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Instructional lecture

Engineering cartilage and bone using human mesenchymal stem cells

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Introduction

Cartilage and bone defects are leading causes of disability. The economic burden of orthopedic repair exceeds 28 billion dollars per year in the United States alone,[1] and the situation is similar in many other countries. Although artificial joints or metal inserts are widely utilized and in most cases work well, cell-based therapies based on tissue-engineered cartilage and bone beckon a new frontier for clinical treatment owing to their biocompatibility and long-term prognosis. Functional tissue engineering[2] involves an integrated use of three components — cells, scaffold material, and a bioreactor (Fig. 1), — in settings that mimic some elements of the in vivo environment. Synergistic interactions of biomimetic cues applied with temporal and spatial regulation influence cell growth and biosynthesis and guide cellular development into functional replacement tissue constructs. This article discusses the design criteria and parameters essential for engineering cartilage and bone grafts as well as the current status and future perspective of the field.

Tissue engineering

Cells

Cells are the actual "tissue engineers," and several considerations guide the choice of cell sources. The cells are ideally immunocompatible, such as autologous chondrocytes for cartilage repair. The use of chondrocytes, however, is limited by their availability and their capacity for expansion in culture, as well as the need for a separate surgery to harvest the cells. The proliferative characteristics of autologous adult mesenchymal stem cells (MSCs) and the less invasive procedures for their procurement are thus considered important advantages. Although MSCs have better expandability than differentiated cells, extensive expansion is known to decrease their biosynthetic capacity and multipotency.[3] Clearly, cells used for tissue engineering need to be biosynthetically active. Mauck and coworkers reported that when bovine chondrocytes and bone-marrow derived MSCs from the same animals were cultured under identical conditions chondrocytes not only created a significantly more functional tissue than MSCs they also maintained the improvement in biosynthetic and mechanical properties after 10 weeks of culture, by which time the MSCs had already reached a plateau.[4] Therefore, optimizing the methods for MSC isolation, expansion, and differentiation remain critical for their effective use in tissue engineering and regenerative medicine.

Scaffold

Scaffolds are designed to support spatially uniform cell attachment and to promote cell differentiation. Chemical properties that determine surface molecules and degradation patterns and structural properties such as pore size, orientation, and void volume are important in this respect. Scaffolds also serve as logistic templates for guiding tissue formation. The incorporation of regulatory molecules such as growth factors, along with the mechanical properties of the scaffolds at various hierarchical levels, plays major roles in molecular and biophysical regulation of cell differentiation. For example, fiber alignment could be optimized to enhance the in vitro formation of intervertebral disc and meniscus tissue constructs.[5,6] Hydrogels, with their ability to maintain cells in their spherical shape, were shown to be beneficial in supporting the chondrogenic phenotype.[7] For cartilage and bone tissue engineering, scaffold materials need to have appropriate mechanical properties that sustain load, and their degradation should correlate with advances in tissue growth. For example, silk

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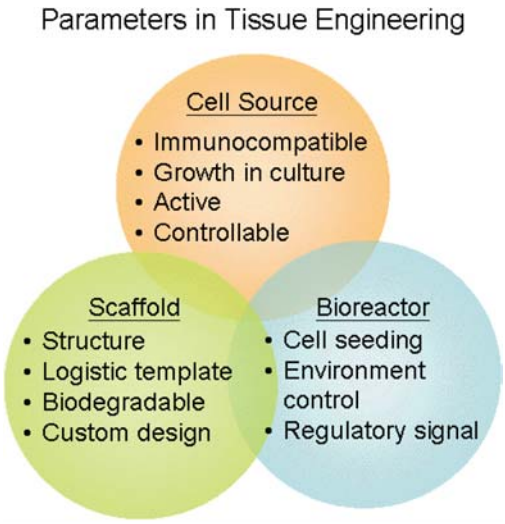

