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A Paradigm for Functional Tissue Engineering of Articular Cartilage via Applied Physiologic Deformational Loading

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Abstract—Deformational loading represents a primary component of the chondrocyte physical environment *in vivo*. This review summarizes our experience with physiologic deformational loading of chondrocyte-seeded agarose hydrogels to promote development of cartilage constructs having mechanical properties matching that of the parent calf tissue, which has a Young's modulus $E_Y = 277$ kPa and unconfined dynamic modulus at 1 Hz $G^* = 7$ MPa. Over an 8-week culture period, cartilage-like properties have been achieved for 60×10^6 cells/ml seeding density agarose constructs, with $E_Y = 186$ kPa, $G^* = 1.64$ MPa. For these constructs, the GAG content reached 1.74% ww and collagen content 2.64% ww compared to 2.4% ww and 21.5% ww for the parent tissue, respectively. Issues regarding the deformational loading protocol, cell-seeding density, nutrient supply, growth factor addition, and construct mechanical characterization are discussed. In anticipation of cartilage repair studies, we also describe early efforts to engineer cylindrical and anatomically shaped bilayered constructs of agarose hydrogel and bone (i.e., osteochondral constructs). The presence of a bony substrate may facilitate integration upon implantation. These efforts will provide an underlying framework from which a functional tissue-engineering approach, as described by Butler and coworkers (2000), may be applied to general cell-scaffold systems adopted for cartilage tissue engineering.

Keywords—Articular cartilage, Chondrocyte, Functional tissue engineering, Osteochondral constructs, Cartilage repair.

INTRODUCTION

The high prevalence of osteoarthritis (OA)[40,41] and the poor intrinsic healing capacity of articular cartilage has led to the development of cell-based strategies for cartilage repair. The successful repair or replacement of articular cartilage using tissue-engineered constructs will require grown tissue possessing the functional properties of the native tissue as well as adequate graft-to-host tissue integration.[2,21,44,50,95,117] The long-term hypothesis of our studies is that a viable strategy for cartilage tissue engineering is to elaborate constructs *in vitro* which achieve mechanical properties and biochemical composition that match those of native tissue, prior to implantation. This hypothesis stems from our belief that the loading environment in a joint is too harsh to allow a fledgling cell or tissue construct to survive and grow *in situ* into a tissue capable of performing a successful load-bearing function. A premise of the evolving discipline termed "functional tissue engineering" (FTE) is the utilization of bioreactors that recreate the physiologic loading environment, to foster the growth of tissue constructs that mimic the apparent mechanical properties and composition of the native tissue, enabling it to perform its load-bearing function *in vivo*.[21,50] Within this context, our laboratory has been exploring the novel application of physiologic deformational loading, a primary component of the chondrocyte physical environment *in vivo*, to precondition bovine chondrocyte-seeded 3D agarose hydrogel constructs in culture (a system well characterized for chondrocyte biology and mechanotransduction[53]). This review article summarizes and synthesizes the results of our recent studies.[62,84,86,89,91,93]

Mechanical load has been demonstrated in many *in vivo*[105,125] and *in vitro*[20,48,52,65,121,122,138,147] investigations to be important for the normal maintenance of articular cartilage. The mechanical environment of the chondrocyte is an important factor affecting the health of articular cartilage and consequently the function of the diarthrodial joint, and the progression of joint degeneration. Static compressive loads (0.001–3 MPa) have been demonstrated to cause a decrease in proteoglycan (PG) synthesis and release from the ECM of articular cartilage[48,52,65,105] in addition to a decrease in protein synthesis.[48] The catabolism activity of PGs has also been shown to be affected by compressive loading.[52,65] Investigators using dynamic or cyclic loading conditions, corresponding to physiological loading, have also reported changes in biosynthetic activity in articular cartilage.[20,69,104,109,121,122] These effects are strongly dependent on magnitude and frequency of the applied load,[53] with slow frequency loading generally resulting in suppression of PG synthesis while more rapid loading frequencies result in stimulated synthesis.[53] For these reasons, we have

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adopted deformational loading at the physiologic frequency of 1 Hz in our studies.

For articular cartilage, deformational loading and hydrostatic pressure are believed to represent two of the most relevant physical forces to chondrocytes *in vivo*.[4] Accordingly, these forces have been widely studied physical stimuli in cartilage basic science[17,54,69,77,89,92,104,108,109,121,122,129,130,138] and seem therefore to be appropriate mechanical factors to be emulated for the design of bioreactors aimed at FTE of articular cartilage. Beneficial effects of applied physiologic loading on tissue biochemical and material properties have been demonstrated for a combination of applied physiologic hydrostatic pressure and perfusion (~3–6 MPa, 0.05 Hz) on chondrocyte-seeded polymer scaffolds by Carver and Heath,[23–25] for applied shear loading of calcium phosphate constructs by Waldman *et al.*,[144] and by our laboratory for applied physiologic compressive deformational loading of chondrocyte-seeded agarose hydrogels.[86,87,89]

Engineered cartilage constructs have also been subjected to applied deformational loading and found to respond with biosynthetic and gene expression changes similar to articular cartilage explants.[18,32,78] In contrast, long-term (up to 12 months) free-swelling cultures are unable to achieve material properties similar to either dynamically loaded constructs or the native tissue.[1] The deformational loading experiments focused on in this review were conducted (as reported previously[86,89,92]) under unconfined compression and displacement control. By controlling the applied deformation (instead of the applied stress) on our tissue-engineered constructs, we are able to modulate the *in vitro* loading environment to avoid excessive compression that may lead to cell death.

