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Tissue Engineering of Articular Cartilage with Biomimetic Zones

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Articular cartilage damage is a persistent and increasing problem with the aging population, and treatments to achieve biological repair or restoration remain a challenge. Cartilage tissue engineering approaches have been investigated for over 20 years, but have yet to achieve the consistency and effectiveness for widespread clinical use. One of the potential reasons for this is that the engineered tissues do not have or establish the normal zonal organization of cells and extracellular matrix that appears critical for normal tissue function. A number of approaches are being taken currently to engineer tissue that more closely mimics the organization of native articular cartilage. This review focuses on the zonal organization of native articular cartilage, strategies being used to develop such organization, the reorganization that occurs after culture or implantation, and future prospects for the tissue engineering of articular cartilage with biomimetic zones.

Introduction

Articular cartilage is a highly organized tissue that normally functions to provide a low-friction, wear-resistant, and load-bearing surface for efficient joint movement. However, the structure and function of the tissue are frequently disrupted or lost in trauma and, more commonly, in degenerative joint diseases such as osteoarthritis, which affects an estimated 27 million Americans.[1] Paramount to the progression of osteoarthritis is the fact that postnatal articular cartilage lacks the ability for complete self-repair through native healing mechanisms. Currently, the most common treatment of advanced osteoarthritis is joint replacement, which is estimated to reach two million knees and hips per year by 2015 in the United States.[2] In this approach, the cartilage is completely removed and replaced by synthetic components of metal, ceramic, and/or plastic. This approach allows for recovery of some function and reduction of pain. However, with the aging of the population, the need for revision surgeries is increased and the difficulty and cost of such surgeries are high.[3,4] Recently, there has been an increase in popularity of metal-on-metal resurfacing procedures in hips of relatively young and active patients. This procedure preserves femoral bone and reduces the complexity of revision surgery, yet the long-term effectiveness of this technique remains to be established.[5]

As a biological alternative to artificial joint replacement or resurfacing, regenerative medicine–based techniques are under investigation.[6] Over the past 20+ years, there has been a progression in these techniques from providing a suspension of cells in the debrided region of a focal defect, as in autologous chondrocyte implantation (ACI),[7] to forming tissues using combinations of cells, scaffolds (porous 3D structures where cells can attach to the free material surfaces, and media can circulate[8]), and matrices (hydrogels[8]) *in vitro* for implantation. However, clinical application of regenerative medicine–based techniques has been limited and has raised questions about the *in vitro* and *in vivo* research on cartilage tissue engineering. This review will focus on issues related to tissue organization and structure. First, we discuss the organizational and structural aspects of native articular cartilage. Second, we address the extent to which such an organized tissue has been developed *in vitro.* Next, we describe the changes in tissue organization (remodeling) induced by application of exogenous stimuli *in vitro* and implantation *in vivo*. Finally, we provide recommendations for future approaches to form cartilaginous tissues that have biomimetic zones and will provide a functional replacement and restoration of articular cartilage in the long run.

Zonal Organization of Articular Cartilage

Articular cartilage is a thick (e.g., 0.9 ± 0.5 mm in human phalanges[9] and 2.4 ± 0.5 mm in human medial femoral condyles[10]), avascular, aneural tissue that lines the ends of long bones and normally allows for efficient function of diarthrodial

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joints. Although articular cartilage was initially proposed as a relatively simple tissue to engineer,[11] it exhibits exquisite organization on several length scales. Over the range of nanometers to micrometers, the cartilage extracellular matrix is arranged as a network of collagen fibers and proteoglycans that allow for cell adhesion, mechanical support, and transduction of chemical and mechanical signals from the surrounding tissue to the cell. Over the range of micrometers to millimeters, there are topographical differences in cartilage thickness and matrix content across the surface of the joint, which are associated with the level of loading.[12,13] Further, the properties of articular cartilage change with depth from the articular surface, resulting in a zonal structure that is typically divided into three zones: superficial (surface to 10–20% of thickness), middle (20–70%), and deep (70–100%) (Fig. 1A). Such a structure is generally consistent between species (bovine, equine, ovine, caprine, porcine, murine, etc.), although significant differences exist in thickness, cell distribution, and matrix properties.[14,15] Finally, over the range of millimeters to centimeters, the organ has a specific shape that allows it to associate with surrounding tissues and function properly, such as the contours of the femoral head cartilage shaped to articulate with the acetabulum.

The various levels of organization *in vivo* all have importance in the mechanical, metabolic, and transport properties of the tissue.[10,16,17] In this review, we focus on the zonal variations in native and tissue-engineered articular cartilage.

Zonal variations in extracellular matrix

One of the most notable zonal variations in articular cartilage is in the organization of the collagen network, which is primarily responsible for the tensile properties of the tissue and also important for the compressive properties.[18] The collagen fibrils are mainly made of collagen type II, but also contain type IX and type XI, which are important in the regulation of fibril size, interfibril cross-linking, and interactions with the cartilage proteoglycans.[19] In the superficial zone, collagen fibrils are oriented parallel to the articular surface and impart high tensile strength to withstand the tensile stresses associated with joint loading. In the middle zone, collagen fibers are randomly oriented and provide a transition to the deep zone, in which the fibers are oriented perpendicular to the subchondral bone. Whereas collagen content per wet weight does not change significantly with depth,[20] depth-dependent increases in hydroxylysine and hydroxylysylpyridinoline, and age-dependent variations in lysylpyridinoline cross-links are present (Fig. 1A), and play a critical role in the mechanical properties of the tissue.[21,22]

After collagen, large aggregating proteoglycans are the most abundant organic constituents of cartilage, and the concentration of these aggregates increases from the articular surface to the deep zone, even in fetal cartilage.[20,23] The aggregates are made of a hyaluronic acid backbone to which are attached aggrecan monomers, which consist of a core protein that is heavily glycosylated with negatively charged chondroitin sulfate and keratan sulfate glycosaminoglycans (GAGs). Significant differences in metabolic properties of the chondrocytes from different zones in the level of GAG production are partially responsible for zonal variations in GAG content (Fig. 1B[24]).[25,26] Associated with changes in the GAG concentration are changes in the mechanical properties of the tissue. Most notably, the compressive modulus decreases by an order of magnitude from the superficial to the deep zone.[27] These mechanical properties allow for efficient distribution and transmission of loads across the joint surface to the subchondral bone.

