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Bioreactor cultivation of osteochondral grafts

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Abstract

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The clinical utility of tissue engineering depends upon our ability to direct cells to form tissues with characteristic structural and mechanical properties across different hierarchical scales. Ideally, an engineered graft should be tailored to (re)establish the structure and function of the native tissue being replaced. Engineered grafts of such high fidelity would also foster fundamental research by serving as physiologically relevant models for quantitative *in vitro* studies. The approach discussed here involves the use of human mesenchymal stem cells (hMSC) cultured on custom-designed scaffolds (providing a structural and logistic template for tissue development) in bioreactors (providing environmental control, biochemical and mechanical cues). Cartilage, bone and ligaments have been engineered by using hMSC, highly porous protein scaffolds (collagen; silk) and bioreactors (perfused cartridges with or without mechanical loading). In each case, the scaffold and bioreactor were designed to recapitulate some aspects of the environment present in native tissues. Medium flow facilitated mass transport to the cells and thereby enhanced the formation of all three tissues. In the case of cartilage, dynamic laminar flow patterns were advantageous as compared to either turbulent steady flow or static (no flow) cultures. In the case of bone, medium flow affected the geometry, distribution and orientation of the forming bone-like trabeculae. In the case of ligament, applied mechanical loading (a combination of dynamic stretch and torsion) markedly enhanced cell differentiation, alignment and functional assembly. Taken together, these studies provide a basis for the ongoing work on engineering osreochondral grafts for a variety of potential applications, including those in the craniofacial complex.

Key words: bioreactor; bone; cartilage; ligament; scaffold; tissue engineering

Introduction

Tissue engineering combines the principles of biology, engineering and medicine to create functional grafts capable of repairing native tissue following a congenital deformity, disease or trauma. Engineered tissues with sufficiently high fidelity would also foster fundamental research by serving as physiologically relevant models for controlled quantitative studies of cells and tissues. The overall objective of tissue engineering is the restoration of normal tissue function. Ideally, a lost or damaged tissue should be replaced by an engineered graft that can reestablish appropriate structure, composition, cell signaling and function(s) of the native tissue. In light of this paradigm, the clinical utility of tissue engineering likely depends on our ability to replicate the *site-specific* properties of the tissue being replaced across different *size scales*, and provide the continuity and strength of the interface with the neighboring host tissues (1).

Craniofacial tissue engineering is a major area of clinical and scientific interest. Craniofacial deformities due to congenital defects, disease and injury have a tremendous impact on the appearance, function, psychological and social well being of patients. For example, temporomandiubular disorders affect 30 million individuals in the US alone, with more than one million new patients added every year (2). The number of different methods currently under investigation reflects both the inadequacy of existing techniques and the need to more faithfully restore the skeleton (3, 4). Tissue loss in the craniofacial complex is currently treated by autologous tissue grafting, a method limited by the harvesting difficulties, donor site morbidity and the clinicians' ability to contour delicate 3D shapes. Novel methods include conduction (by a scaffold) and induction (by bioactive molecules) of cell migration to repair relatively small defects, and cell transplantation (with or without biomaterial) to repair larger defects (5). Even these methods are inadequate for most parts of the craniofacial skeleton because of the complexity of the structures being replaced. Craniofacial defects involve multiple connective tissues incorporating fibrous joints and/or bone-cartilage interfaces. An 'ideal' repair would thus involve tissue grafts that are custom engineered by *directed differentiation of stem cells* to achieve a desired structure and functionality.

As compared to the transplantation of cells alone, *in vitro*-grown tissue constructs offer the potential advantage of immediate functionality along with the capacity for integration with host tissues. Engineered tissues can also serve as *physiologically relevant models for controlled studies of stem cell differentiation and tissue development under normal and pathological conditions.* Studies conducted *in vitro* can be designed to distinguish the effects of specific signals (cell derived, biochemical and physical) from the complex milieu of factors present *in vivo* (host endocrine and immunologic responses, e.g. Ref. 6), and provide useful, complementary information to that obtained *in vivo*. We review here an approach to functional tissue engineering that is based on the directed biophysical regulation of human mesenchymal stem cells (hMSC) on three-dimensional scaffolds using bioreactors. Tissue engineering of cartilage, bone and ligaments discussed in the context of using the same approach to engineer more complex tissue structures, such as osteochondral grafts with gradients of structural and functional properties.