In our approach to physiologic deformational loading, unconfined compressive loading between two impermeable platens was adopted. Under this loading regime, the construct is free to expand laterally (i.e., in the radial direction).[3] Unconfined compressive loading also gives rise to radially-dependent pressure and fluid flow gradients that are largely restricted to the periphery of the sample for physiologic loading rates (i.e. ~1 Hz).[3,20,69,122] Overall, this loading configuration produces more uniform mechanical signals throughout the thickness of a cylindrical cartilage sample than that of confined compression (where radial expansion is prevented). When compared to the analysis of two contacting articular cartilage layers,[107] this uniformity of interstitial fluid pressure through the depth of the sample in unconfined compression more closely resembles the physiologic situation (Fig. 1). Furthermore, unconfined compression produces both compressive strains (along the axial direction) and tensile strains (along the radial and circumferential directions);[16,29,66,71,106,107,135] these signals represent a more physiologic loading environment than confined compression (wherein sharp pressure and deformation gradients exist at the porous indenter). Finally, unconfined compression can produce tissue strains with negligible change in tissue volume (since the disk can expand laterally when compressed axially), while confined compression is always accompanied by loss of tissue volume due to water efflux. *In vivo* measurements of cartilage volumetric changes have been shown to be small (6%) even following strenuous loading.[36] Conversely, the concurrent

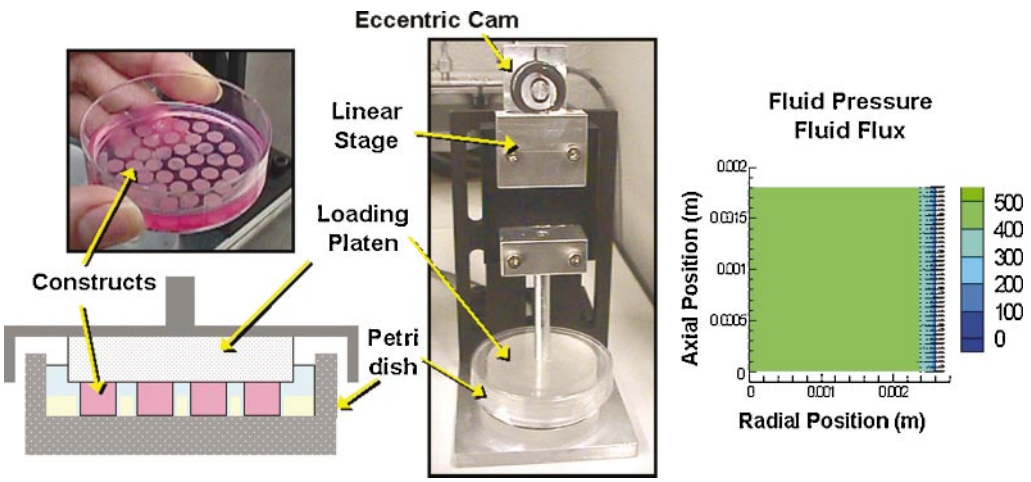

FIGURE 1. (Left) Bioreactor system for dynamic deformational loading. Constructs are placed in the base of a standard Petri dish modified with a custom agarose template (with holes larger than the disk diameter) to maintain positioning during loading. Loading is carried out via a smooth acrylic indenter designed to enter the base of the Petri dish, without interfering with the sidewall. Sinusoidal deformation is prescribed via the revolution of an eccentric cam calibrated to produce a defined displacement of a spring-loaded linear stage follower connected to the loading platen. (Right) Biphasic FEM model prediction of peak hydrostatic pressure and fluid flow (arrows) in a 2% agarose construct undergoing dynamic loading (10% deformation, 1 Hz).

use of a permeable platen with an impermeable platen (in unconfined compression) may provide a means of introducing depth-dependent inhomogeneity in an otherwise relatively uniform gel construct (see discussion of gel–bone constructs below).[76]

For our studies, we have adopted an agarose scaffold, which is a clear, thermoreversible hydrogel made of polysaccharides, mainly the alternating copolymers of 1,4-linked and 3,6-anhydro-α-L-galactose and 1,3-linked β-D-galactose.[7,9,128] Agarose has been used extensively in cartilage biology for maintaining long-term chondrocyte suspension cultures; its ability to promote and maintain the chondrocyte phenotype is well documented (e.g., Refs. 6,9,18,19,72,73,75,86,89). Moreover, agarose hydrogels have been used successfully as cell/drug delivery vehicles and scaffolds for a variety of *in vitro* and *in vivo* tissue-engineering applications (e.g., nerve regeneration,[7,22,151] ocular reconstruction,[133,134] bioartificial pancreases,[150] and protein and peptide delivery[94,145]). In addition, our laboratory and others have adopted agarose for cartilage tissue-engineering applications.[19,60,86,89,101,103,115,119,124,126,127,146] Rahfoth *et al.* and Cook *et al.* have demonstrated that agarose-embedded chondrocytes can be used successfully for repair of articular cartilage and tibial defects *in vivo*.[30,115,146] Agarose has also been used to encapsulate cell–polymer integrates (bioresorbable polymer fleeces) to maintain phenotype and to improve retention and accumulation of ECM components synthesized by isolated human articular chondrocytes.[119,126] Brittberg *et al.* have implied that cell-seeded agarose gels protected by a periosteal flap also support the development of hyaline cartilage.[12]