Zonal variations in cells

In addition to zonal differences in extracellular matrix, cells of each of the different zones of articular cartilage are organized distinctly and express zone-specific markers. In the superficial zone, cells are at a high density, are relatively flattened, and are clustered in a horizontal fashion.[28,29] Here, the cells secrete proteoglycan 4 (PRG4), a large glycoprotein

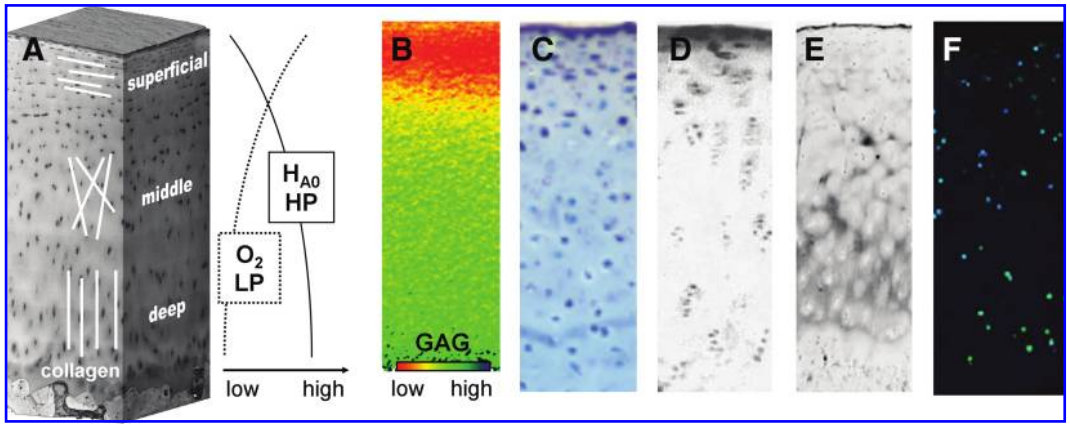

FIG. 1. Zonal organization in normal articular cartilage. (**A**) Three-dimensional histology and schematic showing cell and collagen fibril organization in the superficial, middle, and deep zones. Also depicted are changes in levels of oxygen, collagen cross-links (lysylpyridinoline (LP), hydroxylysylpyridinoline (HP)), and compressive modulus (H_{A0}) through the thickness of the tissue. (**B**) EPIC micro-CT map of GAG distribution in human articular cartilage. (**C**) PRG4 (reprinted from[154] with permission from Elsevier) appears to suitable as a marker for the superficial zone, as does (**D**) developmental endothelial locus-1 (reprinted from[36] with permission form Elsevier). (**E**) Cartilage intermediate layer protein is found in the interterritorial matrix of middle and deep zones,[42] whereas (**F**) Jagged 1 is highly expressed in cells of the middle and deep zones.[46] Color images available online at www.liebertonline.com/ten.

product of the gene *prg4*, that also codes for lubricin[30] and megakaryocyte-stimulating factor (Fig. 1C).[31,32] This zonal marker is functionally important as a boundary lubricant, and mutations in the gene are associated with pathologies.[33] Another potential marker for the superficial zone is clusterin,[34] a molecule that is overexpressed in osteoarthritis.[35] Developmental endothelial locus-1 is also found in the pericellular matrix of the superficial zone but not the deep zone (Fig. 1D), and interacts with the $\alpha_v\beta_3$ integrin, which has been shown to be expressed by superficial zone chondrocytes.[36,37] The role of developmental endothelial locus-1 in the superficial zone remains unclear, however, as it is typically involved in angiogenesis.[38] In mature bovines, Notch1 is expressed solely in the superficial zone of articular cartilage[39]; however, this distribution appears to be species, development, and disease specific.[40,41]

In the middle zone, cells are more spherical and are randomly oriented. Cartilage intermediate layer protein (Fig. 1E) is a potential marker for the middle-deep zones,[42] with apparent autoimmune activity and positive correlation with expression and progression of osteoarthritis,[43] chondrocalcinosis,[44] and lumbar disc disease.[45]

In the deep zone, chondrocytes are larger and organized in vertical columns. Several markers exist in adult cartilage including members of the Notch-Delta signaling pathway, which are expressed throughout cartilage during development, but are localized to the deeper layers in mature mouse tissue,[41] in particular Jagged 1 (Fig. 1F).[46] Additionally, collagen type X, a marker for chondrocyte hypertrophy and indicator of endochondral ossification during development, is expressed in the deepest layers of articular cartilage (but sometimes in the superficial zone, as well[47]).[48]

Engineering Articular Cartilage with Biomimetic Zones

The initial goal for tissue-engineered articular cartilage was to make a homogeneous tissue *in vitro* that mimicked the overall bulk properties of native articular cartilage by combining cells and biomaterial matrices or scaffolds in chondrogenic conditions.[49] While there has been some success using this approach, little information is available on the construct organization on a sub-millimeter scale. In the vast majority of constructs it is not clear whether these tissues develop properties that vary from the surfaces of the tissues, or if these tissues are remodeled *in vivo* to obtain these properties. Given the prevalence and importance of zonal variations in normal articular cartilage, a recent goal has been to engineer tissues with zonal structure, function, or both. The approaches for engineering cartilage with zonal structure and function can be separated into (1) scaffold- and matrix-free, (2) scaffold-based, (3) matrix-based, and (4) hybrid (Fig. 2), and are the focus of the second part of this review. All of these approaches utilize cells, and thus we first discuss potential cell sources for zonal articular cartilage tissue engineering.

Cell sources

Critical to the eventual properties of cell-based tissue-engineered cartilage is the selection of the appropriate cell type or types to be used (Table 1). As the single cell type of

