonavailability of biomaterials capable of controlling cell fate. He stressed that, in nature, there are three pillars of functional regeneration: soluble factors (autocrine and paracrine mechanisms), insoluble factors (ECM), and physical forces (pressure, flow, and shear). For years investigators have sought to use the marriage of biology, materials science, and bioengineering to master the development of tissues either *in vitro* or *in vivo*, but our grip on these three mediating factors is remarkably loose. In particular, the complex interactions between adult stem and progenitor cells and the ECM, and the mechanisms that lead to functional tissue regeneration are poorly defined. Dr. Atala discussed how regenerative cells function to restore injured tissue, and how these mechanisms are being exploited to guide the development of better biomaterials technologies.

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Clinical translation

The term tissue engineering began appearing in the medical research literature as a distinct derivative of its predecessor, cell therapy, in the late 1980s. Although the proceedings from one of the first tissue engineering conferences were published soon thereafter,[258] the potential of tissue engineering to provide tissues and organs to millions of patients suffering from trauma, congenital defects, and chronic diseases has not yet been fully recognized. Unfortunately, few notable successes of clinical translation have been achieved since that time.

People often point to the first tissue engineered product approved for clinical use by the Food and Drug Administration, Dermagraft™, as one of the few examples. Despite the obvious technical and regulatory achievement, Dermagraft has been a commercial disappointment and was sold by the developer, Advanced Tissue Sciences (ATS), to Smith & Nephew for only $12 million in 2002, as part of ATS's bankruptcy liquidation. In October 2005, Smith & Nephew announced that it would exit Dermagraft and related products from the market. The ATS and Dermagraft experience has shown that strong science does not necessarily translate into strong sales. Knowing this, how do we as scientists bring tissue engineering technology from the bench to the bedside?

Interactive biomaterials

In its simplest form, tissue engineering is the combination of biomaterials and cells into a construct that will eventually become the regenerated tissue. In early years, the biomaterial scaffolds were degradable synthetic polymers and naturally derived materials, primarily collagen, that had been used for decades in medical device applications. The view at the time was that if a proper temporary architecture was provided to the cells, functional tissue would form. It was soon determined that cellular interaction with the insoluble environment was as important as it was complex. In an effort to move the science forward, many investigators began to consider the merits of combining the benefits of various types of materials. Specifically, cell binding was considered to be of great importance to the maturation of a construct into functional tissue.

It is well known that cellular recognition is facilitated by the binding of cell surface integrins to specific amino acid motifs of the ECM.[259,260] The predominant ECM proteins are collagen and fibronectin, both of which have been extensively studied with regard to cell binding.[261] Fibronectin contains several regions that support attachment by a wide variety of cell types.[262] Mould *et al.* showed that in addition to the widely known Arginine-Glycine-Aspartic Acid (RGD) motif, the “X”-Aspartic Acid-”Y” motif on fibronectin is also recognized by the integrin a_4b_1, where X represents Glycine, Leucine, or Glutamic Acid, and Y represents Serine or Valine.[263] Remarkably, it was not until well after the discovery of these important cellular interactions that published reports of the efforts of biomaterial scientists to employ synthetic schemes to incorporate protein binding motifs into synthetic polymers first began to appear in the literature.[264–267]

One explanation for this apparent lag in the development of interactive biomaterials is the necessity to employ materials with a proven history of clinical use. Another is that those most knowledgeable about the interactions between growing tissue and its ECM are typically developmental biologists, a group not heavily involved during the nascent stages of the tissue engineering field. Nevertheless, the concept that a combination of natural and synthetic polymers alone will yield successful results in our quest to recapitulate developmental biology is unrealistic. The native ECM is simply too complex to be mimicked accurately.[268–271] This complexity has been shown to be highly cell specific, although most studies fail to elucidate specific mechanisms, particularly with respect to the role ECM plays in recruiting and controlling the differentiation of stem and progenitor cells.[272,273] How then do we overcome

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this circumstance? Do we continue down the current path, looking to the immediate past for solutions to our future, or do we undertake yet another renaissance in biomaterials development? It is apparent that recent advances in stem cell biology compel the biomaterials research community to reexamine its course.

Cell sources

It is widely accepted that almost every tissue in the body, including brain,[274] liver,[275] circulating blood,[276] and heart,[277] as well as skin[278] and fat,[279] contains some type of stem cell or progenitor cell. Preclinical application of these cell sources is beginning to take shape, and as techniques are developed for the isolation, purification, and controlled differentiation of these cells into mature, functional tissues, successful patient treatment paradigms can be envisioned. However, while many differentiated cell types grow well in culture after being isolated from native tissue, stem and progenitor cell characteristics can easily be lost and progeny diverted from their desired terminal fate when cultured under inadequate conditions, that is, without a supportive coating or feeder layer.

Methods to ameliorate this dilemma have been developed in certain cell types and are in routine use at the basic and applied research levels. For example, coating tissue culture plastic with natural biomaterials such as collagen or fibronectin is somewhat effective, and the use of three-dimensional gels such as collagen or Matrigel™ has been successful. However, there is no evidence to suggest that these are optimal biomaterial systems for inducing cell differentiation. On the contrary, recent studies have shown that slight variations in the composition of ECM surrogates can have a dramatic impact on cell growth and differentiation,[280] but have yet to be developed for many cell types. Moreover, several authors have pointed out that ECM composition is more tissue specific than currently appreciated.[281–283] Others have shown experimentally that ECM is able to mediate a regenerative process by attracting stem cells,[279,284] but that these environments are not necessarily optimal for efficient differentiation and functional tissue development. Given that even subtle changes in ECM composition can have a dramatic effect on cellular differentiation,[280,285] it is reasonable to assume that a multitude of possibilities exist and we have merely scratched the surface.