In the context of FTE,[21] the choice of scaffold material and its inherent material characteristics will dictate the nature of loading that can be applied. The properties of agarose permit application of physiologic deformational loading (10% peak-to-peak deformation at 1 Hz) without platen lift off (i.e., separation between the loading platen and construct), permanent construct deformation, or requirement of a prior matrix formation by the encapsulated cells.[89] Additionally, agarose exhibits a similar load-support mechanism as articular cartilage, able to bear greater than 90% of the applied load via interstitial fluid pressurization.[89,131,132] These properties have enabled the chondrocyte deformational loading response (e.g., cell shape, biosynthesis, cell signaling, cytoskeletal remodeling) in 3D culture to be investigated.[43,70,72–75,118] Other scaffold materials, such as polyglycolic acid (PGA), have also proven successful for cartilage tissue-engineering applications; they facilitate significant accumulation of cartilage-like material properties with time in culture in rotating wall and perfusion bioreactor systems.[1,23,34,42,47,110,142,143] Such scaffolds, however, require significant matrix elaboration by cells before sufficient mechanical properties exist to allow for dynamic compressive loading. For example, 3-day cell-seeded PGA constructs are too fragile to be mechanically tested.[142]

In this paper, we review our findings to date examining the ability of applied physiologic compressive deformational loading to promote development of functional constructs for articular cartilage repair. Through a series of iterative studies, our results suggest that an optimal combination of deformational loading, growth factors, and cell-seeding density may foster tissue growth having properties approaching that of articular cartilage. Since the successful repair or replacement of articular cartilage using tissue-engineered constructs will require living constructs possessing functional properties of the native tissue (so as to minimize premature failure) as well as adequate graft-to-host tissue integration,[21,44] we also present extensions of our research that include the development of osteochondral constructs with the vision that an anatomically shaped osteochondral construct may ultimately serve as a biologic alternative to total joint replacement with plastic and metal prostheses.

MATERIALS AND METHODS

Cell Isolation

Articular cartilage was harvested from bovine carpometacarpal (CMC)[89] or patello-femoral joints[86] of 3–5 freshly slaughtered 4–6 month old calves obtained from a local abattoir. From our experience, 4×10^8 cells per CMC joint and 2×10^9 cells per patello-femoral joint can be isolated. Cartilage was rinsed in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10 or 20% fetal bovine serum (FBS), amino acids (0.5× minimal essential amino acids, 1× nonessential amino acids), buffering agents (10 mM HEPES, 10 mM sodium bicarbonate, 10 mM TES, 10 mM BES), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The cartilage chunks were then combined and digested in DMEM (5 ml/g tissue) with 2.5 mg/ml pronase (Calbiochem, San Diego, CA) for 1 h at 37°C with gentle stirring, followed by 0.5 mg/ml collagenase type II (Sigma Chemicals, St. Louis, MO) for 4 h at 37°C with gentle stirring. The resulting cell suspension was then filtered through a 30-μm pore size mesh to isolate individual cells, which were then sedimented in a benchtop centrifuge for 10 min at 1000*g*. After rinsing the pellets, cells were resuspended in 10 ml of DMEM, and viable cells counted using a hemacytometer and trypan blue. For the preparation of chondrocyte/agarose constructs, one volume of chondrocyte suspension (at 20, 40, or 120×10^6) were mixed with an equal volume of 4% low-melt agarose (Type VII, Sigma) in Phosphate Buffered Saline (PBS) at 37°C to yield a final cell concentration of 10, 20, or 60×10^6 in 2% agarose. After mixing, the chondrocyte/agarose mixture was cast into slabs between sterile glass plates separated by spacers, and cylindrical constructs cored using a dermal trephine, or

alternatively poured into sterile stainless steel molds (for osteochondral and anatomic constructs). Typical dimensions for the cylindrical constructs are 0.476 cm diameter and 0.2 cm thickness ($\sim$0.04 cm^3). Constructs were maintained in culture for up to 70 days, with daily changes of growth medium (with variable FBS concentration). Cultures were maintained in growth medium consisting of DMEM supplemented as indicated above with 50 μg ascorbate/ml.

In some studies, biochemical stimulants of matrix biosynthesis found in articular joints, insulin-like growth factor-1 (IGF-1)[58,137,141] and transforming growth factor-β1 (TGF-β1),[15,58,79,96–98,102,139] were added to the culture medium. The concentration of TGF-β (10 ng/ml)[8,97,113] and IGF-1 (300 ng/ml)[120,121] were adopted from ranges found to yield optimal results in the literature.

Deformational Loading Bioreactor

A custom bioreactor, described previously,[89] was used to dynamically load constructs (Fig. 1). Briefly, it consists of a loading platen (acrylic, 44.7 mm diameter) in line with a linear translation stage (New England Affiliated Technologies, Lawrence, MA). This linear stage is driven by an eccentric circular cam that provides an adjustable downward displacement of 10% of the initial construct thickness. The cam is driven by a stepper motor (Superior Electric, Model M062CS09, Bristol, CT) whose rotation speed is controlled by a signal generator (BK Precision, Model 4017, Placentia, CA). The loading platen is constructed to enter a standard 60 mm petri dish (Becton-Dickinson, Franklin Lakes, NJ) and incorporates a petri dish lid to maintain sterility during loading. The cell-laden disks sit in the bottom portion of a petri dish, and are constrained from gross lateral movements by an agarose (2%, type IB, Sigma Chemicals, St. Louis) template with circular holes of a larger diameter than the disks to allow for free diffusion. Loading is therefore unconfined in nature, between two impermeable platens. Dynamic deformation is applied with the bioreactor described above, at a physiologic 10% peak-to-peak compressive deformation and a frequency of 1 Hz. This loading regimen was carried out 5 days a week, for 3 h of loading per day (i.e., three consecutive cycles of 1 h loading and 1 h rest).[92] Loading occurs in a humidified 5% CO_2 incubator at 37°C. Free-swelling control disks were positioned adjacent to the loading device in the incubator during loading periods.