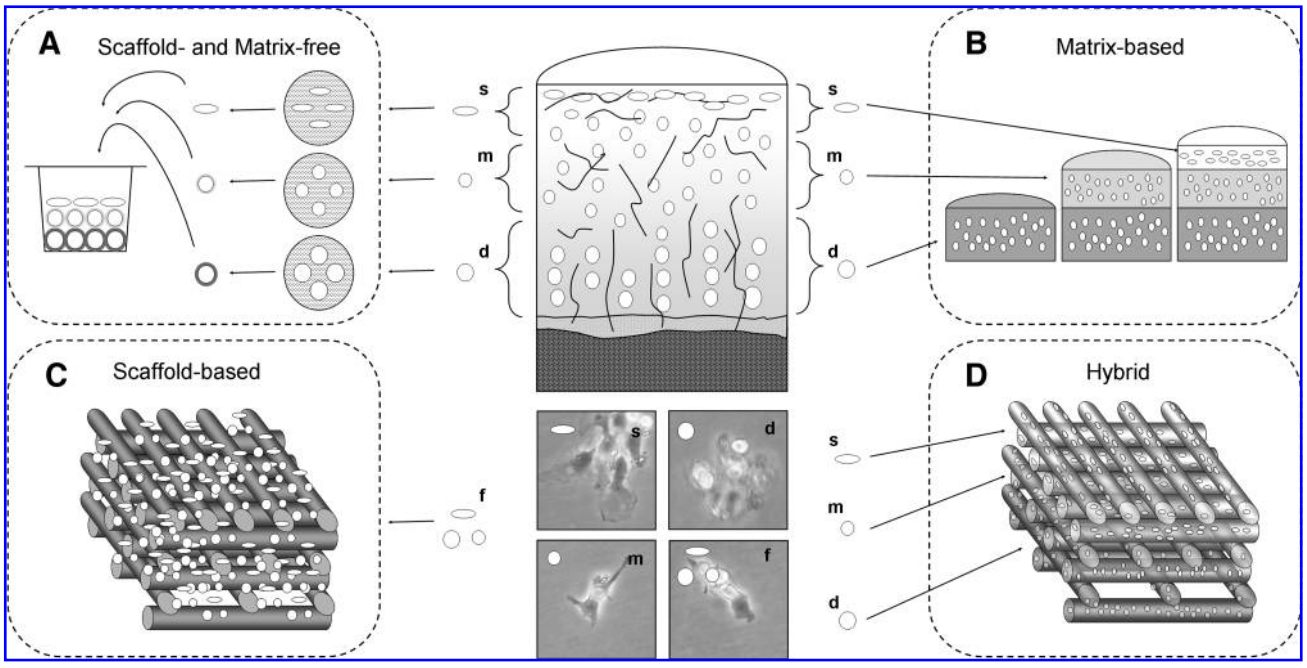

FIG. 2. Schematic of methods that can be applied to zonal cartilage tissue engineering. Cells from different zones (s, m, d) of articular cartilage are isolated and, for example, (**A**) cultured in alginate, released from the alginate and layered in a scaffold- and matrix-free approach.[82] Alternately, (**B**) cells are suspended in a hydrogel matrix and sequentially polymerized in layers to localize zonal cells[119] and/or provide zonal variations in matrix. (**C**) To provide zonal structural differences, scaffolds can be printed with a gradient of pore sizes[93] and seeded with full-thickness (f) cells. Finally, (**D**) a hybrid approach uses different cell populations within distinct hydrogel matrices that are printed to give zonal cellular, structural, and matrix properties.

Table 1. Cell Sources for Tissue Engineering Articular Cartilage with Biomimetic Zones

Cell type	Source	Abundance	Ease of cell harvest	Expansion capacity	Cost effectiveness	Zonal matrix production	References
Chondrocytes	Full-thickness	++	+	+	+	+	7, 49, 50, 78, 80, 81, 91, 93, 94, 96, 99, 101, 105, 108, 111, 112, 115–117, 122, 126, 139–143, 145, 146, 148–153, 156, 158, 161, 166
	Zonal	±	±	+	±	+++	23, 25, 51, 77, 83–86, 119, 123, 124, 144, 147, 157
Progenitor cells	Cartilage	±	−	++	±	++	54, 55, 57–62
	Bone marrow	++	++	+++	+	+	56, 64, 65, 88, 97, 98, 101, 109, 116, 136
	Adipose	+++	++	+++	+	??	63, 66, 67, 72
	Synovium	++	+	++	±	+	31, 68, 69, 73–75
	Umbilical cord	±	+++	+++	+	??	70, 71

Qualitative scoring for each characteristic and cell type is shown from lowest to highest (−, ±, +, ++, and +++; ?? indicates untested).

articular cartilage, chondrocytes are a natural choice for the fabrication of *de novo* cartilage. Chondrocytes are typically isolated from full-thickness cartilage and expanded *in vitro* to obtain sufficient cell numbers (e.g., ~12 million cells for ACI[50]), which can lead to chondrocyte dedifferentiation before formation of a construct or injection of a cell suspension into a defect site. While this approach has had some success, one potential problem is the disorganization of the cells from different zones and the relatively homogeneous matrix that is produced. If a native cartilage-like structure is to be attained, biochemical and biomechanical signals must be applied appropriately and the mixture of chondrocytes from different zones must respond appropriately to these signals. There is some evidence that differentiated cells from different zones are unable to produce zone-specific molecules under various conditions, and thus may not be appropriate for developing the zonal structure of articular cartilage.[51,52]

Rather than isolating full-thickness chondrocytes from the articular cartilage, cells can be isolated from the different zones directly and constructs reconstructed to exhibit key features of the articular cartilage structure and function. As mentioned previously, cells from the different zones exhibit distinct biosynthetic activities,[25,26,53] and therefore may be required to be localized in their respective regions of a tissue-engineered construct to develop native-like matrix distribution.[10]

Alternatively, chondroprogenitor/stem cells that are found within articular cartilage could be used. While no single definitive marker has been found, a group of cells within the cartilage have stem cell characteristics such as tri-lineage potential[54] and colony-forming ability.[55] These cells have been isolated with fluorescence-activated cell sorting using surface markers such as $CD9^+/CD90^+/CD166^+$,[56] chondroitin sulfate sulfation motifs,[57] Jagged1, Notch1,[58] and Delta,[46] or alternatively the exclusion of Hoechst 33452.[59] When $CD105^+/CD166^+$ cells were isolated and cultured under appropriate differentiation media, they exhibited adipogenic, osteogenic, and chondrogenic potential.[60] Fibronectin-adhesive chondroprogenitor cells of the superficial zone of bovine articular cartilage have a higher propensity to form colonies, expand *in vitro*, and differentiate *in vivo* than cells from the other zones. Chondroprogenitor cells have also been found in full-thickness human OA cartilage (7.5% of total cells)[60–63] as well as normal cartilage (3.5% of total cells).[46,60]

A variety of tissues contain mesenchymal stem/progenitor cells (MSCs), which have the capacity to form all (or some, depending on their potency) types of mesenchymal tissue,[64] and thus may have the ability to form all zones of cartilage. These cells can be isolated from several locations in the body, such as bone marrow,[64,65] fat,[66,67] synovium,[68,69] and umbilical cord,[70,71] in procedures that are less invasive than cartilage harvest.[72] One promising source for chondrogenic stem cells is the synovium, based on the similar expression profiles of the synoviocytes to chondrocytes and their differentiation capacity, which is particularly induced with BMP-2.[68,73,74] The stem cell population can be isolated by $CD14^-$ selection, and tissues engineered from these cells have been shown mechanically similar to articular cartilage and to express and accumulate collagen II but not collagen X.[69,75] In addition, they show a functional characteristic of the superficial zone, expression of PRG4,[76] and thus may be particularly useful in regenerating the upper layers of articular cartilage.