Tissue engineering paradigm

It is thought that the cell function *in vitro* can be modulated by the same factors known to play a role during embryogenesis. *In vivo*, the processes of cell differentiation and tissue assembly are directed by multiple factors acting in concert and according to specific spatial and temporal sequences. These include: 1) a structural and logistic template for cell attachment and tissue development, 2) physiological milieu, 3) availability of supply of nutrients, oxygen and biochemical regulatory factors, and 4) physical regulatory signals (Fig. 1). Importantly, the context around the cell matters, such that the effects of factors are interactive, dependent on the length scale, time and pattern of their application. A 'biomimetic' approach to tissue engineering is based on the biophysical regulation of the cell (the actual 'tissue engineer') by exogenous signals generated by the scaffold and the bioreactor. One model system based on the above assumptions which is discussed here (Fig. 2) involves the *in vitro* creation of immature but functional tissues by the integrated use of:

- *Cells* that can be selected, expanded, and transfected to express the genes of interest;

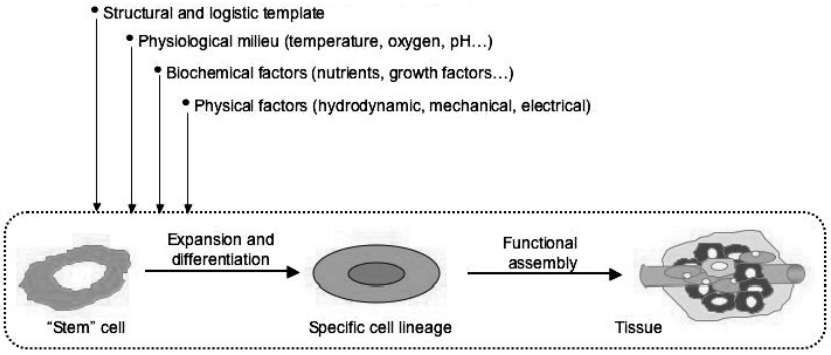

Fig. 1. Developmental paradigm. Tissue development and remodeling, *in vivo* and *in vitro*, involves the proliferation and differentiation of stem/progenitor cells, and their subsequent assembly into a tissue structure. Cell function and the progression of tissue assembly depend on: 1) the availability of a structural template for cell attachment and tissue formation, 2) the maintenance of physiological conditions in cell/tissue environment, 3) supply of nutrients, oxygen, metabolites, and growth factors, and 4) presence of physical regulatory factors.

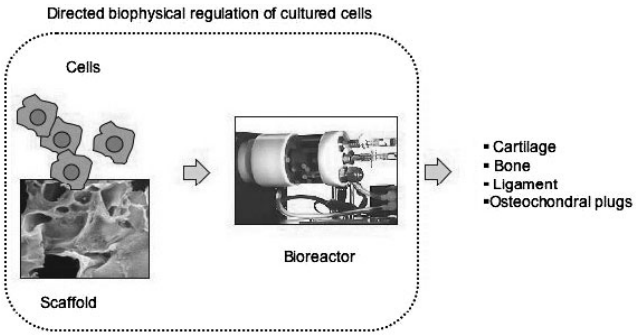

Fig. 2. Tissue engineering paradigm. The regulatory factors of cell differentiation and tissue assembly depicted in Fig. 1 can be utilized *in vitro* to engineer functional tissues by an integrated use of isolated cells, biomaterial scaffolds, and bioreactors. The cells themselves (either differentiated or progenitor/stem cells seeded onto a scaffold and cultured in a bioreactor) carry out the process of tissue formation, in response to regulatory signals. The scaffold provides a structural, mechanical, and logistic template for cell attachment, and tissue formation. The bioreactor provides the environmental conditions and regulatory signals (biochemical and physical) that induce, enhance or at least support the development of functional tissue constructs.

- *biomaterial scaffolds* that serve as a structural and logistic template for tissue development and biodegrade at a controlled rate;
- *bioreactors* that provide environmental conditions necessary for the cells to regenerate a functional tissue.

Cells used in previous studies included articular chondrocytes, osteoblasts, and bone marrow-derived precursors (6–11). One representative cell source is hMSC, derived from bone marrow (Fig. 3).

Scaffolds were porous biodegradable synthetic polymers (8), benzylated hyaluronan (12), porous collagen (9,13), and porous silk (9,13–15). Scaffolds have been seeded with cells dynamically in stirred flasks (16), by using hydrogel as a cell delivery vehicle (17), or by medium perfusion. Some representative biomaterial scaffolds are shown in Fig. 4.

Bioreactors used for the cultivation of cell-polymer constructs included mixed flasks (18), rotating vessels (7,19), perfused cartridges (20), and bioreactors with mechanical stimulation (14,17). Constructs were evaluated structurally (composition, ultrastructure) and functionally (metabolic and differentiation markers, mechanical behavior, capacity for integration), both *in vitro* and *in vivo*.

Tissue engineering bioreactors

Ideally, a bioreactor should provide an *in vitro* environment for rapid and orderly tissue development starting from isolated cells and three-dimensional scaffolds. Bioreactors are designed to perform one or more of the following functions:

- Establish spatially uniform concentrations of cells seeded onto clinically sized biomaterial scaffolds;
- control conditions in culture medium (e.g. temperature, pH, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules);
- facilitate mass transfer between the cells and the culture environment;
- provide physiologically relevant physical signals (e.g. interstitial fluid flow, shear, pressure, compression, stretch) (19,21).