Fig. 1. Main components of tissue engineering

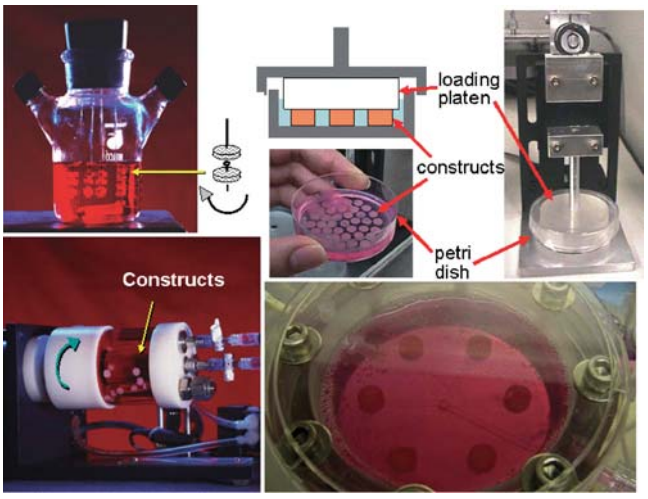

Fig. 2. Examples of bioreactor designs. *Clockwise from top left*: Spinner flask bioreactor with constructs threaded to long needles and on a magnetic spinning plate. Dynamic deformational loading device with an eccentric cam that loads samples in a 60-mm culture dish. (Courtesy of Dr. Clark Hung) Perfusion bioreactor with six wells, each perfused at a well-defined flow rate. (Courtesy of Dr. Chris Cannizzaro) Rotating vessel bioreactor where the vessel rotation maintains the cultured tissue constructs in a state of free suspension and exposed to dynamic changes in fluid pressure and velocity. (Courtesy of Sam Ogden)

fibroin can be modified to have pore sizes[8] ranging from 470 to 940 µm and can be functionalized, mineralized, or used as a hydrogel.[9] Custom-designed materials with appropriate structural, chemical, mechanical, and signal transmission properties are currently being developed. Further advances are being made by incorporating controlled delivery of multiple growth factors or genes.

Bioreactor

In addition to scaffolds, bioreactors provide a biomimetic environment for optimizing cell functions (Fig. 2). They enhance nutrient transport and waste removal and supply the necessary regulatory signals, such as dynamic compressive loading for cartilage and hydrodynamic shear for bone. For example, fluid shear has been shown to promote osteopontin and osteocalcin gene expression in MSCs,[10] and perfusion bioreactors have been designed to provide mass transport and shear in cultured constructs to promote osteogenesis. Bioreactors can also facilitate seeding in three-dimensional scaffolds where uniform distribution would otherwise be difficult.[11] When designing bioreactors, one also has to consider environmental factors such as gas exchange, temperature control, and long-term sterility. The ability for online evaluation of tissue development is also beneficial, such as noninvasive imaging[12,13] and mechanical testing.[14] For cartilage and bone tissue engineering bioreactors, the magnetic resonance imaging (MRI) and microcomputed tomography (micro-CT) are among the most favored imaging options.

Cartilage tissue engineering

To engineer "successful" cartilage replacements, several outcome measures are important for determining the functionality of the tissue. Cartilage provides lubrication and sustains load by its ability to pressurize fluid within its matrix. The charged macromolecules [proteoglycans and glycosaminoglycans (GAGs)] provide hydration and electrostatic load, whereas the collagen network provides tension that keeps in balance the tissue swelling that would result from pressurization. Therefore, functional indicators of engineered cartilage grafts include the appropriate GAG and collagen contents, adequate compressive moduli, and frictional coefficients. It is also important to monitor the spatial and temporal development of the tissue matrix to achieve mechanical competence along with the capacity for integration with host tissues. The following sections provide some examples of the design considerations for scaffold materials and bioreactors.

Scaffold

Naturally occurring biomaterials, such as collagen, provide appropriate biological cues to guide cell fate. However, collagen scaffolds are impaired by their poor mechanical integrity. Meinel et al. found silk scaffolds to be more chondrogenic than collagen scaffolds, possibly because of their higher porosity and better maintained structure and mechanical properties[15] (Fig. 3A). Porous scaffolds can also be combined