If growth environments can be designed to direct MSCs toward specific chondrocyte lineages before construct formation, a single starting pool of MSCs could be used throughout the construct. Such an approach of zonal differentiation has shown potential for maintaining the zonal phenotype of chondrocytes isolated from different zones using aggrecan- and collagen-coated plastic.[77] Alternatively, combinations of stem/progenitor cells and chondrocytes may provide advantages. For example, MSCs could form a surface layer atop a layer of deep zone chondrocytes.

Scaffold- and matrix-free approaches

Scaffold- and matrix-free approaches for cartilage tissue engineering employ either chondrocytes or stem/progenitor cells with the potential to form cartilage under chondrogenic conditions without the support of exogenous scaffolds or matrices (Fig. 2A and Table 2). As such, these approaches mimic chondral condensation and development and avoid

Table 2. Approaches for Tissue Engineering Articular Cartilage with Biomimetic Zones

Approach	*Biological properties*	*Mechanical properties*	*Ease of biological modification*	*Efficiency of cell seeding*	*Cost effectiveness*	*Zonal organization*	*References*
Scaffold and matrix free	+++	−	−	+++	++	+	7, 23, 50, 51, 63, 65, 69, 78, 80, 81, 83–86, 88, 99–101, 157, 161, 166
Scaffold based	±	++	+	±	+	++	49, 90–94, 96–101, 103–109, 140–143, 145, 146, 149, 150, 155, 158, 162
Matrix based	++	±	++	+++	+	+++	74, 77, 79, 103, 104, 111, 112, 114–119, 121–124, 126, 127, 136, 139, 147, 148, 151–153, 156, 162

potential complicating issues of biomaterial degradation and scaffold design. The simplest such *in vitro* approach, micromass pellet culture, is used as a standard method for analyzing the ability of cells to form cartilage.[78] Pellets are formed by centrifuging a large number of cells (250,000+) and subsequently culturing the cell pellet in chondrogenic medium containing TGF-β(1, 2, or 3) for up to 4 weeks. Such pellets do develop a zonal organization of cells and matrix, with GAG content typically increasing from the outer surface to the center. While this method is useful for assessing chondrogenic potential through histological and quantitative biochemical assays, it is not directly applicable for clinical applications, as the pellets are small (~1–2 mm and shrinking for some cells), the zonal variations are spherical rather than depth dependent, and the required cell numbers are too high to restore a reasonably large defect of $>1\,cm^2$.

As an alternative, cells can be seeded into hanging cell culture inserts (e.g., Transwell®) and are able to form relatively thick tissue (order 1 mm) over the entire insert surface.[65] These tissues are much more substantial than pellets seeded with similar numbers of cells, in terms of wet weight and matrix deposition. Additional preculture of cells in alginate allows for re-differentiation of expanded chondrocytes and matrix accumulation around the cells[79]; the cells and newly formed matrix can then be recovered with nonenzymatic dissolution of the alginate and seeded onto cell culture inserts (Alginate-Recovered Chondrocyte method[80]). The tissues generated with this method contain more matrix and are thicker, and thus they may potentially be useful for future clinical tissue engineering applications.[81]

Cells isolated from the superficial and middle zones of bovine cartilage produce cartilage constructs with different levels of matrix and PRG4 (lubricant) production[82] (Fig. 2A). When cells from the two zones were layered, cells at or near the superficial-seeded surface expressed PRG4, resulting in a tissue with a structure that resembled the upper zones of native articular cartilage and with lubricant production that could be tailored by varying the ratio of cell types and expansion conditions.[51,82] Additionally, cells from the middle and deep zone have been seeded into hanging cell culture inserts or atop calcium polyphosphate substrates to produce a tissue similar to the deep zone/calcified cartilage transition to the bone.[83,84] Hydroxyapatite mineralization in such tissues is localized to the layer in contact with the substrate, whereas the region further from the substrate is noncalcified and hyaline in nature.[85,86] Layering cells from the appropriate may be important, as labeled newborn bovine chondrocytes from the individual zones mixed with cells from the other zones did not show evidence of cell sorting to the appropriate layers of the construct.[87] However, in these experiments, the location of the cells with respect to the construct or membrane surfaces was sufficient to initiate the cell differentiation at that depth and produce a zonally correct tissue. Cells expanded over several passages did not retain these properties,[87] and the formation of such tissues with adult human tissues remains to be investigated.

MSCs from bone marrow can also form cartilaginous tissue *in vitro* when cultured in serum-free chondrogenic media in micromass pellet cultures[64] and cell culture inserts.[65] In addition to *in vitro* studies, clinical applications of the cells, in principle, have taken place for decades in attempt to repair damaged cartilage by penetrating the subchondral bone in microfracture and subchondral drilling procedures.[88] These procedures result in a fibrocartilaginous repair tissue that achieve outcomes similar to the more challenging and expensive ACI technique.[7,89] The signals in the joint appear to be inadequate for consistent genesis of the three-zone structure of native cartilage starting from bone marrow.