An overview of representative tissue engineering bioreactors is shown in Fig. 5. Bioreactors enhance the effectiveness of tissue engineering scaffolds (12), and

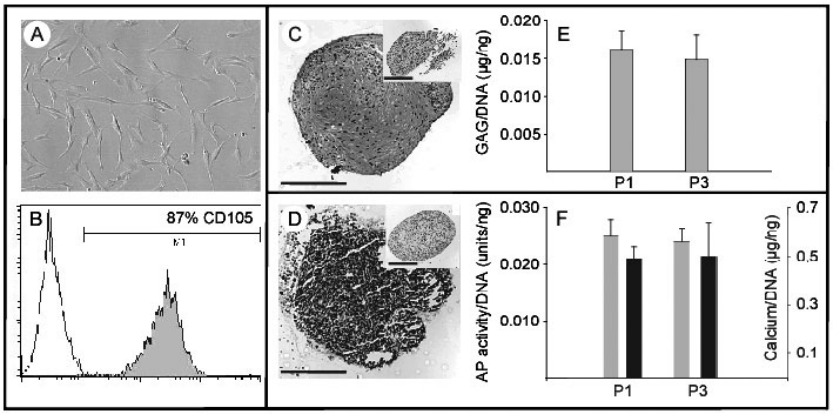

Fig. 3. A representative cell source: human bone marrow-derived mesenchymal stem cells (hMSC). (A) Cell selection by their ability to adhere to substrate. Phase-contrast photomicrograph of passage 2 (P2) cells at 60–70% confluence. (B) Cell characterization by fluorescence activated cell sorting (FACS). The expression of CD105 surface marker in P2 MSC. (C) Characterization of the ability for selective cell differentiation into mesenchymal lineages. Chondrogenic differentiation of P2 hMSC in pellets cultured either in chondrogenic medium (large image) or in control medium (insert). Scale bar: 1 mm. (D) Osteogenic differentiation of P2 hMSC in pellets cultured either in osteogenic medium (large image) or in control medium (insert). Scale bar: 1 mm. Stain: von Kossa (mineralized matrix is shown in black). Corresponding deposition of (E) GAG (μgGAG/ngDNA) and (F) calcium (μg calcium/ngDNA, black) and alkaline phosphatase activity (units/ng DNA, grey) in P1 and P3 MSC in pellets cultured for 4 weeks in chondrogenic and osteogenic medium, respectively (9).