Anthony Atala summarized that the application of developmental and stem cell biology to biomaterials development offers an astounding number of possibilities, yet our understanding of this interplay in the context of tissue engineering remains incomplete. Any textbook on the composition of ECM will almost universally contain the same basic chapters: collagens, fibronectin, elastin, laminin, and perhaps another section on proteoglycans. A recent literature search of the key words "extracellular matrix" and "biomaterial" yielded 1,432 hits. If one adds the term "biglycan," a critical ECM proteoglycan in bone formation, the number of hits drops to only three. This begs the question, are collagen, fibronectin, laminin, and elastin the most widely studied ECM-related biomaterials because they are the most important, or because they are the most easily studied?

Most approaches to biomaterials development for tissue engineering fail to take into account the tremendous complexity of the ECM. This is understandable given that most analytical tools for studying the composition of the ECM are solution-based techniques, with the exception of immunostaining methods. Also, given the fact that complete digestion of the ECM without significantly disrupting the integrity of the constituent molecules is extraordinarily difficult, it is not surprising that biomaterials development has fallen so far behind advances in cell and molecular biology. In order to overcome these inherent challenges, efforts need to be focused on the application of existing tools in ways that have never been tried and the development of new tools that will allow investigators to completely reverse engineer the ECM. Only through a complete understanding of the compositional gradients, temporal changes, and remodeling

of even the most minute constituents of the ECM will we be able to use the solid cellular environment to drive tissue and organ morphogenesis.

Acknowledgments

Antonios Mikos thanks Simon Young and Mark Wong for help with the preparation of this article, and the National Institutes of Health (NIH) for funding support (R01 DE15164). Susan Herring thanks Patricia Emry and Frank Starr for help with the procedures, and the National Institute of Dental and Craniofacial Research (NIDCR) for funding support (PHS award DE08513). Helen Lu thanks Kristen Moffat for help with the preparation of this article, and the Whitaker Foundation, the Wallace H. Coulter Foundation, the NIH (NIAMS AR052402), and the National Science Foundation (GK-12 Graduate Fellowship) for funding support. Rita Kandel thanks M. Grynpas, M. Hurtig, and R. Pilliar for help with the preparation of this article, and the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council, and the Arthritis Society for funding support. Mehmet Toner thanks Alex Fowler for help with the preparation of this article. David Mooney thanks NIDCR and the NHLBI for funding support.

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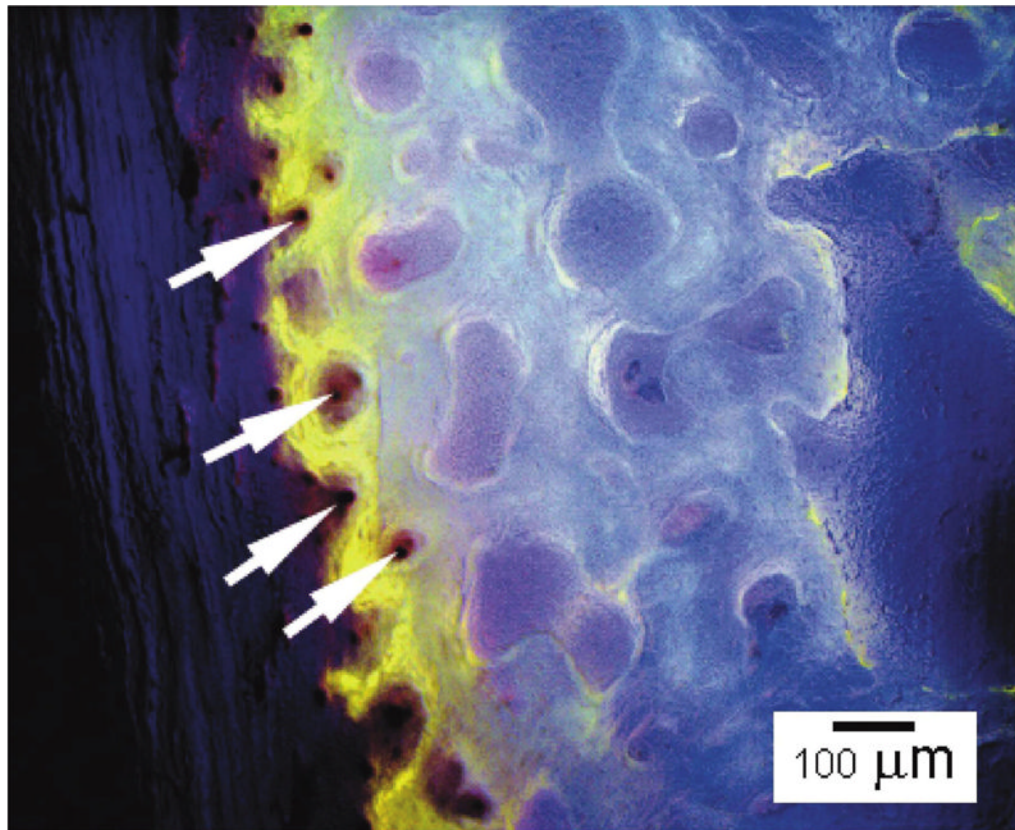

FIG. 1.

Lateral surface of the zygomatic bone, coronal section, epifluorescent illumination. Light color is the 3-hour calcein label in a continuous layer of newly mineralized matrix. Vascular fill (red) is seen in the periosteal vessels and in cross-sectioned vessels within the calcein-labeled layer (arrows). Color images available online at www.liebertpub.com/ten.