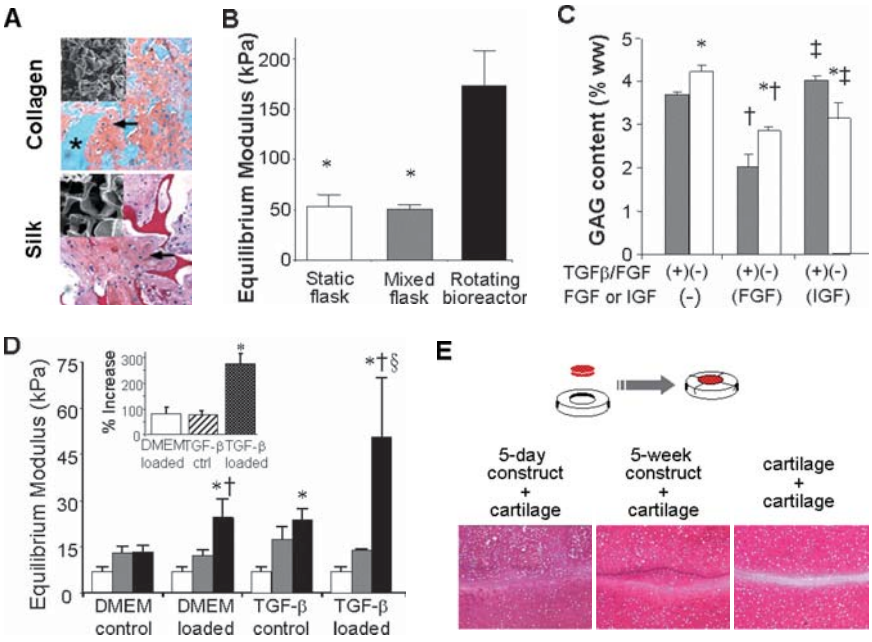

Figure 3. Cartilage tissue engineering. **A** Effects of scaffold material.[15] **Inset** SEM image of the scaffold. **B** Effect of mechanical stimulation on compressive modulus[21] (*$P < 0.001$ vs. rotating vessel bioreactor). **C** Effects of growth factor on GAG production.[19] *Significance of transforming growth factor-β1/fibroblast growth factor-2 [(+)TGFβ1/FGF-2]. †Significance of FGF-2. ‡Significance of IGF. **D** Interaction of growth factors and mechanical stimulation. *Open bars*, day 0; *gray bars*, day 14; *black bars*, day 35. *Significant difference from day 0. †Significant difference of loaded constructs in the same group at the same time point. §Significant difference from the control construct at the same time point.[38] **Inset** Percentage increase at day 35. *Significant difference from other groups. **E** Integration properties of engineered construct with cartilage explant[23]

with hydrogels to create heterogeneous cartilage constructs.[16]

Nutrient supply and growth factors

Although cartilage maintains chondrocytes in a relatively anaerobic environment, studies have demonstrated that a sufficient supply of oxygen and nutrients is essential for chondrocyte anabolism.[17,18] Furthermore, growth factors modulate the expansion and chondrogenic phenotype in both chondrocytes and MSCs. Pei and coworkers demonstrated that initial treatment with fibroblast growth factor-2 (FGF-2) and transforming growth factor-β1 (TGFβ1) dedifferentiated bovine articular chondrocytes and promoted cell expansion, whereas insulin-like growth factor-1 (IGF-1) redifferentiated these cells and promoted the chondrogenic phenotype[19] (Fig. 3B). This sequential treatment of growth factors appears to be important in promoting the chondrogenic phenotype, as it was shown by Byers et al. that transient exposure of TGFβ3 enhances the mechanical property of chondrocyte-laden hydrogels.[20]

Mechanical regulation

To mimic the physical environment in vivo, many groups have developed bioreactors to apply mechanical stimulation to the tissue-engineered constructs. Providing laminar shear flow with rotating vessel bioreactors enhanced biosynthesis as well as mechanical properties[21] (Fig. 3C). Bioreactors also improved mass transport in the constructs. It appears that the increased nutrient supply can interact synergistically with the mechanical signals to advance tissue growth. For example, by combining dynamic deformational loading and TGFβ1, chondrocyte-laden agarose demonstrated an almost threefold increase in compressive modulus over free swelling controls[22] (Fig. 3D).

Integration

To remain functional in vivo, an engineered construct has to integrate with the host tissue. Mature articular cartilage, however, does not integrate well with host tissue, which may lead to construct degeneration following implantation. Obradovic and coworkers tested the integration properties using an in vitro explant ring model where the engineered construct (or cartilage explant) was inserted into an explant ring (Fig. 3E) and monitored in culture.[23] It was found that developing constructs (i.e., within 1 week of culture) integrated markedly better with the explant rings than did more mature constructs (i.e., after 5 weeks of culture). Further examination indicated that proliferating cells at the edge of developing constructs were important for the remodeling of the integrating interface and for the formation of the tissue bond that provided integration.