Scaffold-based approaches

While the scaffold- and matrix-free approaches described above rely on specific cell populations and matrix synthesis to result in a particular structure, scaffold-based biomaterial approaches have the opportunity to provide structure, mechanical strength, and organization directly via the scaffold design (Fig. 2C and Table 2).[90] Similar to initial scaffold-free approaches, the goal of initial scaffold-based approaches was a homogeneous tissue. Thus, traditional fabrication technologies were used to create uniform scaffolds. These methods include fiber bonding, melt molding, and particle leaching. Optimization and control of pore-size and interconnectivity proved to be a challenge with these methods, and as a result it proved difficult to seed the scaffolds evenly with cells. Thus, the resulting constructs typically exhibited a high density of cells at the periphery and low cell density in the

center, which could be overcome with dynamic seeding protocols.[49,91]

Alternative methods based on rapid prototyping technology have subsequently been used to form scaffolds with 100% interconnected porous networks and defined geometry. Parameters such as fiber diameter, fiber angle, material, and pore-size can be controlled using solid free-form fabrication (SFF), allowing for control of the structure on the scales of tens of microns to centimeters.[92] Associated with the structure are mechanical properties, and anisotropic properties have been formed by varying the structure of each layer.[93] Using a different fabrication technique, woven scaffolds exhibiting tension–compression nonlinearity have also been created[94]; however, questions remain about the low porosity and interconnectivity of such scaffolds. Thus, several of the important mechanical properties of articular cartilage can be incorporated into the constructs from the initial seeding until the scaffolds are degraded and replaced by newly formed matrix. Additionally, printed fiber scaffolds with an open pore structure allow for simplified homogeneous cell seeding and reduction of nutrient limitations throughout the construct.

One drawback of printed fiber scaffolds is the relatively large fiber diameters (hundreds of micrometers), which present themselves to cells as essentially 2D surfaces, and thus can have a negative influence on cell function. Another drawback is the expense. Nanofiber meshes (nonwoven scaffolds with fiber sizes $<1\,\mu m$) are easily fabricated by electrospinning and can incorporate natural and synthetic materials, such as collagen and poly(ε-caprolactone).[95] Such meshes have been shown to induce chondrogenesis better than large fiber scaffolds.[96] The nanofiber mesh network is said to resemble the collagen network of articular cartilage, although cells incorporate into this network in distinct ways. MSCs seeded atop poly(ε-caprolactone) nanofiber meshes appear to infiltrate the mesh and remodel the matrix in a cartilage-like manner[97] through an unknown mechanism. Nanofiber meshes are useful for providing signals and mechanical stability to seeded cells and could be useful for generating zonally organized engineered cartilage constructs by seeding cells of different phenotype on either side. Meshes fabricated with aligned polymer fibers may be useful in forming tissues with oriented mechanical and cellular properties, such as the superficial zone in which collagen fibers are normally aligned.[98] Such scaffolds have been shown to result in preferential orientation of MSCs up to 5 weeks in chondrogenic differentiation culture,[98] but the cells have not been characterized for expression of zonal markers and should be regarded as preliminary proof-of-concept studies.

Another scaffold-based approach that has been implemented in clinical repair of articular cartilage defects is the so-called matrix-induced ACI technique. In this approach, a bi-layered membrane of collagen types I and III, with one cell-impermeable layer and one highly porous layer, replaces the periosteal flap of traditional ACI. This technique has shown similar positive outcomes in patients with focal defects and fewer complications with graft hypertrophy, and is technically easier than traditional ACI.[99] Histological and MRI-based follow-up studies have shown that zonal organization of the *de novo* tissues can range from disorganized (Fig. 4D[100]) to zonally developed in terms of T2 relaxation times,[101] which are related to the collagen network.[102] Assessment of zonal markers has not been determined for these tissues.

There are several additional scaffold-based approaches that develop physical (pore size, porosity, stiffness, etc.) and chemical (adhesion sites, enzymes, growth factors, etc.) gradients in scaffolds,[103,104] with potential application to zonal cartilage tissue engineering. However, only a few studies have investigated these gradient approaches for use in cartilage tissue engineering. Gradients in pore size have been formed using the TheriForm fabrication process to generate osteochondral scaffolds with localized chondrocyte distribution.[105] Scaffolds with protein gradients have also been generated using sintered microparticles containing enzymes or growth factors.[106,107] Nonetheless, the utility of gradient scaffolds in engineering articular cartilage with biomimetic zones remains to be determined.

Matrix-based approaches

Hydrogel matrices offer several opportunities for producing organized tissues (Fig. 2B and Table 2). They can be formed in various shapes and polymerized using light, enzymes, or chemical cross-linkers from a wide variety of material precursors. As they are a liquid form to begin with, they take the shape of the mold, which could be the cartilage defect or potentially an entire articular surface,[108,109] and offer the opportunity of injectable engineered tissues. The fiber size created in such gels is in the order of the natural extracellular matrix (e.g., $\sim 8\,nm$ diameter for agarose[110]), and with incorporated biological signals can provide a more biologically relevant environment for chondrogenic differentiation than macroscopic polymer scaffolds. Additionally, it is much easier to generate a construct with uniformly seeded cells in a suspension of hydrogel than in a fibrous scaffold. A vast array of hydrogels have been investigated, falling under the general classes of natural (fibrin, alginate, gelatin, agarose, hyaluronic acid, Matrigel, etc.), synthetic [poly(ethylene glycol) (PEG), poly(propylene fumarate), Pluronics, etc.], and natural–synthetic combinations.

Natural hydrogels have been used for decades in studies of chondrocyte function and generally show maintenance of chondrocyte phenotype[111,112] and are promising for cartilage tissue engineering, due in part to their inclusion of biologically relevant substrates. Collagen gels are an intuitive choice based on the abundance of collagen and the collagen network importance in the function of native cartilage. Such gels have the advantages of incorporated bioactive sites and matrix metalloproteinase degradability. These gels have been used *in vitro* as well as *in vivo* and clinically for several types of tissues, including cartilage, although there are concerns over immunogenicity of animal-derived matrices, especially those containing collagen type II.[113] A recent method suggests a cell-recruitment-based approach with potential for zonal tissue development using a cell-free collagen gradient hydrogel scaffold to recruit MSCs.[114]

Other natural materials from nonmammalian sources have been studied extensively in cartilage tissue engineering research, and have also been applied to generate matrix-based constructs with zonal properties. Agarose gels of different concentrations have been seeded with chondrocytes as a model for the depth-dependent mechanical properties of articular cartilage.[115] The compressive moduli of the different

layers started as homogeneous within the layer and ended as a gradient with smoother transition between the layers after 4 weeks in culture.[115] MSCs encapsulated in alginate disks expressed PRG4 when cultured with TGF-β, indicating their potential for zonal function, yet further work is required to show reduction in frictional properties of such constructs.[116] While studies using agarose and alginate have been primarily laboratory based, recent clinical trials implanting chondrocyte-seeded medical-grade alginate–agarose hydrogels in chondral and osteochondral defects have shown hyaline cartilage in biopsies of 8/13 patients.[117] In these biopsies, normal zonal organization was not evident.