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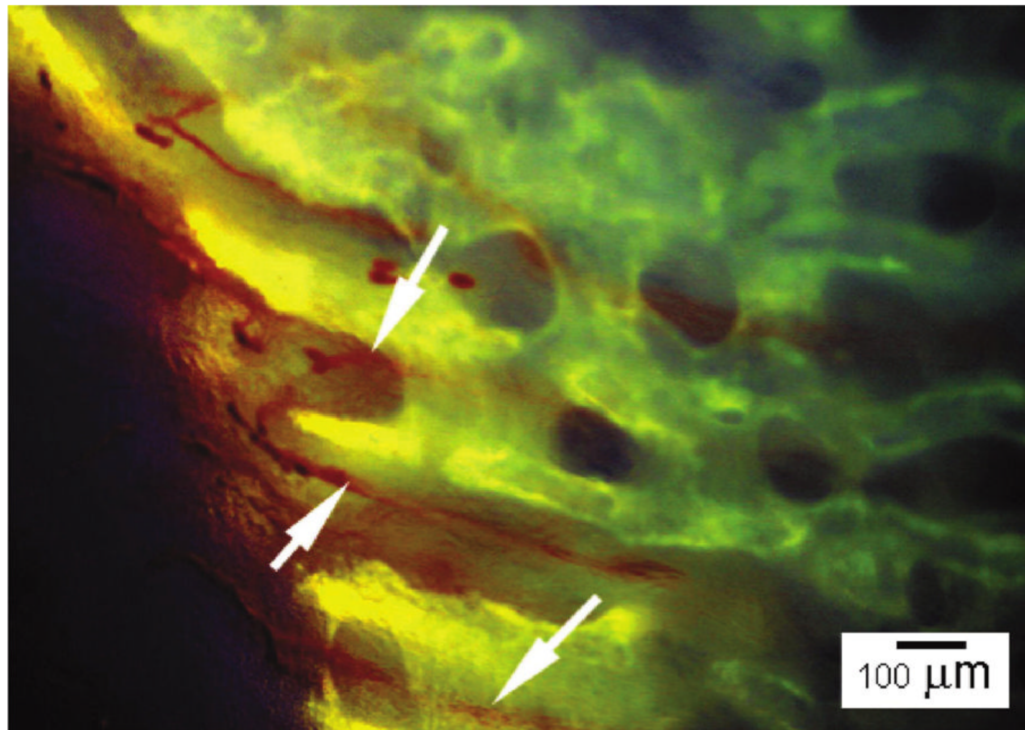

FIG. 2.
Lateral surface of the temporal bone, coronal section, epifluorescent illumination. The 3-hour calcein label is discontinuous and forms the tips of bony spicules. Vascular fill (red) shows that the radially arranged vessels of this layer are continuous with intraosseous vessels (arrows) and differ in orientation from the periosteal vessels. Color images available online at www.liebertpub.com/ten.

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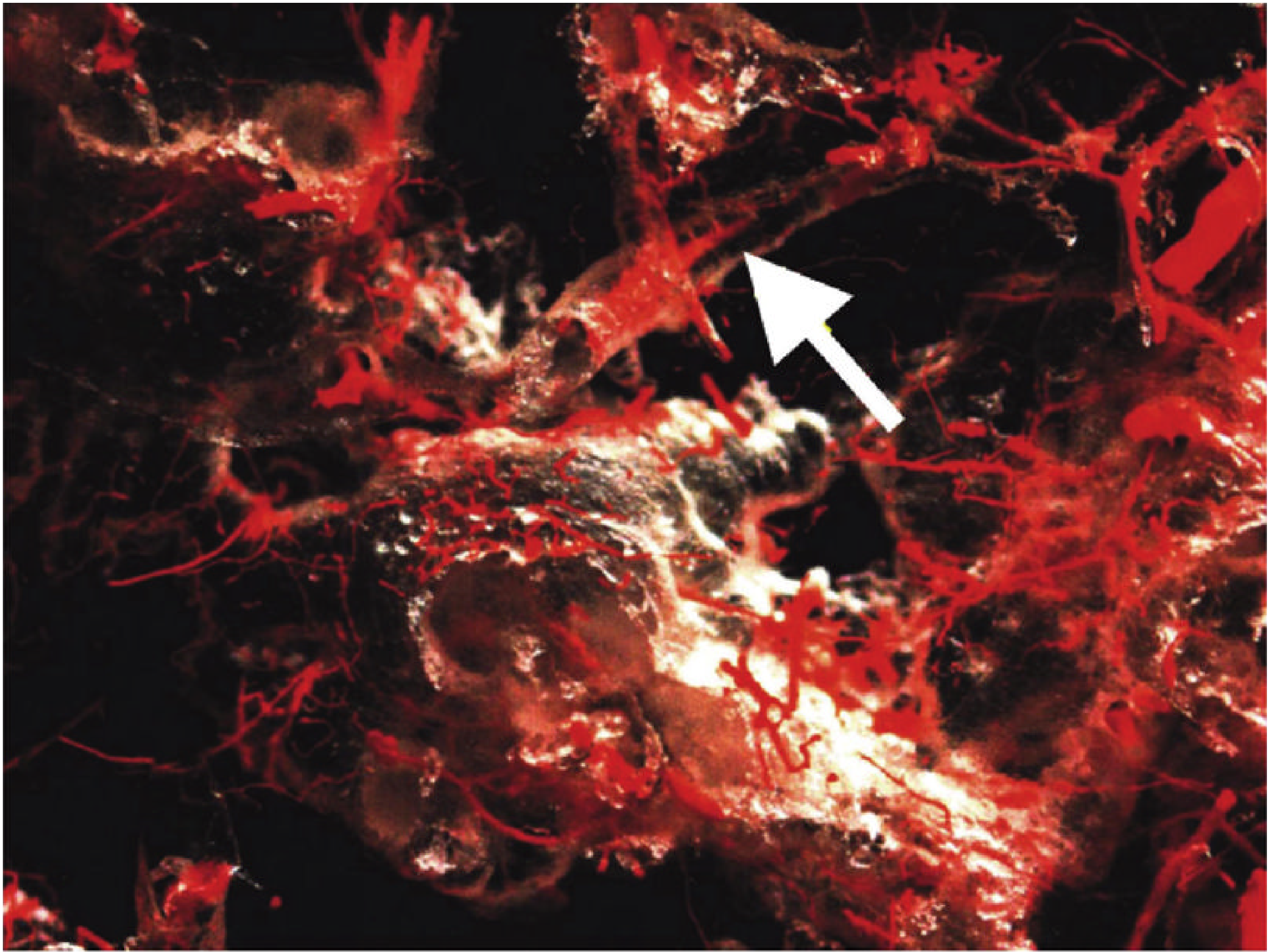