Bone tissue engineering

The clinical need for bone grafts has motivated the development of biomaterials and bioreactor systems that maximize the potential of mesenchymal stem cells to exhibit a differentiated osteoblastic phenotype and

form functional bone tissue. Various studies have demonstrated that cell differentiation and the quality of the engineered graft are influenced by the scaffold properties and application of biophysical stimuli.

Scaffolds

Scaffold biochemistry and degradation properties critically influence the osteogenic capacity of MSCs. For example, various studies have demonstrated that incorporating mineralized components such as hydroxyapatite into scaffold structure increased the expression of osteoblastic markers. When nonmineralized scaffolds were used, human MSC-seeded collagen sponges exhibited considerably less mineralized deposits than silk scaffolds because they degrade more quickly and are unable to provide the proper structure for tissue development over time.[24] Silk substrates with varying pore sizes were used to determine the effect of scaffold microarchitecture with bone marrow-derived human MSCs.[25] Upon exposure to osteogenic factors, the MSCs produced mineralized nodules in all silk scaffolds; however, the size and distribution of these nodules were influenced by the initial pore structure of the silk scaffolds. Specifically, in scaffolds with pore sizes of 100–200 μm the trabecular nodules were very small and highly connected. When pore sizes of 400–500 μm were used the sizes of the nodules increased, and they exhibited an open structure that more closely resembled that of native bone. Intermediate pore sizes resulted in trabeculae with transitional architecture (Fig. 4A).

Mechanical regulation

The quality of tissue-engineered bone grafts is affected by the uniformity of cell growth and mineral distribution throughout the scaffold. Limited nutrient transfer in static culture results in significantly inhomogeneous cell distribution. Bioreactors improve nutrient transfer to cells by providing media perfusion through the constructs. An intrinsic effect of convection is the shear stress, a biophysical stimulus imparted to cells. Various studies done in two-dimensional systems demonstrate that shear stress can be mechano-transduced by osteoblasts, osteocytes, and undifferentiated stem cells to result in increased mineralization and up-regulation of osteogenic gene expression.[26] Similarly, higher flow during culture improves the quality of engineered bone grafts. To decouple the effects of increased nutrient transfer from increased shear stress, Sikavitsas et al. utilized dextran molecules to obtain culture media of varying viscosities.[27] Rat osteoblasts demonstrated enhanced mineralization in tissue constructs subjected to increased shear (with comparable levels of nutrient transfer) (Fig. 4B). It should be noted, however, that despite the improvement in graft sizes and homogeneity using perfusion bioreactors, it is not yet possible to grow grafts more than several millimeters thick.

In vivo studies

A major consideration for repairing in vivo defects is related to the forces to which the tissue engineered grafts would be exposed. Non-load-bearing sites are generally considered more easily treatable as the mechanical competence of the graft is not critical to its functionality. Meinel et al.[28] treated the rat calvarium using human MSC-seeded silk scaffolds. There was significant improvement in the quality of bone regeneration when tissue-engineered constructs were used relative to cell-seeded scaffolds or scaffolds alone (Fig. 4C). In the latter two cases, there appeared to be little integration between the host tissue and the graft. In contrast, when a tissue-engineered graft was used to treat a rat femoral defect, micro-CT analysis suggested complete graft–host integration (Fig. 4D), indicating that the biochemical or mechanical differences in the environment also influence this aspect of repair.[29]

Osteochondral tissue engineering

In vitro studies

Practical applications seldom require engineered grafts that are compositionally homogeneous. Rather, it may be necessary to obtain grafts that approximate the natural tissue stratification and hierarchical organization or that achieve integration between different tissue types in vivo. Hence, osteochondral grafts are designed to serve as complex tissues that exhibit a transient of properties between articular cartilage and the underlying trabecular bone. One motivation for this approach was to circumvent poor cartilage–cartilage integration and take advantage of the native healing properties of bone.