Gene Expression in Short-Term Loading Studies

For short-term studies, chondrocyte-seeded constructs (10 million cells/ml) were either loaded (as described above) or maintained in free-swelling culture for a period of 3 days. To isolate RNA from agarose, disks were first digested in QX1 buffer (Qiagen Inc.) at 50°C for 5 min. RNA was then extracted using Tripure isolation reagent (Boehringer Mannheim) according to the manufacturer's protocol and was further purified with the Rneasy Kit (Qiagen Inc.). The samples were reverse transcribed using oligo$(dT)_{15}$ primers and Superscript II reverse transcriptase (Gibco). For each PCR reaction, 3 μl of cDNA was amplified using Taq polymerase (Promega) and intron-spanning primers designed for bovine aggrecan or GAPDH.[138] PCR products were electrophoresed on a 2% agarose gel, and the intensities of the bands were quantified using NIH Image (Version 1.62). Relative gene expression was determined by normalizing the aggrecan signal intensity to the corresponding level of GAPDH.

Anatomic Molds

Using a CAD system, molds were designed which reproduce the 3D surfaces of both the articular surface and the underlying subchondral bone of an articular layer.[62,85] G-code was generated from the CAD models to drive a CNC milling machine, and surface molds were milled from stainless steel. Using these molds, chondrocyte-seeded agarose was cast to produce anatomic constructs in the shape of the human retropatellar articular layer from a patellofemoral joint. Patellar molds had a surface area of 11.7 cm^2, and an anatomic cartilage layer volume of 4.02 cm^3 with an average thickness of 0.34 cm.

Osteochondral Constructs

Devitalized bone disks were prepared by coring cylindrical specimens from bovine tibia trabecular bone. Trabecular bone is sufficiently porous to permit agarose infiltration, and may be a more compatible material for bony ingrowth.[59,111] Disks were cut with a diamond blade saw to a thickness of 2 mm, cleaned of marrow with a water pick, and sterilized in 70% ethanol for 4 h. The cell–agarose suspension was loaded onto the bone discs in a custom mold that produced a final construct thickness of 4 mm (2 mm agarose +2 mm gel/bone). To cast disks, molds were placed on a rigid surface overlaid with filter paper wetted with sterile PBS. A predetermined volume of molten cell-laden agarose was poured into the mold, followed by the insertion of the bone cylinders. With this technique, agarose can be cast to penetrate the underlying bone to a desired depth. After gelling for 15 min at room temperature, constructs were transferred to DMEM with 10% FBS and cultured at 37°C and 5% CO_2. To create anatomically shaped osteochondral constructs, trabecular bone blocks were obtained from the distal end of 1–2 year old bovine femurs using a bandsaw. These bone blocks were cleaned of marrow and affixed to a Plexiglas plate with epoxy to facilitate clamping and machining. They were then contoured down to the proper surface topography with CNC milling, using the same G-code used to create the subchondral component of the stainless steel patella mold. After milling, bone blocks were cut from their Plexiglas base and cleaned. A final soaking (4 h) in absolute ethanol was carried out to ensure sterility. To create a bilayer composite construct, the milled bony layer was

used in place of the stainless steel bony molding surface. The articular surface of the patellar mold and collar were assembled and positioned with the articular mold facing up. Cell-laden agarose was then poured onto the articular surface molding piece, and the bony surface placed on top to permit controlled interpenetration of gel into the bony region.

Material Testing

Cylindrical constructs were tested in confined compression or unconfined compression using a custom computer-controlled testing system.[131] After equilibration under a tare load of 0.02 N, stress–relaxation tests were conducted to 10% strain at a ramp rate of 1 μm/s. The aggregate modulus (H_A) or Young's modulus (E_Y) of the construct, for confined and unconfined testing respectively, were calculated from the equilibrium stress and initial cross-sectional area. For bilayered constructs, the magnitude of strain was calculated based on the thickness of the gel region only, as the bony region is much stiffer and does not deform.[67] In some studies, unconfined dynamic modulus was measured after stress-relaxation to 10% strain and equilibrium, by superimposing a 2% strain with frequencies ranging from 0.005 to 1 Hz (as in Ref. 93).

Biochemical Content

The biochemical content of each sample was assessed by first measuring the sample wet weight, lyophilizing overnight, and measuring dry weight. Gross water content was then determined from the difference. Once dry, the samples were papain digested overnight at 60°C, as described previously.[86,89] Aliquots of digest were analyzed for GAG content using the 1,9-dimethylmethylene blue dye-binding assay.[38,39] Additional aliquots were analyzed for DNA content using the Hoechst dye assay[68] against a standard curve of calf thymus DNA (Sigma Chemicals, St. Louis, MO). A further aliquot was acid hydrolyzed in 12 N HCl at 110°C for 16 h, dried over NaOH, and resuspended in assay buffer (24 mM citric acid monohydrate, 0.012 v/v glacial acetic acid, 85 mM sodium acetate trihydrate, 85 mM sodium hydroxide, pH 6.0). Ortho-hydroxyproline (OHP) content was then determined via a colorimetric assay by reaction with chloramine T and dimethylaminobenzaldehyde,[136] scaled for microplates. OHP content was converted to total collagen content using the conversion of 1:10 ratio of OHP:Collagen.[142] Each biochemical constituent (DNA, GAG, and collagen) was then normalized to the tissue wet weight to correct for differences in construct size.