FIG. 3.
Lateral portion of the zygomatic bone, parasagittal section. An intraosseous vessel near the surface can be seen encased in a bony tube (arrow). Color images available online at www.liebertpub.com/ten.

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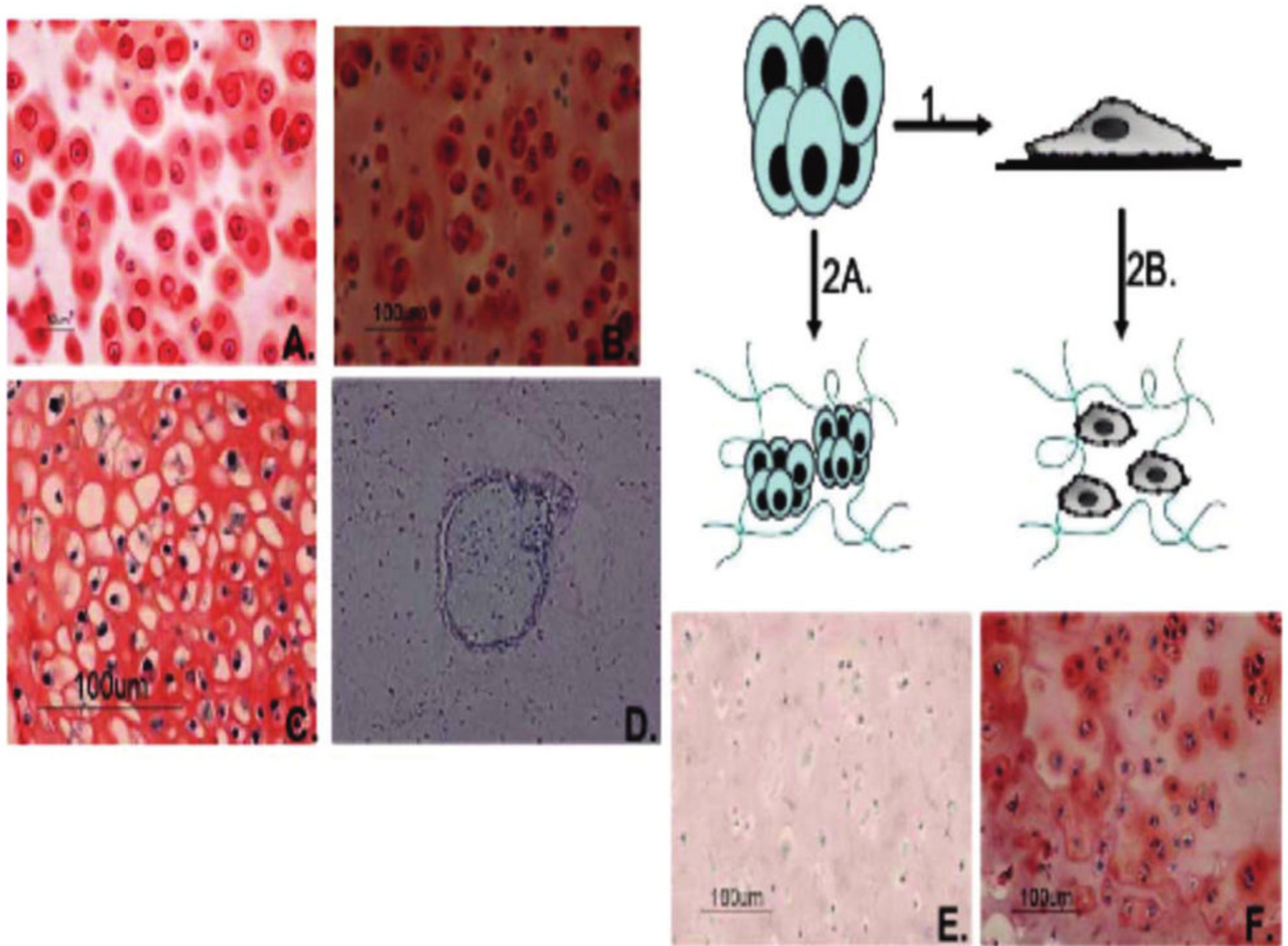

FIG. 4.