Synthetic hydrogels have the advantages of less variability and greater flexibility in mechanical and biochemical properties over natural hydrogels. Also, certain hydrogels have the advantage of being photopolymerizable, which would allow for suspensions of cells and hydrogel to be injected and polymerized *in situ*.[118] Already, photopolymerizable hydrogels (e.g., PEG diacrylate) have been used as a model system for studying zonal organization by layering cells from different zones and have showed good cell viability, retention in respective layers, and differential cartilage matrix expression.[119]

Hydrogels have also been designed to incorporate specific biological activity (such as adhesion or growth factors) and degradation sites through a combination of natural and synthetic molecules.[120,121] Synthetic hydrogels with site-specific design have the potential of providing signals necessary for chondrogenesis, and when combined with a layering approach could provide distinct signals to cells in different regions/zones of the construct. One approach uses a peptide-based hydrogel that self-assembles, and has been shown to form a functional cartilaginous matrix with time in culture when seeded with immature bovine chondrocytes.[122] Another approach that incorporates specific cartilage matrix components (including hyaluronic acid and chondroitin sulfate) into a photopolymerizable PEG diacrylate hydrogel has shown that chondrocytes from different zones respond differently in terms of matrix production and gene expression.[123] Thus, when used in a layering or gradient system,[119,124] customized hydrogels offer multifaceted control over construct zonal properties.

Hybrid approaches

Hybrid approaches based on bioprinting combine the deposition of specific cell populations with the simultaneous deposition of biomaterials[125] and will aid the further development of zonal cartilaginous grafts (Fig. 2D). As discussed previously, using SFF printing technologies, a certain degree of organization can be obtained by the connected porous polymer network.[93] However, because of the toxic solvents and high temperatures used in most SFF techniques, this approach encounters a number of nonphysiological conditions and is hence not compatible with living cells. Using hydrogels, a more physiological environment can be created. Indeed, cell suspensions can be mixed into *in situ* crosslinkable hydrogels (e.g., gelatin, agarose, alginate, or PEG) in a cartridge and subsequently printed following a programmed 3D pattern.[126,127] Also, the water-based bioinks can contain biologically active components, such as proteins, peptides, DNA, hormones, extracellular matrix molecules, and natural or synthetic polymers,[128] to further enhance the behavior of the cells. Thus, these approaches combine the scaffold- and matrix-based approaches to create hybrid and more complexly organized constructs.

The organization provided via both scaffold design and controlled deposition of cells at predefined locations can potentially accelerate the organization of cells into a functional tissue. Hence, a number of hydrogel-based bioprinting approaches are currently being developed for a variety of tissues, including bone[127,129] and cartilage.[126] For example, using chondrocytes embedded in alginate, hydrogel constructs have been created with heterogeneous geometries and high cell viability ($94 \pm 5\%$).[126] In addition, by printing layers of chondrocytes isolated from the deep, middle, and superficial zones of cartilage, embedded in a polymer hydrogels, this technology further allows the production of a graft with controlled architecture that also mimics the topographical organization of cells in the native tissue.

The research field of bioprinting has only recently emerged,[125] and bioprinting technology is still in its infancy. As a consequence, a number of areas of investigation may aid in the translation of bioprinting to clinical application. Technical issues, such as the effects of dispensing pressure and nozzle diameter, have marked effects on cell survival.[129,130] Also, it remains to be determined if a printed chondrocyte-laden construct would outperform a chondrocyte cell suspension or homogeneously seeded scaffold *in vivo*. Moreover, it is unclear if placing cells and matrix in the native orientation would enhance biological properties or mechanical stability.

Tissue Remodeling

All cartilage tissue engineering approaches rely on cell-mediated tissue remodeling to achieve the desired final tissue organization and structure. As cells are responsive to their environment, controlled application of exogenous biochemical and biomechanical signals may be a key to producing a preimplant tissues with appropriate structure and function. After implantation, constructs are subjected to a complex *in vivo* environment that is less controlled; understanding the *in vivo* remodeling process will help facilitate development of long-term functional tissues with biomimetic zones.

In vitro *exogenous stimuli*

Various biochemical signals have been applied *in vitro* with varied effects on cartilage matrix production and remodeling.[131] Biochemical factors have great influence on zonal marker expression, and thus could be important to integrate into specific parts of the construct. For example, PRG4 is upregulated by factors such as TGF-β(1–3), IGF-I, PDGF, and BMP7,[132–134] whereas inflammatory cytokines interleukin-1 and tumor-necrosis factor-α generally inhibit expression of PRG4 in various culture systems.[132,134,135] Application of such factors to specific regions of the engineered tissue could be implemented through incorporation of growth factors into a hydrogel network[136] or through site-specific incorporation of growth-factor-containing microparticles.[106]

Biomechanical signals can be applied in a controlled manner using bioreactors to impart shear, compression, tension, pressure, or a combination of these loads on the growing tissues.[131] Chondrocytes are quite responsive to mechanical signals, remodeling the matrix according to the

loads, and thus the choice of loading regime is important for regulating development of a structure similar to native cartilage. Fluid-based bioreactors include spinner flasks,[137] laminar flow bioreactors,[138] hydrostatic pressure bioreactors,[139] and direct perfusion bioreactors,[140] which result in markedly different tissues. After prolonged incubation *in vitro*, constructs cultured in spinner flasks develop a zone of fibrous tissue at the periphery. This type of layered structure, which resembles (to a certain extent) native cartilage when viewed from the center of the construct to the surface, has been predicted based on changes in cell proliferation and nutrient consumption.[141] Laminar flow bioreactors have been successfully used to produce tissues with excellent bulk mechanical properties and GAG content in long-term cultures,[142] and can also result in tissues with a peripheral zone of low GAG content.[143] Hydrostatic pressure bioreactors increase GAG content and also affect chondrocytes from different zones differently, with middle zone chondrocytes eliciting the greatest calcium response and matrix production.[144] Direct perfusion bioreactors force fluid through the tissue and hence apply a complex combination of hydrostatic pressure, fluid shear stress, and compression that can enhance DNA, GAG, and collagen content compared to static controls, but application to zonal cartilage constructs remains to be investigated.[145,146]