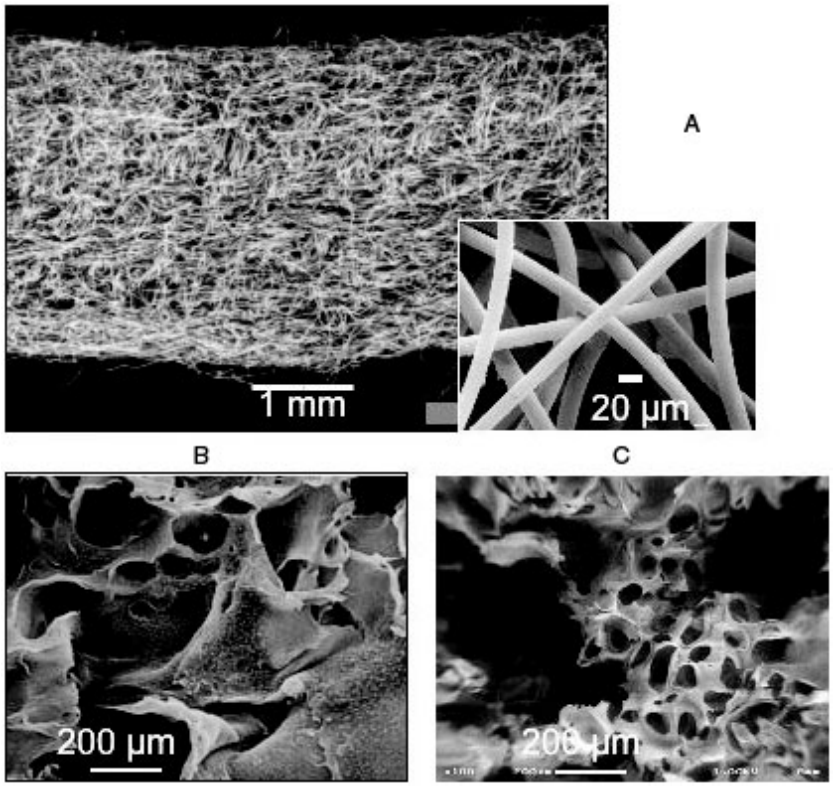

Fig. 4. Representative scaffolds for tissue engineering. Various modes of preparation and use of biomaterials scaffolds include: (A) Fibrous mesh made of polyglycolic acid (95–97% void volume; 13 μm diameter fibers (6). (B) Collagen sponge in form of discs punched from sheets of Ultrafoam® collagen hemostat (Davol Inc., Cranston, RI), a water-insoluble, partial HCl salt of purified bovine dermal (corium) collagen formed as a sponge with interconnected pores. (C) Silk sponge with macro- and micro-porous structure, prepared by salt leaching from soluble, biodegradable silk (40). From Annals of Biomedical Engineering Vol. 32, 2004 pp. 116, Meinel L, Kareourgiou V, Fajardo R, Snyder B, Shinde-Patil V, Zichner L, Kaplan D, Langer R and Vunjak-Novakovic G. Figure 1. With kind permission of Springer Science and Business Media.

support the maintenance of differentiated cell function (19,21).

In the *rotating bioreactor* (Fig. 5A), up to 12 tissue constructs are suspended in the 110ml annular space between two cylinders, gas is exchanged via a silicone membrane, and mixing is provided by construct settling in the rotating flow. Mixing enhanced mass transport at construct surfaces, while transport within the tissue constructs still occurred by molecular diffusion only. These vessels gave excellent results for tissue engineering of cartilage, a tissue with relatively low metabolic requirements (7,10,12,22,28,31,36).

Perfused cartridges are 1.5 ml vessels designed to provide interstitial fluid flow through the construct