Cartilage has been engineered using a number of different cell types. Primary bovine chondrocytes (**A**), caprine MSCs (**B**), mouse EBs (**C**), and human EBs (**D**) demonstrate Safranin-O positive proteoglycan production in polyethylene glycol (PEG) gels with the exception of human EBs, which did not undergo chondrogenesis. Human embryonic stem (ES) cells were aggregated into EBs and either encapsulated directly (2A) or disaggregated and cultured for five passages (1) before encapsulation (2B). The hEBs that were encapsulated into PEG gels did not stain positive for Safranin-O (**D**). Cells derived from the hEBs (1) encapsulated in PEG gels also did not stain positive for Safranin-O when cultured in chondrogenic medium with TGF-β1 (**E**). Incorporation of the adhesion peptide sequence YRGDS into the PEG gels promoted homogenous differentiation and formation of cartilage-like tissue from hES-derived cells (**F**), confirming the unique requirements for human ES cell differentiation. Reproduced with permission from *Annals of Biomedical Engineering*. Color images available online at www.liebertpub.com/ten.

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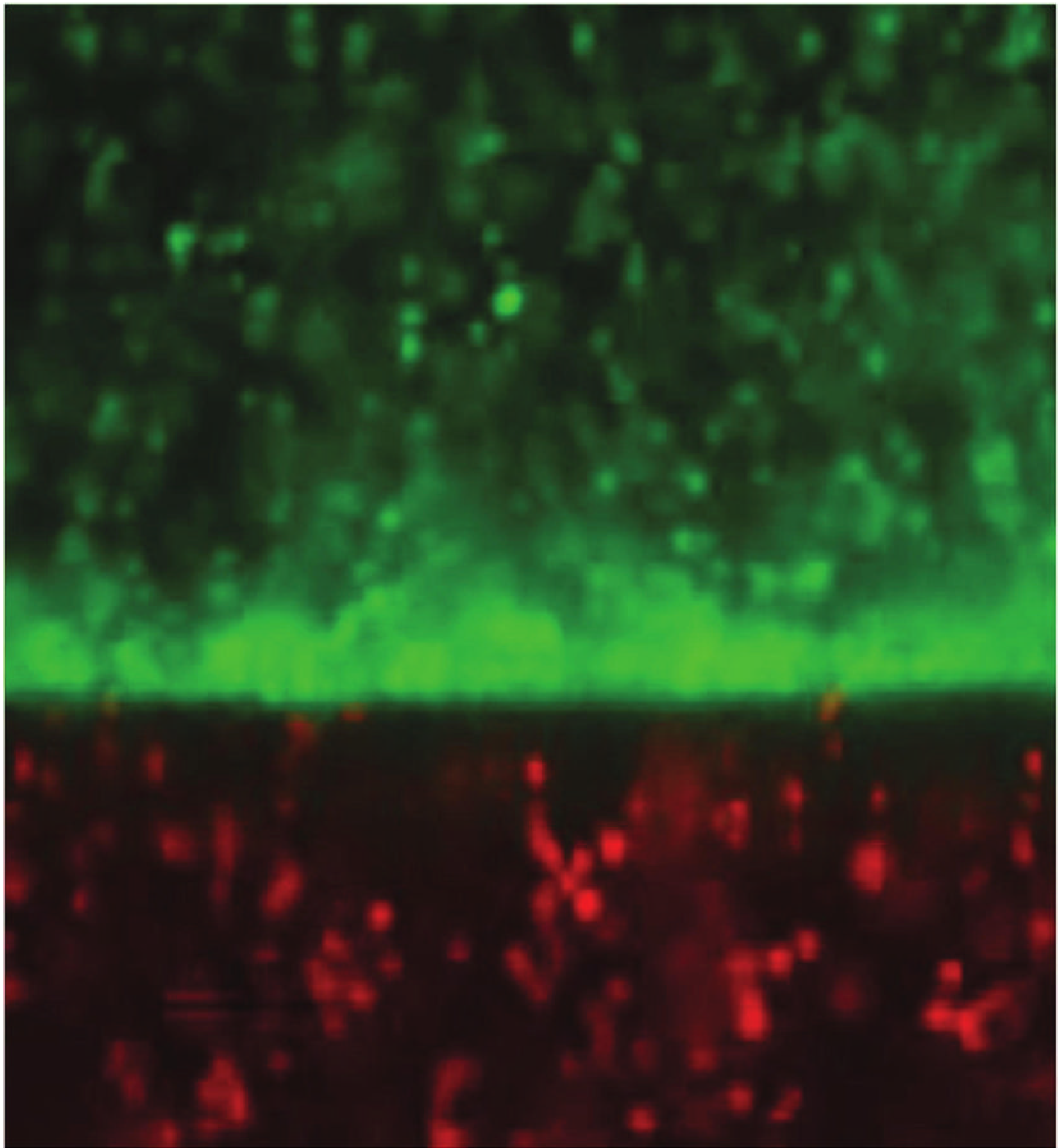

FIG. 5.
Cells labeled with colored dyes encapsulated in a bilayered hydrogel. Organized tissue can be created or interactions of coculture environments can be studied. Reproduced with permission from *Annals of Biomedical Engineering*. Color images available online at www.liebertpub.com/ten.

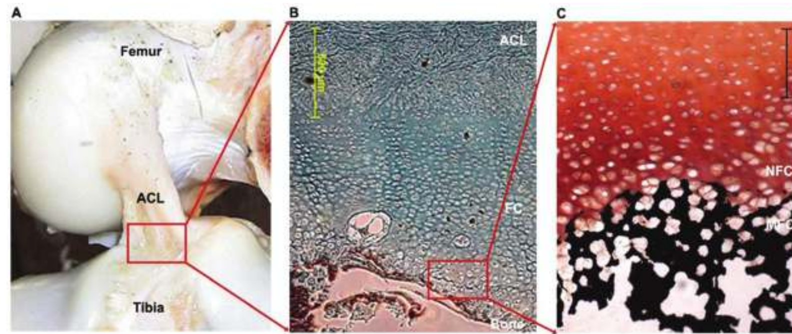

FIG. 6.