In recent studies, chondrocyte-laden hydrogels were physically infused into an underlying, acellular, bony substrate that could be used as an attachment device.[30] MSCs are particularly advantageous for the formation of osteochondral constructs by virtue of their ability to differentiate into both cell types. Predifferentiated MSCs, under separate optimizing conditions for bone or cartilage, may be sutured together and cultivated in culture medium containing supplements essential to the development along both lineages. Tuli et al.[31] reported the growth of osteochondral constructs from hMSCs by press-fitting high-density chondrifying hMSCs into a scaffold and seeding the remaining regions of the scaf-

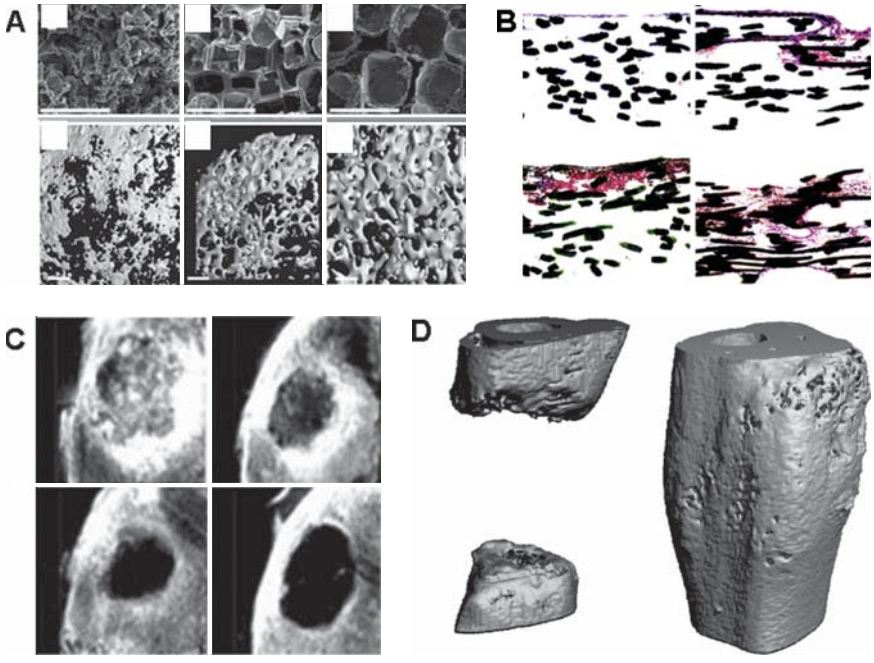

Fig. 4. Bone tissue engineering. **A** *Upper panel* (from left to right): Silk scaffolds of small, intermediate and large pore sizes. *Lower panel*: Corresponding trabecular structure of bone mineral formed by mesenchymal stem cells (MSCs) in the silk scaffolds. Trabecular microarchitecture more closely approximates that of native bone with the larger pore sizes. **B** Histological sections of rat stromal osteoblasts cultured in static conditions (*upper left*), flow with 0% dextran (*upper right*), 3% dextran (*lower left*) and 6% dextran (*lower right*) indicating that increased shear increases the cellular response. (Adapted from Sikavistas[27]). **C** Repair of a rat calvarial defect with tissue-engineered bone (*upper left*), human MSCs (hMSCs) seeded scaffolds (*upper right*), scaffolds only (*lower left*), or untreated (*lower right*).[28] **D** Rat femoral defect: untreated (*left*) or treated with tissue-engineered construct (*right*)[29]