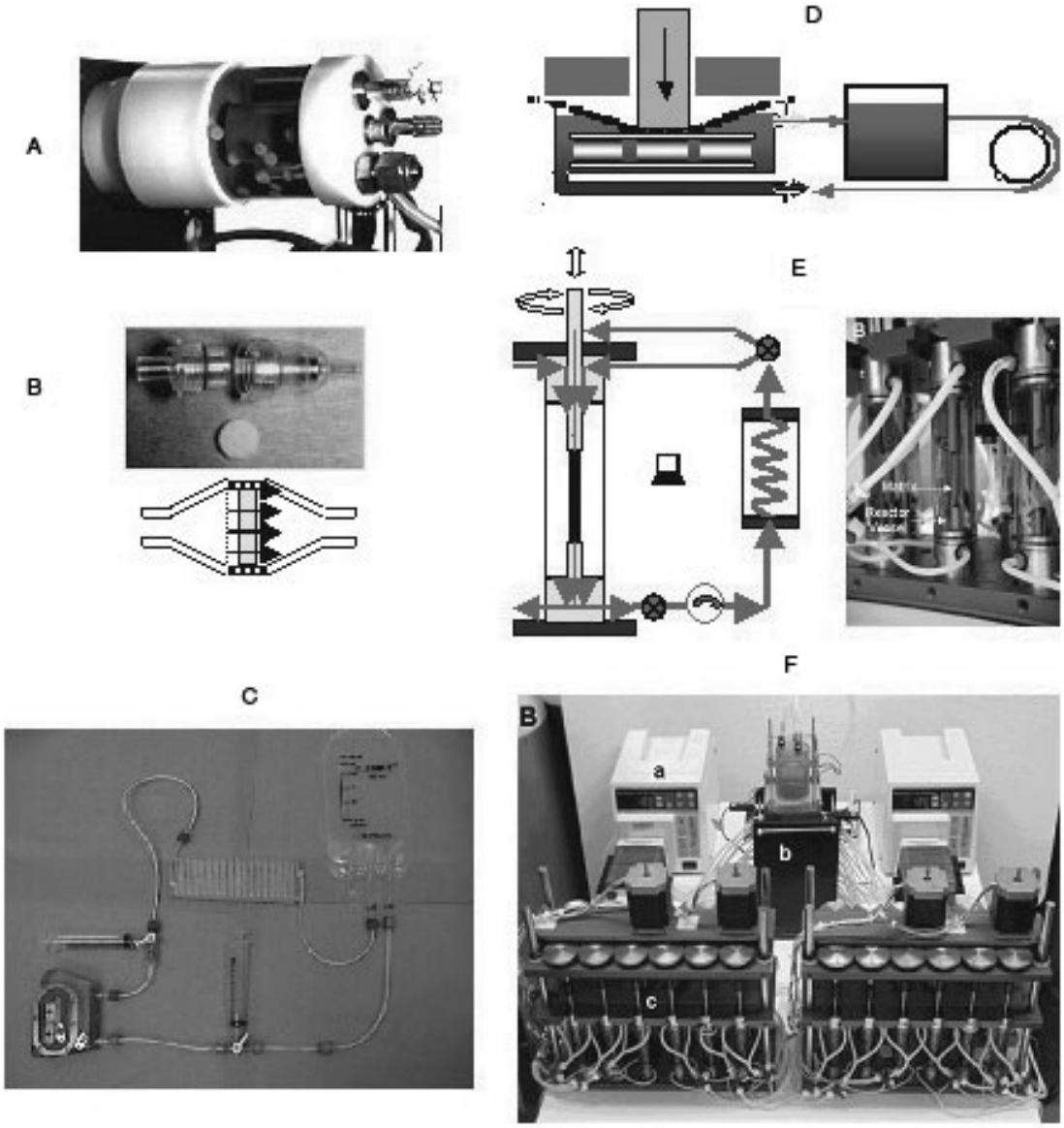

Fig. 5. Bioreactors: (A) Rotating bioreactor in which tissue constructs are cultured freely suspended in dynamic laminar flow (7) (B) Photograph (courtesy of N. Dunkelman) and schematic of perfused cartridge. Interstitial flow of culture medium (arrows) provides the contact between culture medium and the construct interior with minimal diffusional distances and thereby enhances mass transport throughout the construct interior (23). (C) Perfused chamber within a 'portable' single loop containing a gas exchanger (to provide control of oxygen and pH levels in culture medium), bag with fresh culture medium, and sampling port. (D) Perfused chamber system that provides mechanical loading to a construct (24). (E) Schematics and a photograph of columns providing medium perfusion around the cultured tissue in conjunction with the application of multidimensional mechanical loading during. Cultured constructs are subjected to a combination of dynamic stretch (or compression) and dynamic torsion, applied either continuously or intermittently (14,25). (F) Photograph of the modular bioreactor system employing columns shown in (E) (two sets of n = 12 columns each) (14).

within; gas is exchanged by medium recirculation through an external gas exchanger (Fig. 5B) (23). These cartridges enabled local environmental control in engineered bone and thereby enabled the cultivation of thick layers of viable, metabolically active tissue. *Perfused chambers* (Fig. 5C) are 3, 10 or 30 ml volume vessels designed to provide medium flow around tissue constructs or cultured cells. They are connected to an external, loop containing gas exchanger, a miniature pump, and a small reservoir with fresh medium, and designed for use either in an incubator or as a 'portable' system for transferring engineered tissue constructs (e.g. to the imaging facility).

Perfused chambers with mechanical loading (n = 5 chambers per culture system) that can accommodate up to five engineered constructs apiece (Fig. 5D); gas is exchanged via an external device, mixing is provided by recirculation, and loading is provided by a mechanical

spectrometer capable of applying a variety of mechanical stimulation regimes. This system was successfully used for long-term studies of engineered cartilage (24).

One of the most advanced bioreactor systems (Fig. 5E, F) is a modular bench-top device that contains 24 tissue culture vessels with medium perfusion and mechanical loading. Each cartridge (Fig. 5E) is connected to an independent perfusion loop and can be subjected to multidimensional mechanical loading (dynamic compression/tension and torsion). This system was successfully used to engineer ligaments (14,17,25).

The composition, morphology and mechanical properties of engineered tissues grown in mechanically active environments are generally better as compared to those grown in static environments, presumably due to enhanced mass transport at tissue surfaces (molecular diffusion remained the dominant mechanism of mass transport within the tissue) (19). Although transport limitations may not be a significant problem in the engineering of cartilage, an avascular tissue with low cellularity that can be cultured to thicknesses exceeding 5 mm (7), diffusional mass transport has severely curtailed efforts to engineer bone, which could be cultured only to thicknesses of approximately 200 μm (8,26,27). Thus, the distributions of cells and ECM in non-perfused engineered tissues are generally non-uniform. In native tissues, this problem is alleviated by vascularizatioin as well as loading-induced interstitial flow of fluid. In bioreactors, the interstitial flow of culture medium can be utilized to minimize concentration gradients and thereby avoid the non-uniformities in tissue structure and function.

Cellular responses can be mediated by specific cytokines, growth and transcription factors, including oxygen and IGF-I for cartilage (10,28) and members of the TGFβ/BMP superfamily for bone and periodontal tissues (29). Physical factors utilized to improve tissue development include fluid flow (30), hydrodynamic shear and pressure (22) and mechanical compression (32,33). *In vitro*, dynamic but not static compression enhanced synthesis of proteoglycans in cartilage explants (34), improved mechanical function of engineered cartilage (32,33) and enhanced chondrogenesis of chick limb bud cells (35).

In conclusion, mechanical environment and growth factors independently modulate the development and functional properties of engineered cartilage, bone and ligaments, interact to produce results not suggested by the independent responses, and produce tissues superior to those obtained by utilizing the same factors individually (1,36). Our approach to tissue engineering of clinically sized and mechanically functional grafts involves the *in vitro* utilization of factors normally present *in vivo*: interstitial flow at physiological velocities, mechanical loading, and regulatory molecules (1,14,15,17).