Anatomy and matrix organization of the ligament-to-bone insertion site. (**A**) The anterior cruciate ligament (ACL) connects to the femur and tibia through two insertion sites (posterior view). (**B**) The multitissue organization of the tibial insertion, transiting from the ACL to fibrocartilage (FC) region, and then to the bone region (modified Goldner's Masson Trichrome, *bar* = 500 μm).[49] (**C**) The fibrocartilage interface is further divided into the nonmineralized fibrocartilage (NFC) and mineralized fibrocartilage (MFC) zones (von Kossa, *bar* = 200 μm). Color images available online at www.liebertpub.com/ten.

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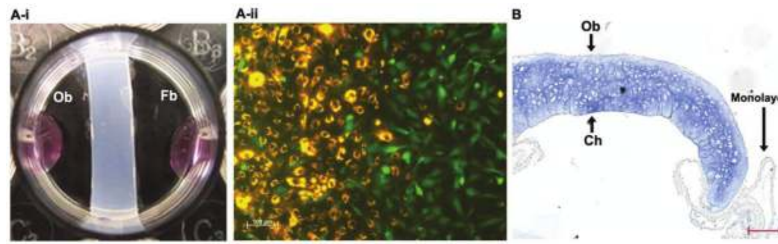

FIG. 7.

Coculture models to evaluate interaction of interface-relevant cells. (**A-i**) *In vitro* coculture model of fibroblasts (Fb) and osteoblasts (Ob) permit heterotypic and homotypic cell-cell interactions.[88] (**A-ii**) Fibroblast (CFDA-SE, green) and osteoblast (CM-DiI, orange-red) distribution at day 7, *bar* = 100 μm. (**B**) *In vitro* coculture model of chondrocytes (Ch) and osteoblasts (Ob), established by forming an osteoblast monolayer on top of the chondrocyte micromass. Glycosaminoglycan distribution was restricted to the chondrocytes micromass during coculture (day 10, Alcian blue, *bar* = 100 μm). Color images available online at www.liebertpub.com/ten.

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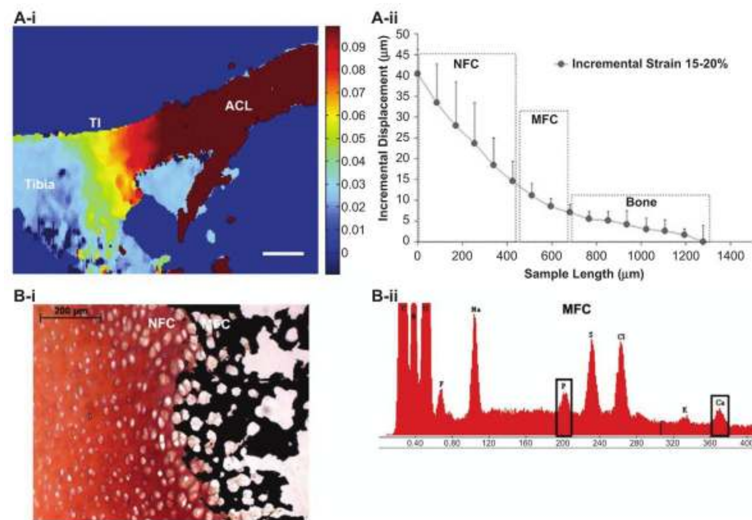

FIG. 8.

Structure-function relationship at the ligament-to-bone insertion. (**A-i**) Elastographic analysis of the ACL-to-bone insertion (TI) under applied uniaxial tension.[97] Displacement map calculated from ultrasound radio frequency data (Increase in magnitude in mm: blue to red, *bar* = 5 mm). A region-dependent decrease in displacement is a result of increasing tissue stiffness from the ligament to fibrocartilage region and to the bone region. (**A-ii**) Microcompression testing of the ACL-to-bone insertion also revealed region-specific increase in tissue stiffness from the nonmineralized (NFC) to mineralized fibrocartilage (MFC) and to bone.[102] In the displacement curve, slope of the curve in each region represents the strain, with a less steeper slope for MFC, indicating decreased strain compared to the NFC zone. (**B**) The increase in tissue stiffness across the interface may be related to the higher calcium phosphate distribution from the NFC to MFC, and to bone (**i** von Kossa and **ii** Elemental analysis of the MFC revealed presence of Ca and P at the insertion[104]). Color images available online at www.liebertpub.com/ten.