Histological Analysis

For histology, samples were fixed overnight at 4°C in acid formalin ethanol, and decalcified (when a bone region was present) in 22.5% formic acid, 0.68 M sodium citrate for 1–4 days with daily changes of decalcifying solution. These fixed samples were then dehydrated with a graded series of ethanol and embedded cross-sectionally in paraffin blocks (Tissue Prep, Fisher Scientific). Paraffin blocks were cut on a rotary microtome (Leica, model 2030) into 8 μm thick sections, and then affixed onto glass slides with a 0.1% solution of Elmer's glue (Elmer's Products, Columbus, OH). Once on slides, specimens were deparaffinized with Citrosolv, rehydrated, and stained with either Safranin-O (1% in dH_2O, pH 6.7) to view PG distribution or with hematoxylin and counterstained with eosin Y alcoholic (Sigma, St. Louis, MO) to determine cell distribution. To view collagen distribution, sections were stained with Picrosirius Red (0.1% in picric acid) for 45 min followed by counterstaining with Alcian Blue (1% in 3% w/v acetic acid) for 30 min. Samples were imaged with a color CCD camera mounted onto an inverted microscope (Olympus, Japan, model CK-40), utilizing the Metaview software (Universal Imaging, Downingtown, PA). Sections for immunohistochemical staining were deparaffinized as above, digested with 0.5 mg/ml hyaluronidase for 30 min at 37°C, and swelled overnight in 0.5 M acetic acid at 4°C. Sections were washed with PBS, and blocked with 10% normal goat serum (NGS, in PBS) for 10 min at room temperature. After blocking, samples were incubated with monoclonal antibodies to either type II collagen (II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa, 1:1 dilution of supernatant in 10% NGS) or to type I collagen (MAB3391, Chemicon International, Temecula, CA, 25 ug/ml in 10% NGS) for 1 h at room temperature. Sections were then washed with PBS, and incubated with Alexa 488 conjugated goat anti-mouse 2° antibody (Molecular Probes, Eugene, OR) at 5μg/ml in 10% NGS for 1 h at room temperature. After extensive washing with dH_2O, samples were then treated with propidium iodide (Molecular Probes, Eugene, OR) at 10 ug/ml for 5 min to visualize cell nuclei, washed three times with dH_2O, and coverslipped with Gel/Mount (Biomedia, Foster City, CA). On each slide, one section was maintained as a nonimmune control, following the procedure described above with 10% NGS substituted for primary antibody. Sections were viewed using an inverted microscope (Olympus, Japan, model IX-70) equipped with a confocal imaging system with dual wavelength excitation at 488 and 568 nm, and multiple high NA objectives.

RESULTS

To assess the efficacy of the physiologic loading protocol (three cycles of 1 h dynamic loading at 10% deformation/1 Hz followed by 1 h of rest), the aggrecan gene expression response of chondrocyte-seeded constructs seeded at 10×10^6 cells/ml was examined after 3 days of applied loading[78] (Fig. 2(A)). It was found that dynamic loading leads to a stimulatory ~3-fold increase in aggrecan gene expression ($p = 0.0345$, Fig. 2). This response is observed before significant changes in construct material

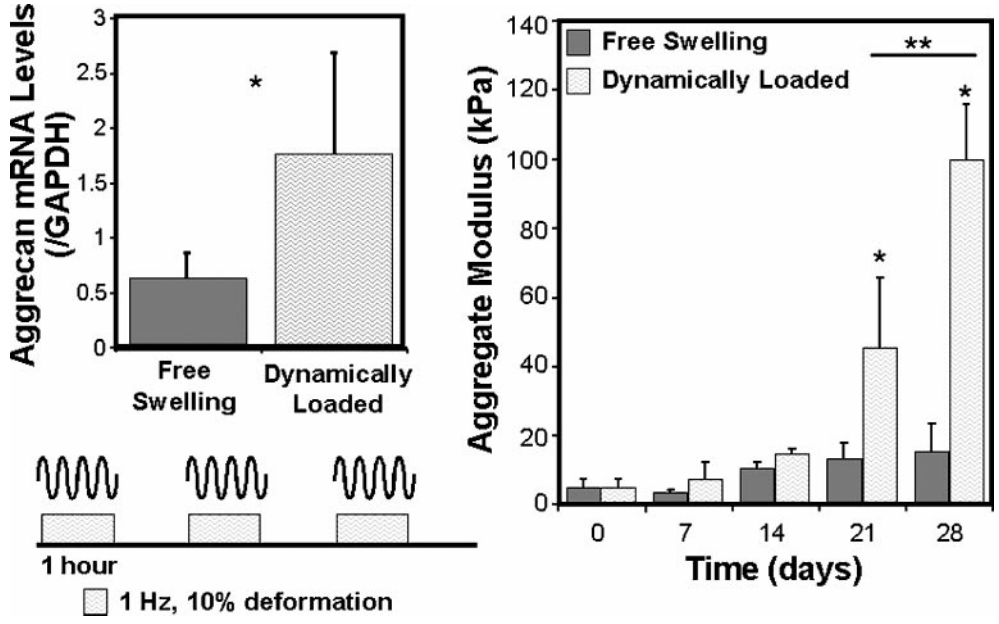

FIGURE 2. (Left) Aggrecan gene expression (normalized to expression of GAPDH) of chondrocyte-seeded (10 million cells/ml) agarose constructs grown for 3 days in free-swelling culture or with dynamic deformational loading (three consecutive cycles of 1 h loading at 1 Hz, 10% deformation, followed by 1 h free-swelling culture per day). *indicates $p < 0.0$-4, $n = 3$-5. (Right) Aggregate modulus of constructs grown as in (Left) over a period of 28 days. *indicates $p < 0.05$ vs. FS controls at each time point, **indicates $p < 0.05$ of DL construct vs. previous time point DL construct. Each data point represents the mean and standard deviation of 3–4 samples.

properties or biochemical constituents are detectable. Physiologic loading was then applied to constructs over a 28-day period[89] (Fig. 2(B)). Measurements of the confined aggregate modulus revealed that free-swelling cultures increased ~4-fold whereas dynamically loaded constructs increased ~20-fold ($H_A = 100$ kPa).