Direct mechanical loading can also be applied in custom or commercial bioreactor systems to improve matrix production and affect zonal construct characteristics (Fig. 3). Tensile bioreactors differentially influence proteoglycan production by zonal chondrocytes, and may therefore be useful in further developing zonal construct properties when using zonal chondrocytes (Fig. 3B).[147] Dynamic compression bioreactors are more commonly used to increase extracellular matrix synthesis in tissue-engineered cartilage (Fig. 3A).[148–150] Dynamic compression applied to layered constructs with varying mechanical properties (2% agarose vs. 3% agarose) imparts depth-dependent strain and fluid flow and results in stiffer tissue in the initially softer, more permeable layer.[151] These results emphasize that simply prescribing variations in scaffold/matrix properties may be insufficient to determine mechanical properties of engineered cartilage when generally beneficial mechanical signals are included. Dynamic compression of biphasic (bone/cartilage) constructs results in depth-varying stress distribution in the engineered cartilage layer, and thus may provide appropriate signals for zonal matrix production and remodeling.[152] Dynamic compression along with shear at the articular surface most closely approximates *in vivo* loading, and bioreactors based on hip simulators have been shown to be beneficial for lubricant production in small constructs (Fig. 3C).[153] Joint-scale bioreactors could also be useful in producing large joint surfaces with properties influenced by realistic joint loading, and such a bioreactor with femoro-tibial loading induced by a continuous passive motion device has been shown to increase PRG4 production during culture of knee joints.[154] Bioreactors such as these could be important in developing zonal variations in the collagen network of tissue-engineered cartilage.

In vivo *remodeling*

Despite meticulous efforts to design a zonal structure *in vitro*, the *in vivo* environment can have a major influence on the final structure and organization of tissue-engineered cartilage. Initial experiments with tracking cells implanted in polylactic acid scaffolds[155] or alginate matrices[156] into rabbit cartilage defects showed that ~85% of implanted cells were lost over the first 4 weeks of implantation. These experiments indicate that cells can persist in the defects after implantation, but may be significantly reduced in number. Issues to address after implantation include cell proliferation, cell death, and cell reorganization. Recent studies with zonal constructs formed using the Alginate-Recovered Chondrocyte method additionally show that immature and delicate constructs do not maintain their zonal structure shortly after

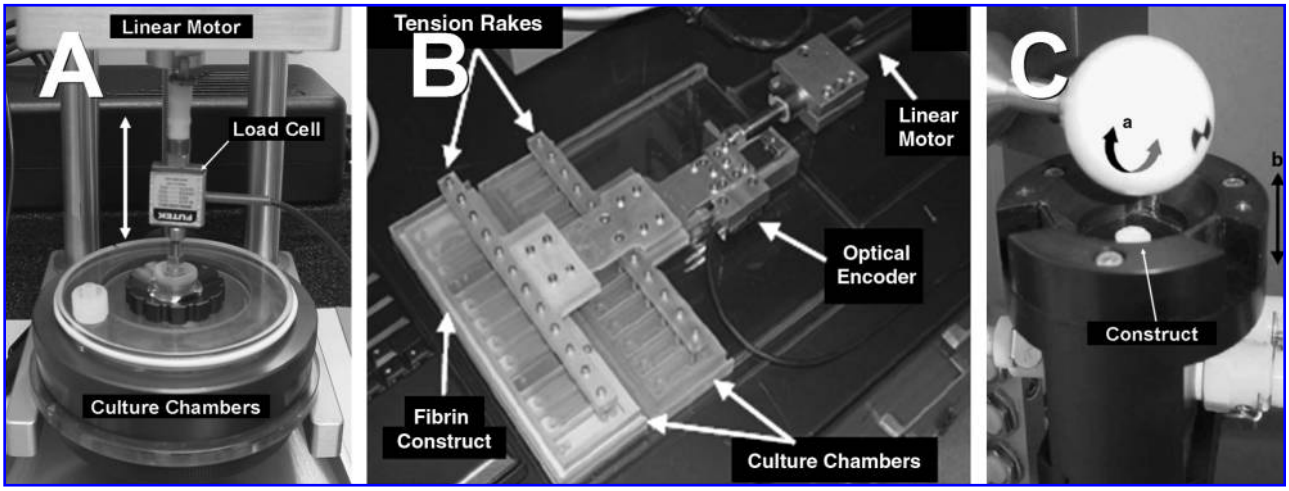

FIG. 3. Bioreactors for *in vitro* tissue conditioning. (**A**) A commercial dynamic compression bioreactor (Tissue Growth Technologies, Minnetonka, MN, www.tissuegrowth.com) allows for controlled simultaneous dynamic compressive loading and perfusion of up to 12 constructs. (**B**) A custom tensile bioreactor allows for tensile loading of 12 constructs, and has been used to show different responses from chondrocytes of different zones.[147] (**C**) A hip simulator-based bioreactor applies surface motion as well as dynamic compression, and has been shown to enhance the surface properties of the engineered cartilage.[153]