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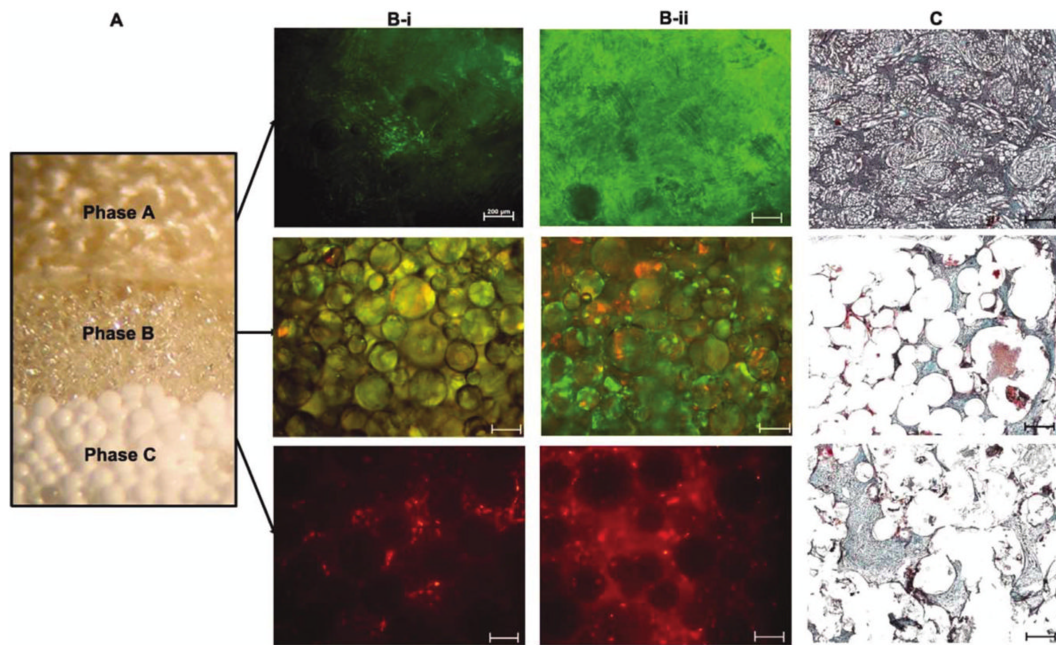

FIG. 9.

Biomimetic multiphasic scaffold for interface tissue engineering: Design, *in vitro* and *in vivo* testing. (**A**) Triphasic scaffold modeled after the three regions of the interface[112]: Phase A for soft tissue, Phase B for the fibrocartilage region, and Phase C for bone. Phase A consists of knitted degradable polymer mesh, Phase B of sintered degradable polymer microspheres, and Phase C of osteointegrative polymer-ceramic composite microspheres.[11] (**B**) *In vitro* coculture of fibroblasts and osteoblasts on the triphasic scaffold resulted in phase-specific cell distribution and controlled matrix heterogeneity.[112] Fibroblasts (Calcein AM, green) were localized in Phase A and osteoblasts (CM-DiI, red) were found in Phase C at day 1 (**i**) and day 28 (**ii**). Both osteoblasts and fibroblasts migrated into Phase B by day 28. (**C**) *In vivo* evalution of the triphasic scaffold cocutlured with fibroblasts and osteoblasts revealed abundant tissue infiltration and matrix production at 4 weeks postimplantation.[113] (modified Goldner's MassonTrichrome, *bar* = 200 μm). Color images available online at www.liebertpub.com/ten.

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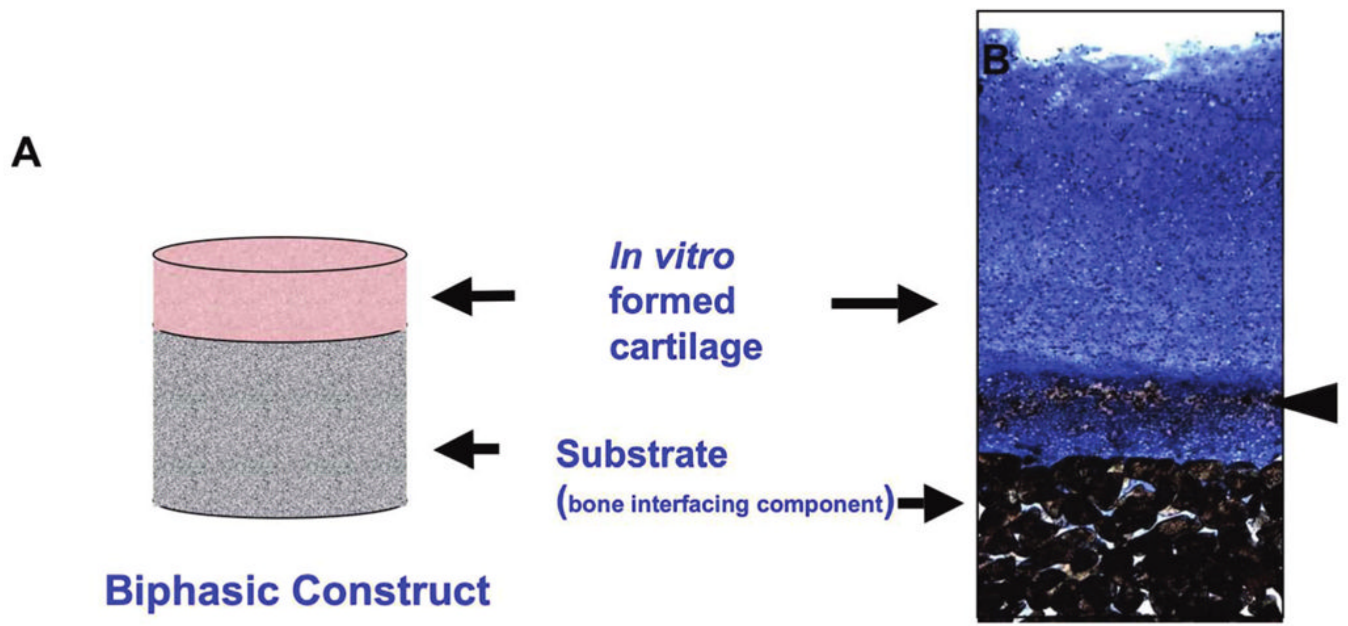

FIG. 10.
(**A**) Line drawing of biphasic construct. (**B**) Histological appearance of biphasic construct at 8 weeks of culture. The cartilage is integrated with the upper aspect of substrate and has a nonmineralized zone and a mineralized zone (arrowhead) adjacent to the substrate (von Kossa and toluidine blue, ×50 original magnification). Color images available online at www.liebertpub.com/ten.