The effect of 10% deformational loading and growth factors (TGF-β1 and IGF-1) on the development of functional articular cartilage properties in agarose-seeded constructs was then investigated.[84] Although the presence of growth factor and mechanical loading each enhanced matrix synthesis and development of stiffer tissues, the combination of a growth factor with physiologic deformational loading yielded synergistic results, i.e., more than the linear summation of each stimulus individually. Specifically, deformational loading enhanced the development of the aggregate modulus on day 35 by 83%. TGF-β1 increased the modulus by 76% whereas IGF-1 increased the modulus by 44% when normalized to respective FS controls. The linear sum of the loading and TGF-β1 is 159%, but experimental results showed a 277% increase in tissue stiffness (Fig. 3(A)). Similarly, the linear sum of mechanical loading

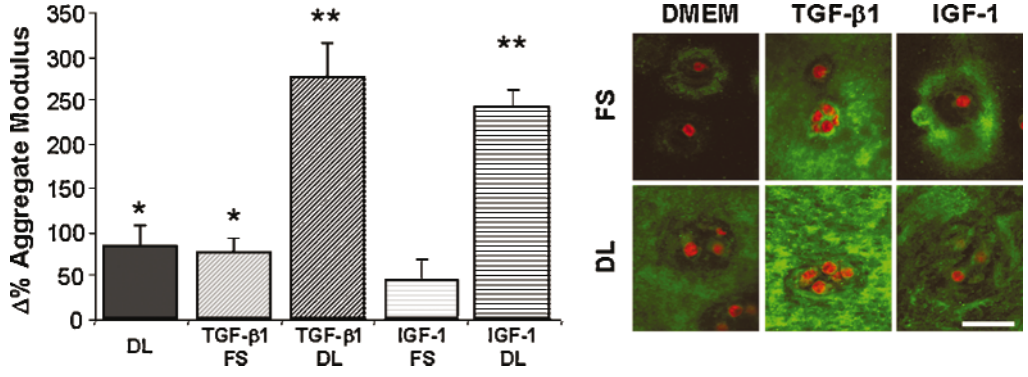

FIGURE 3. (Left) Percent increase normalized to day 35 FS in aggregate modulus (H_A), with different culture conditions on day 35. *($p < 0.02$) and ** $p < 0.0001$ indicate significant difference from "Control" constructs at same time point. Each data point represents the mean and standard deviation of 3–4 samples. (Right) Type II collagen staining of constructs after 5 weeks of culture in control media (fully supplemented DMEM with 10% FBS), in control media supplemented with 10 ng/ml TGF-β1, or in control media supplemented with 300 ng/ml IGF-1. FS: Free Swelling, DL: Dynamically Loaded; Scale bar = 20 μm.

and IGF-1 would predict an increase of 127%, whereas an enhancement of 245% was observed experimentally. Immunofluorescent staining for type II collagen further indicated that applied deformational loading increased levels of cell biosynthetic products and enhanced their distribution (Fig. 3(B)). Staining for type I collagen revealed only weak staining localized to the cell peripheries (not show).

In studies designed to explore the effects of cell-seeding density on growth, agarose constructs, seeded at 10, 20, and 60×10^6 cells/ml were grown in free-swelling culture in medium supplemented with 10% FBS (Fig. 4(A)). Parallel increases of aggregate modulus and GAG content were observed for the 10 and 20×10^6 cells/ml constructs. Constructs seeded at 60×10^6 cells/ml reached a stiffness ~2.5-fold greater than those seeded at 10×10^6 cells/ml after 35 days,[86] plateauing despite an increasing trend of GAG content over the same time. This finding was interpreted to likely reflect nutrient insufficiency, as these experiments used a cell-to-media ratio of $>3.7 \times 10^6$ cells/ml feed medium/day. In subsequent deformational loading experiments of constructs seeded at 60×10^6 cells/ml, the effect of nutrient availability on construct growth can be clearly seen by varying the amount of serum available to growing constructs, either by increasing feed medium volume and/or increasing serum concentration (Fig. 4(B)).