Case studies

Tissue-engineered cartilage

In recent studies (13) we established methods for tissue engineering of cartilage using hMSC and protein scaffolds. The hMSC were isolated, expanded in culture, and characterized with respect to the expression of surface markers, and their ability for chondrogenic and osteogenic differentiation. Cells were seeded on highly porous scaffolds that were made either of Type-I collagen (fast degrading material with $\sim$100 μm pores and poor mechanical properties, Fig. 6A), or silk (slowly degrading material with $\sim$200 μm pores and excellent mechanical properties, Fig. 6B), The resulting constructs were cultured for 4 weeks in either control medium (DMEM with 10% fetal bovine serum) or chondrogenic medium (further supplemented with chondrogenic growth factors).

The hMSC formed cartilaginous tissues on all scaffolds, but the extent of chondrogenesis was substantially higher for hMSC cultured on silk than on collagen. Degradation of collagen resulted in a breakdown of the support lattice structure, whereas silk scaffolds retained their structural integrity throughout the duration of culture (Fig. 6C, D). The deposition of GAG and Type-II collagen were markedly higher for hMSC cultured on silk (Fig. 6E, G) than on collagen scaffolds (Fig. 6F, H). In collagen-based constructs, the areas of continuous ECM were only approximately 100–200 μm in length (Fig. 6F), in contrast to 500–700 μm long interconnected areas of cartilaginous tissue observed within silk scaffolds (Fig. 6E). These results suggest that silk scaffolds are particularly suitable for tissue engineering of cartilage starting from hMSC, presumably due to their high porosity, slow biodegradation and structural integrity.