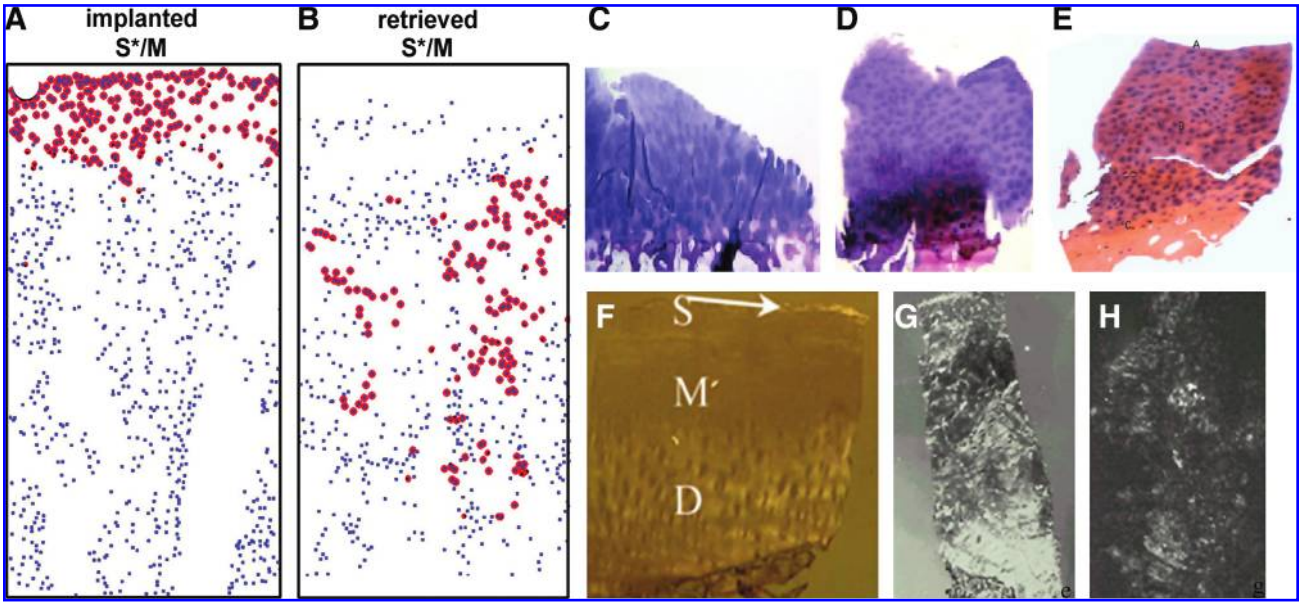

FIG. 4. Cell re-organization and matrix remodeling *in vivo*. Zonal porcine cartilage constructs with fluorescently labeled superficial cells showed (**A**) well-defined layers after 4 weeks of *in vitro* culture, but (**B**) this structure was lost after 1 week *in vivo*.[157] While no clinical trials have been performed with zonal engineered cartilage, several clinical approaches for regeneration result in tissues that generally lack the zonal organization of native articular cartilage. (**C**) ACI and (**D**) microfracture 2 years after implantation[89] lack the zonal architecture, similar to (**E**) matrix-induced ACI 1 year postoperative.[100] In rare cases, polarized light microscopy shows (**F**) good collagen alignment in ACI repaired regions, as opposed to (**G**) mixed hyaline-fibrocartilage and (**H**) more typical fibrocartilage formation.[166] ACI, autologous chondrocyte implantation. Color images available online at www.liebertonline.com/ten.

implantation (Fig. 4A, B).[157] While further *in vitro* maturation of the construct is likely to improve cell retention and zonal structure, constructs that are too mature may not integrate with surrounding tissues.[158]

In addition to retention of cell structure and localization, the organization of the extracellular matrix and degradation of matrix within the constructs should be considered. The joint presents a variety of enzymes, such as aggrecanases (ADAMTs) and matrix metalloproteinases, that may degrade the *in vitro*–generated matrix.[159] Several methods have been and are currently being developed to assess the changes to implanted constructs after surgery, including cell tracking, histology, synovial fluid analysis, and noninvasive imaging.[160,161] It is apparent from correlative histological and noninvasive T2-weighted MRI of equine repair tissue that microfracture, a procedure that does not prescribe any order to the reparative cells (MSCs from the synovium and bone marrow), results in tissue that lacks the collagen organization of native tissue,[102] whereas human repair using the matrix-induced ACI technique shows zonal organization as indicated by T2 relaxation times.[101] Hence, further *in vivo* studies are needed on homogeneous and zonal tissue-engineered constructs to better understand which aspects of the original construct are maintained and which are simply lost during the *in vivo* remodeling process.

Future of Zonal Tissue-Engineered Cartilage

Engineered zonal cartilage tissues may have utility in two distinct arenas. First, the tissues can act as more accurate models (*in vitro* or *in vivo*) of cartilage organization, development, and disease. These models would be particularly useful in testing novel pharmaceutical treatments. Second, they may be applied clinically to repair and/or replace damaged tissues.

Representative in vitro *models*

It is becoming increasingly clear that standard tissue culture models (monolayer cultures) are not representative of the native cellular environment and that these models can provide misleading results. Thus, there has been a push toward developing three-dimensional culture systems to better represent the native environment and cellular response.[162] Tissue-engineered cartilage is, by nature, a three-dimensional culture system, and thus provides the potential of more biologically relevant signals than monolayer cultures. Extending the organization of the tissue-engineered cartilage to include the zonal variations in cells, matrix, and mechanical properties could provide an even better model to understand chondrocyte interactions, cartilage development, and disease.[163]

Several of the engineered cartilage systems recapitulate the high cell density and low matrix content of fetal or immature cartilage, and applying specific mechanical and/or biochemical stimuli while monitoring the zonal development could provide key insight into normal cartilage development and what is needed to form *de novo* organized cartilage in the adult/aged human. Especially considering the importance of the integrity of the superficial zone in the progression of arthritis,[164] including this zone and subsequent zones could help with development of realistic *in vitro* models of cartilage disease and the effects of pharmacological and/or mechanical inputs on the progression of the disease. These models could reduce the reliance on animal models of cartilage disease. This is important as significant differences are seen

between the activity of human and animal chondrocytes and in the structure (e.g., thickness) of the articular cartilage. The model systems also allow more detailed noninvasive monitoring of zonal properties, using laboratory-based imaging systems such as micro-CT[24] and MRI.[165]

Clinical applications

While development of improved *in vitro* models is important, the ultimate aim of cartilage tissue engineering, including zonal cartilage tissue engineering, is in the development of a tissue that can serve as a long-term biological treatment for cartilage disease. There are several reasons to pursue zonal constructs for this purpose. To function properly, articular cartilage has developed an exquisite organization, and it is likely that a simple homogeneous tissue cannot replace the functions of native cartilage. Current clinical engineered tissues do not commonly attain such zonal organization. The provision of zonal properties from the outset may allow for less remodeling *in vivo* to achieve the desired zonal structure, and thus a shorter rehabilitation time. This would be beneficial from a patient and an economic viewpoint. Zonal constructs also can address the difficult problem of neocartilage–cartilage integration[158] by matching the zonal construct mechanical properties to the surrounding tissues to reduce strain discontinuities at the interface.

Conclusions

While progress has been made in the development of zonal engineered cartilage, a number of challenges remain to be addressed. There are now numerous choices in terms of cell sources (Table 1) and methods for *in vitro* zonal organization (Table 2 and Fig. 2). Choosing the appropriate cell type(s) and appropriate methods for their isolation and expansion are required for reproducible production of *in vitro* model systems and clinically applicable engineered cartilage tissue. A number of clinical challenges have already been addressed by the ACI technique, but the formation of a zonal construct makes the process somewhat more difficult. Thus, simplification of zonal techniques needs further attention. There is limited information on remodeling of the engineered zonal tissues. *In vitro*, mechanical signals are likely required for significant matrix remodeling, but it remains to be seen whether or not these need to be applied before implantation or if a rehabilitation schedule can be designed to apply to the appropriate signals after implantation. Finally, additional techniques are needed to characterize the *in vivo* remodeling and comparative studies with established tissue engineering approaches are required.

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