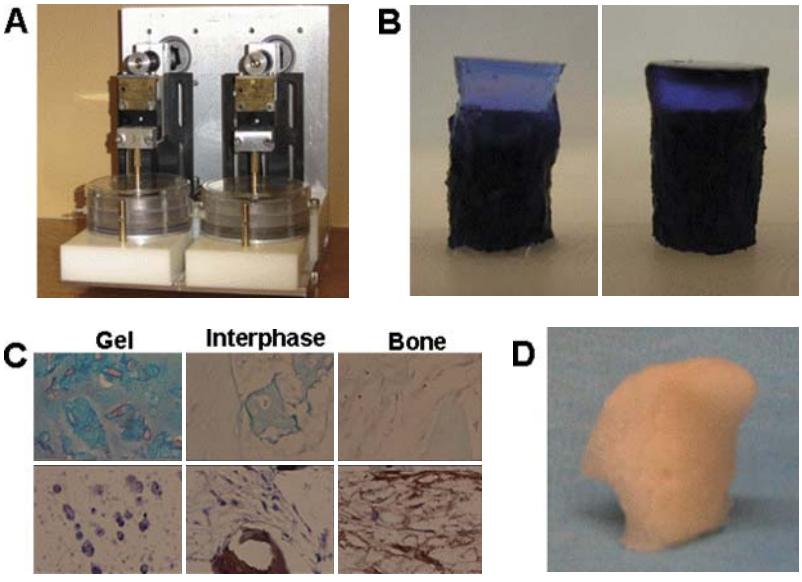

Fig. 5. Osteochondral tissue engineering. **A** Advanced bioreactor system capable of providing spatially regulated perfusion and dynamic compression to hMSC-seeded biphasic osteochondral grafts. **B** Unseeded biphasic (gel/scaffold) construct cultured with dye for 30 min in a bioreactor with perfusion only (*left*) or perfusion and compression (*right*), demonstrating transport to the gel region with compression. **C** Undifferentiated MSCs were cultured in biphasic scaffolds for 5 weeks in chondroinductive medium. Alcian blue stains show chondrocytic differentiation only in the gel region (*upper panel*). Type I collagen staining indicate osteogenic differentiation only in the scaffold region (*lower panel*). **D** Anatomically shaped human mandibular condyle made from trabecular bone

fold with osteo-induced hMSCs resulting in single-unit grafts with a gradient of properties.

A biomimetic approach to osteochondral graft formation should utilize biphasic constructs to recapitulate the structural and mechanical differences in the two tissue types. The sensitivity of hMSCs to their microenvironment facilitates spatial control of lineage commitment within a single construct and potentially circumvents the need for predifferentiation of the cells. Human MSCs may be seeded into a gel (for cartilage) or solid porous scaffold (for bone) and subsequently be exposed to different mechanical stimuli (compression and perfusion respectively) for biophysical stimulation and improved nutrient transport. These multiple functions are achieved using advanced bioreactors (Fig. 5A). As a result, there was expression of lineage-specific markers in different regions of biphasic grafts (agarose and decellularized trabecular bone) where human MSCs were seeded into the constructs[32] (Fig. 5C).

In vivo studies

Various animal models have investigated the plausibility of using osteochondral plugs for repairing focal defects in articular cartilage.[33,34] These studies have

traditionally used terminally differentiated cell types (which produce better functional grafts than stem cell populations). However, Solchaga and coworkers[35] attempted to heal osteochondral defects in rabbits using autologous bone marrow vacuum-seeded into hyaluronan-based biomaterials and implanted directly into defect site without in vitro culture. Their method depended on microenvironmental cues to guide cell differentiation and resulted in the formation of regions of bone and cartilage. However, unseeded scaffolds also elicited a healing response, indicating that the bone marrow may have simply accelerated a process that was mediated by the body's normal healing mechanisms. Currently, there remains a scarcity of publications demonstrating the application of stem cell-derived osteochondral constructs for repairing in vivo defects.

Anatomically shaped osteochondral grafts

There have been several meritorious approaches to recapitulating the complex, anatomically correct shapes in developing osteochondral constructs. The temporomandibular joint (TMJ) condyle has been used extensively as a model system because of its relatively small size and highly complex structure. A promising approach was reported by Alhadlaq et al.[36] wherein rat MSCs were predifferentiated along different chondrogenic and osteogenic lineages prior to seeding into a stratified gel molded in the shape of a cadaveric mandibular condyle. Another strategy is to combine clinical imaging technology such as CT or MRI to obtain patient-specific anatomical shapes that can then be molded into a biomaterial seeded with autologous cells (Fig. 5D). Such an approach circumvents issues of graft rejection via immune responses and is patient-specific. It also requires the use of highly advanced bioreactor designs to cultivate functional properties prior to implantation. For instance, Hung's group[37] obtained anatomically shaped patella grafts with bovine chondrocytes in the cartilaginous phase and an acellular bony support. A bioreactor capable of imparting physiologically relevant stimuli to cells in the developing cartilage region is required for in vitro cultivation to improve the mechanical characteristics.

Conclusions and future perspectives

Much progress has been made in understanding the conditions required for directing cell differentiation and assembly into functional tissue structures. Studies of cells cultured on biomaterial scaffolds (providing structural and logistic templates for tissue formation) using bioreactors (providing environmental control, efficient mass transport, and the necessary molecular and physical signals) played a major role in advancing our ability to direct cell behavior. The next step is to apply technologically advanced, biologically inspired tissue engineering systems to cells that are easy to obtain, well characterized, and of interest for clinical use. It is likely that the increasing interactions between the fields of tissue engineering, developmental and adult biology, and medical sciences will help address the challenges ahead of us.

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