Highlighting the best performing group of Fig. 4, the 60×10^6 cells/ml group with 20% FBS and high medium volume group (maintained at <1.8 million cells/ml feed media/day), the construct Young's modulus was observed to increase steadily during a 56-day culture period (reaching

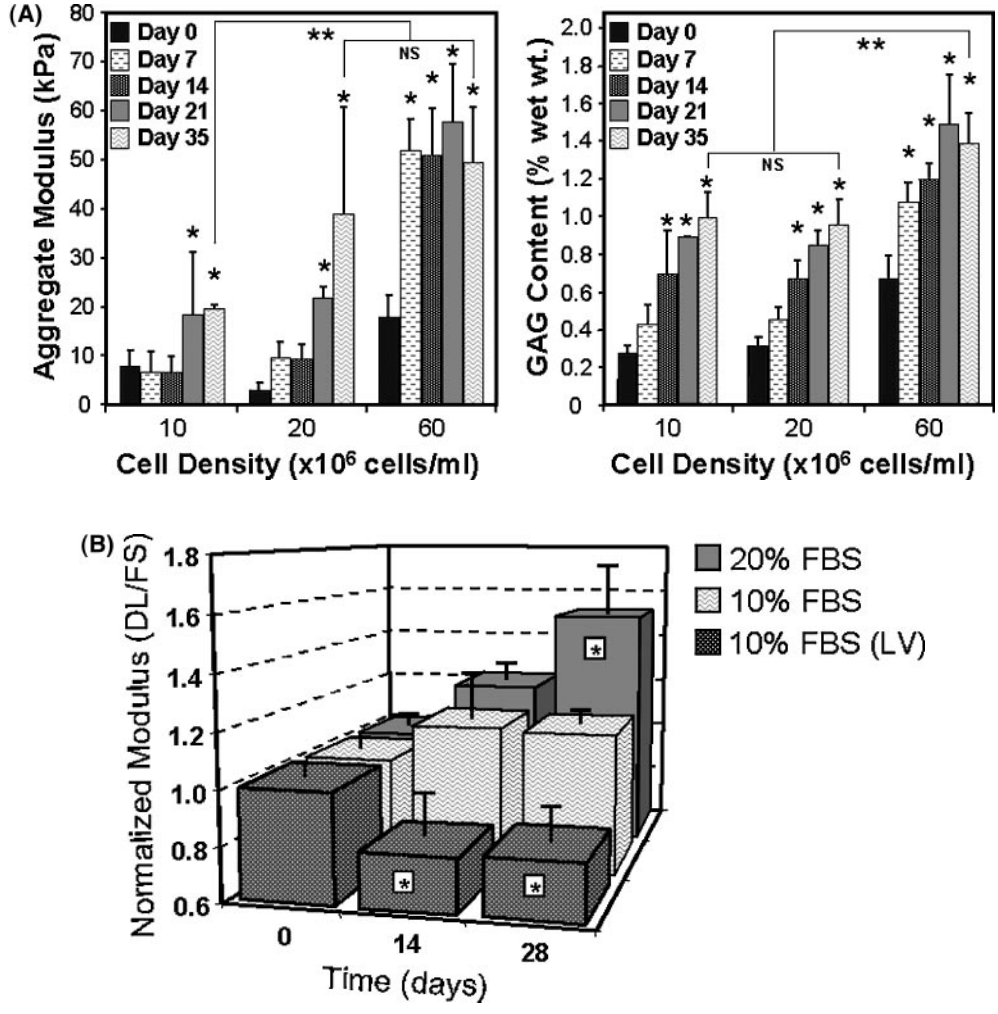

FIGURE 4. (A) Aggregate Modulus (left) and GAG content (% ww, right) over time in free-swelling culture with increasing seeding density. NS: not significant, *indicates significant difference from day 0 within group ($p < 0.05$). **indicates significant difference between groups ($p < 0.05$). Each data point represents the mean and standard deviation of 3–4 samples. (B) Young's modulus of dynamically loaded (DL) constructs ($n = 3$–4) at 60 million cells/ml normalized to free swelling (FS) controls over 1 month culture period in different media conditions. Ten percent FBS (LV): constructs cultured with >3.7 million cells/ml feed media/day in 10% FBS containing media; 10% FBS: constructs cultured with <1.8 million cells/ml feed media/day in 10% FBS containing media; 20% FBS: constructs cultured with <1.8 million cells/ml feed media/day in 20% FBS containing media. *indicates significant difference ($p < 0.05$) of dynamically loaded construct from free-swelling control within the same group at the same time point.

~2/3 of native tissue) and the dynamic modulus exhibited a frequency-dependent increase similar to the native tissue (reaching ~1/8 of the value) (Fig. 5, top). Interestingly, GAG content and Safranin-O staining as well as collagen content and Picrosirius Red/Alcian Blue staining was similar for loaded and free-swelling disks (Fig. 5, bottom).[92] Application of dynamic loading also significantly decreases swelling of the construct.

Bilayered cylindrical agarose-bone composites, with the potential to integrate with the underlying subchondral bone, were also studied. For osteochondral constructs, infusion of 60×10^6 cells/ml agarose resulted in fully penetrated agarose trabecular bone composites, with both halves of the construct remaining interconnected over the entire culture period (Figs. 6 and 7). Mechanical properties of cylindrical osteochondral constructs increased nearly 4-fold over a 28-day culture period ($p < 0.05$ versus day 0, $n = 3$–5). Construct stiffness was accompanied by steadily increasing GAG levels (~12-fold from day 0) (Fig. 6). While osteochondral constructs exhibited steady growth, the magnitude of properties achieved over a 4-week period was several-fold lower than compared to gel-alone constructs in our other studies.[92] These findings may suggest that natural bone, as prepared and cultured in this study, may have some detrimental effect on chondrocyte biosynthetic activities. In an earlier study, vital staining showed that chondrocytes remained viable in the upper gel and lower bone–gel halves of the construct and assumed a round morphology, with cells also exhibiting type II collagen deposition (data not shown[62]). In efforts to develop therapies where entire articular layers may be replaced, potentially serving as a substitute to arthroplasty, anatomically shaped patellar gel and gel–bone constructs were produced (Fig. 7).[62] Both retained their shape after 35 days in culture, and produced a GAG-rich extracellular matrix that stained with Safranin-O, with the spatial distribution of PG generally greater at the periphery.