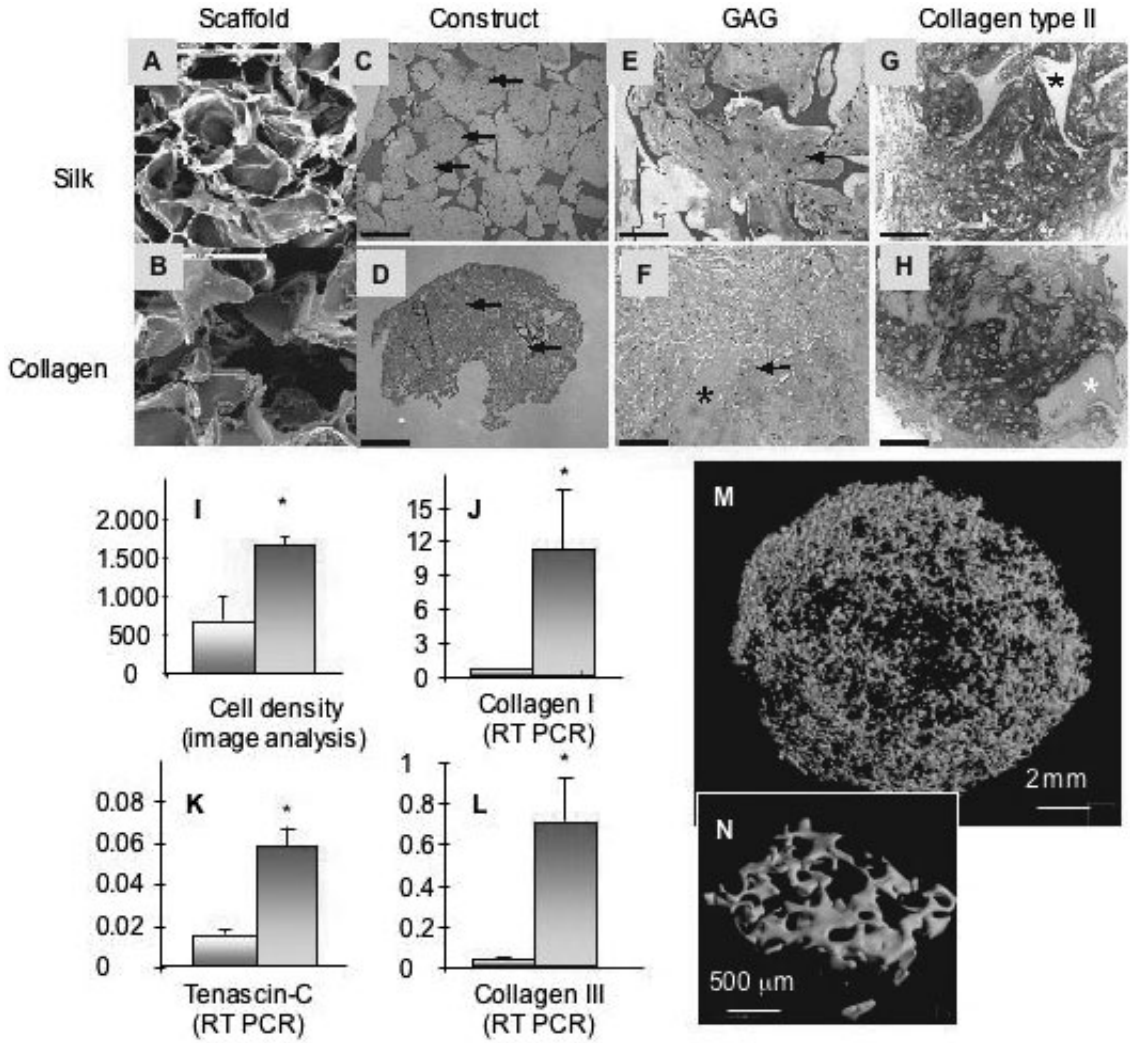

Fig. 6. Tissue engineering of cartilage, bone and ligaments using human MSC and protein scaffolds. (A–H) Engineered cartilage. Deposition of tissue matrix in 4-week constructs based on hMSC cultured on silk (A,C,E,G) or collagen scaffolds (B,D,F,H). Stains: safranin-O for GAG (red), an antibody for type II collagen (brown) (25). (I–L) Engineered ligament. hMSC were cultured for 2 weeks in collagen gel, with the application of mechanical stimulation designed to mimic that in a human knee. (I) cell density (determined by image processing), and the marker transcripts for (J) collagen I, (K) tenascin C and (L) collagen III were markedly higher for stimulated (red bars) than nonstimulated (grey bars) ligaments (3). (M–N) Engineered bone. After 4 weeks of cultivation, bone constructs cultured on silk scaffolds were ~1 cm in diameter and 2mm thick and contained dense bone–like matrix (24). A microcomputerized tomography (μCT) of a representative construct is shown.

Tissue engineered ligaments

We recently reviewed various approaches to biologically based ACL replacements (25). Passive tension within the engineered ligament-induced fibroblast elongation and alignment, and the structural reorganization of the extracellular matrix (37). The application of cyclic mechanical loading during cultivation promoted the proliferation of smooth muscle cells and the assembly of newly synthesized ECM (38), and the synthesis of Type-I collagen by ACL fibroblasts (17). The cultivation of human ACL fibroblasts in collagen gel attached to two bone anchors and exposed to cyclic stretch resulted in ligament-like tissue architectures which depended on the application of mechanical stimuli (14).

Cultured hMSC can be directed to undergo functional assembly of into engineered ligaments that have the architecture and mechanical properties resembling those of native ligaments, in an environment designed to resemble some aspects of that *in vivo* (e.g. 14,17). Engineered ligaments were characterized *in vitro* (molecular, structural and mechanical properties) and *in vivo* (phenotype stability, capacity for survival and vascularization in a goat model).

Mechanical stimulation-induced cell alignment in the direction of the resulting force (concurrent axial and torsional strain over 21 days of culture), in contrast to random cell orientation in unstimulated controls (17). Mechanical stimulation fostered cell growth (Fig. 6I), and the expression of collagen Type I (Fig. 6J), tenascin C (Fig. 6K), and collagen Type III (Fig. 6L). At the same time, no up-regulation of bone or cartilage cell markers was observed. These studies suggest that tension and torsion applied during construct cultivation directed hMSC differentiation into a ligament cell lineage in preference to alternative paths – bone or cartilage.

Tissue-engineered bone

Tissue engineering of bone was studied by culturing hMSC on porous scaffolds (partially demineralized bone, unmodified collagen, cross-linked collagen, silk) in bioreactors (stirred flasks, perfused cartridges, bioreactors with mechanical loading) (9,39). With time in culture, the size and DNA content of collagen-based constructs decreased, to approximately 25% of initial after 4 weeks of culture, in contrast to silk-based constructs which maintained their initial size and DNA content, such that the amount of DNA was $>80\%$ of initial after 4 weeks of culture. Importantly, the loss of DNA in collagen-based constructs (75% of initial) matched the loss in construct weight (75% of initial); the maintenance of DNA content in silk-based constructs was consistent with the relatively slow degradation of silk scaffolds (9).