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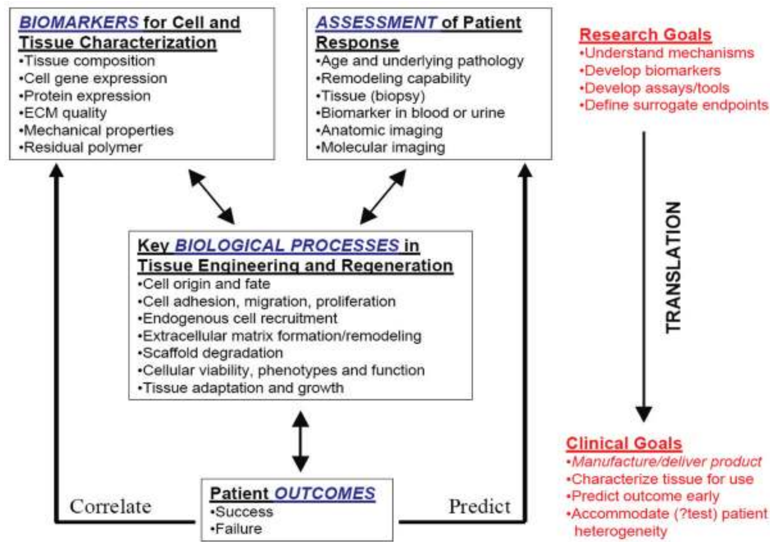

FIG. 11.

Paradigm for translating research in heart valve tissue engineering from the laboratory to the clinic. Biomarkers for cell and tissue characterization in conjunction with structural, chemical, and molecular information obtained via *in vitro* and *in vivo* models are necessary for understanding key biological processes in tissue engineering and regenerative medicine. These concepts and data can be used to predict and measure patient success and failure. Data from clinical experience further informs the development of appropriate biomarkers, which may result in reassessment of the appropriate characterization parameters. Reproduced and modified with permission from Mendelson *et al*.[141] Color images available online at www.liebertpub.com/ten.

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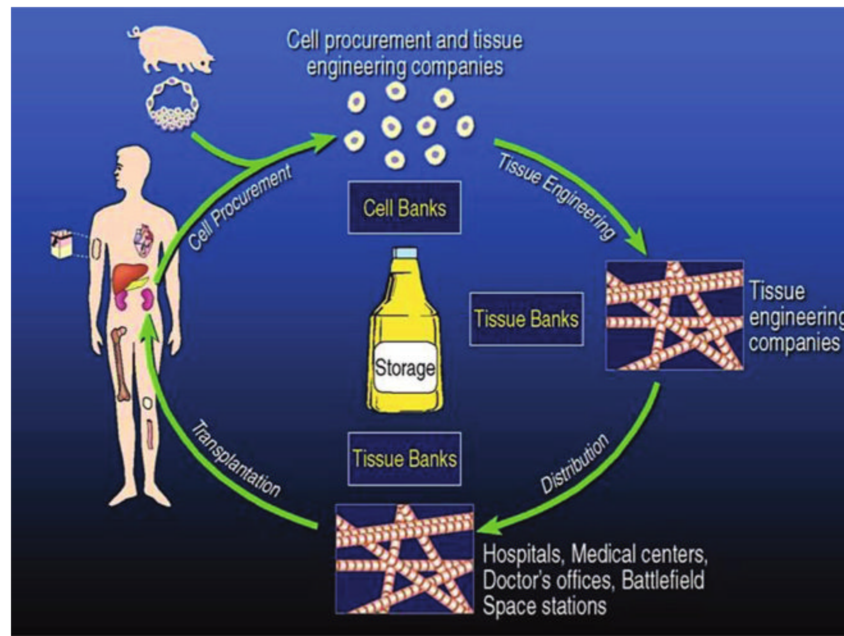

FIG. 12.
Long-term storage of cells is critical for the successful applications of tissue engineered products. Cell and tissue banks are needed at various steps in the development of tissue engineered products. Reprinted with permission from Acker, J.P., Chen, T., Fowler, A., and Toner, M. Engineering dessication tolerance in mammalian cells: tools and techniques. In: Fuller, B.J., Lane, N., and Benson, E.E., eds. Life in the Frozen State. Boca Raton, FL: CRC Press, 2004. Color images available online at www.liebertpub.com/ten.

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Table 1

Key Aspects of Tissue Engineering of Oral and Craniofacial Tissues

Tissue Engineering Aspect	Challenge
Wound healing environment	• Regeneration of lost tissues in essentially “contaminated” conditions.
Scaffold design requirements	• Injectability. • Ability to encapsulate cells. • Ability to harden to a state which mimics the mechanical properties of bone. • Degradation at a rate fast enough to allow the in-growth of surrounding tissue.
Cell-surface interactions	• Ability to selectively promote the adhesion of specific cell populations while excluding the invasion of others.
Growth-factor delivery	• Increased efficiency by loading physiological amounts of growth factors into a scaffold allowing for improved cost-effectiveness. • Spatial and temporal control over the release of multiple growth factors using multiple kinetic rates.
Assessment	• Development of clinically-relevant animal models for *in vivo* qualitative and quantitative assessment of implanted materials.
Scale-up	• Ability to use tissue engineered constructs in large defects where diffusional limitations may limit the viability of encapsulated cells. • Ability to determine the translatability of results seen with *in vivo* animal models in the clinical setting.
Specific clinical issues which remain unaddressed	• Control over the morphology of regenerated bone. • Periodontal ligament anchorage and effective gingival seal around titanium dental implants. • Regeneration of entire teeth with supporting structures in extraction sockets.