The bioreactor hydrodynamics markedly affected the progression of osteogenesis and the resulting microstructure of engineered bone. In spinner flasks (convective transport at construct surfaces, molecular diffusion inside constructs) the mineralized matrix formed at the outer rim of the construct, as seen in microcomputerized tomography (μ-CT) images of whole constructs (Fig. 6M). The length of interconnected bone rods was between 0.5 and 1 mm, a distance that corresponds to the convective penetration depth of fluid into the highly porous scaffolds. In perfusion bioreactors (convective transport throughout the construct, diffusion over short distances), the mineralized matrix was randomly distributed throughout the construct volume. The individual rods were oriented in the direction of flow, and in some cases spanned from one to the other surface of the construct, but remained smaller than those in spinner flasks, and did not connect to a form a lattice. Osteogenesis in silk-based constructs was markedly better than in collagen-based constructs. Importantly, the architecture of silk scaffolds (shape, size, and connectivity of the pores) determined the architecture of engineered bone. Constructs based on hMSC and silk scaffolds maintained their initial size and shape, supported osteogenesis at levels not observed in collagen scaffolds, and had remarkably well developed microstructure, resembling that of native trabecular bone (Fig. 6N).

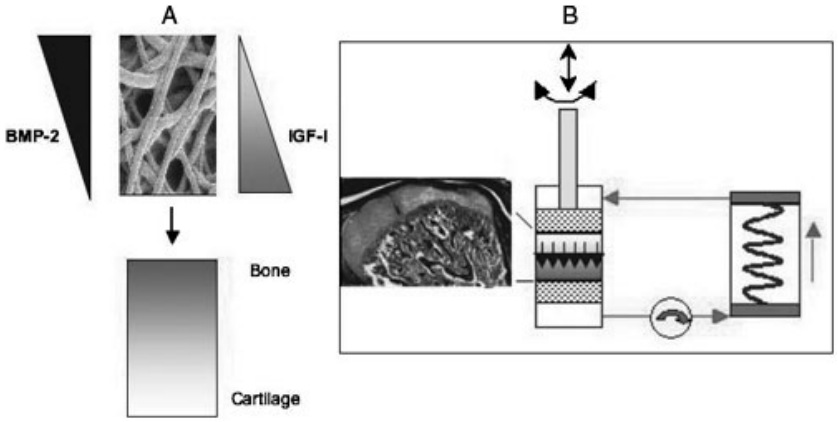

Fig. 7. Tissue engineering of osteochondral grafts. (A) A scaffold fabricated from biodegradable silk protein is designed to mimic, structurally and mechanically, the properties of the native tissue being engineered. The scaffold is functionalized by covalently binding growth factors, with opposing gradients of a chondrogenic factors (e.g. IGF-I) and osteogenic factors (e.g. BMP-2). (B) Scaffolds are seeded with hMSC and cultured in a modular bioreactor shown in Fig. 5F. Each cartridge has a diameter of 10 mm and a working height of 2–7 mm, and contain one construct placed between two porous platens serving as flow distributors. Culture medium is perfused through the constructs, either continuously or intermittently. Cultured constructs are subjected to mechanical loading, as explained in Fig. 5E, F.

Tissue engineering of osteochondral grafts

To date, tissue engineering is still largely focused on single tissue structures or tissue types, and this limits its scientific, and clinical utility. Tissue engineering in the craniofacial complex would particularly benefit from stratified tissue grafts. For example, osteochondral grafts for the replacement of mandibular condyle are of significant clinical interest and at the same time an excellent model for controlled studies of stem cell differentiation and functional assembly.

One approach to engineering complex tissue grafts with gradients of molecular, structural, and functional properties are depicted in Fig. 7. Scaffolds of interest are made of highly porous silk protein ($\sim$100–500 μm pores, $\geq$90% void volume) (40), using a layered approach, to generate gradients of structural and mechanical properties. Subsequently, scaffolds are functionalized by covalently binding growth factors, either at spatially uniform concentrations, or in form of spatial concentration gradients. One combination of particular interest involves the opposite gradients of two different growth factors (e.g. IGF-I and BMP-2) in the same scaffold, in order to mimic an 'ideal' concentration of IGF-I for cartilage at one end and an 'ideal' concentration of BMP-2 for bone at the other end. To minimize the diffusional distances for mass transfer within constructs, the interstitial flow of culture medium is utilized, at velocities physiological for native cartilage and bone (10–100 μm/s). To enhance hMSC differentiation and functional assembly, engineered tissues are subjected to dynamic intermittent loading.

Conclusion

The paradigm discussed here is that the restoration of normal tissue function can be achieved by transplanting engineered tissue grafts grown *in vitro* that are immature but functional, and have the ability to integrate with the adjacent native tissues. Craniofacial defects involve multiple tissues, and/or bone-cartilage interfaces, a situation that translates into the requirement of engineering 'custom-designed' tissues with gradients of structural and functional properties. One approach to tissue engineering of such complex grafts involves *directed differentiation of stem cells by an integrated use of scaffolds and bioreactors*. Engineered tissues of sufficiently high fidelity can also serve as high-fidelity models for basic studies of cells and tissues. We have discussed the design and operation of representative bioreactors that have been used to engineer articular cartilage, bone and ligaments, and outlined some of the ongoing work on tissue engineering of osteochondral grafts.

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