DISCUSSION

Using an FTE approach, our goal has been to develop constructs with functional properties similar to the parent calf tissue, which has a Young's modulus (E_Y) of 277 kPa and unconfined dynamic modulus (G^*) at 1 Hz of 7 MPa.[80] To date, for high seeding density constructs (60×10^6 cells/ml), cartilage-like properties have been achieved with dynamic deformational loading alone with $E_Y = 186$ kPa and G^* that exhibits a frequency-dependent response (with $G^* = 1.64$ MPa at 1 Hz) approaching those of native calf articular cartilage, after 8 weeks in culture.[92] For these constructs, the GAG content reached 1.74% wet weight and collagen content 2.64%

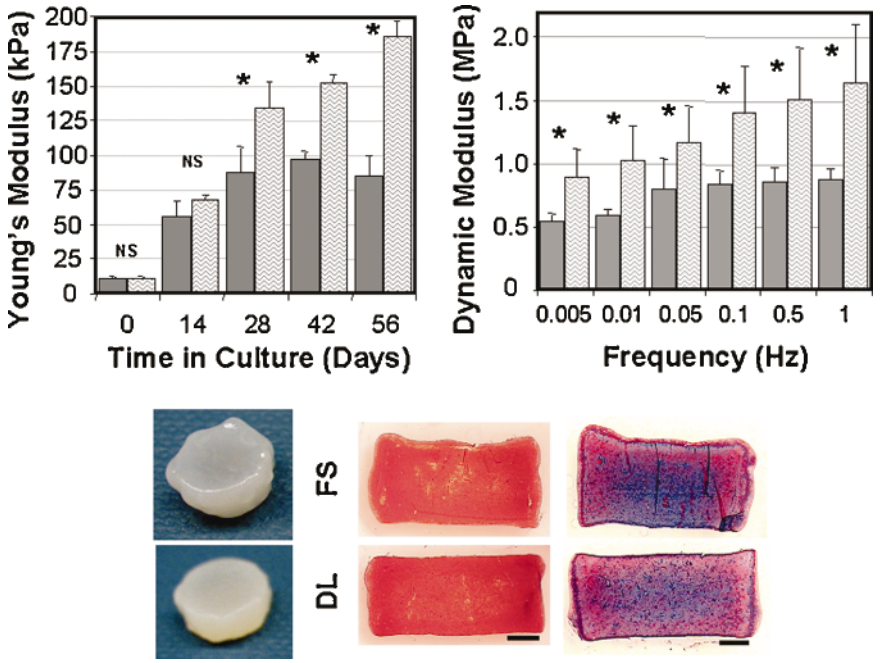

FIGURE 5. (Top) Equilibrium Young's modulus (left) with time in culture and dynamic modulus (right, day 56) measured between 0.005 and 1 Hz for constructs seeded at 60×10^6 cells/ml cultured in 20% FBS containing media cultured with (DL) or without (FS) dynamic deformational loading. Data represent the mean and standard deviation of 3–4 samples. NS: not significant ($p > 0.05$), *indicates significant difference from free-swelling control at same time point and culture condition ($p < 0.05$). (Bottom) Gross appearance of constructs on day 56 of culture (left) and staining on day 42 with Safranin-O (for PGs, middle) or Picrosirius Red/Alcian Blue (for collagen, right). Scale bar: 1 mm.

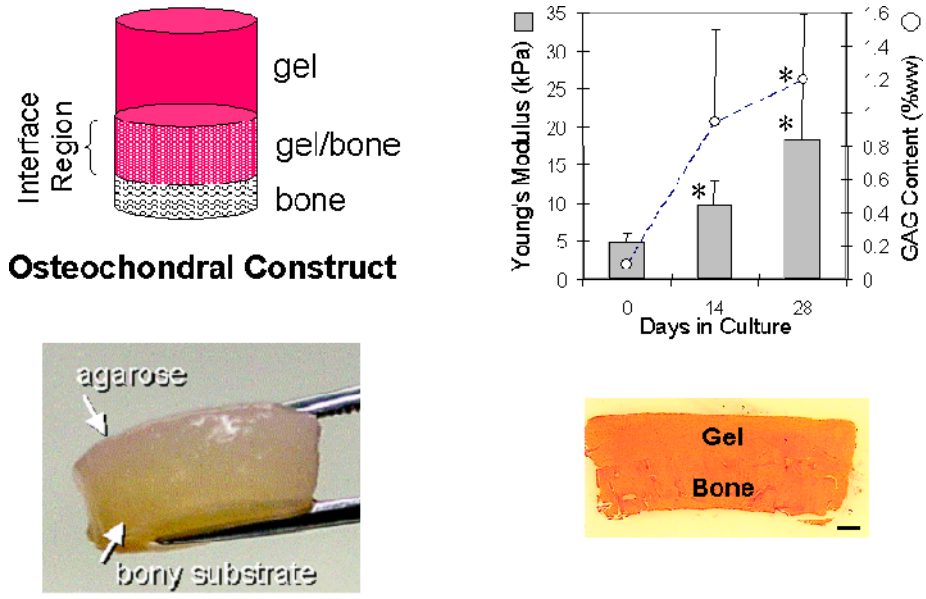

FIGURE 6. Schematic of chondrocyte-seeded osteochondral (OC) construct regions (top left). OC construct gross appearance (bottom left) and Safranin-O staining of OC construct (bottom right, scale bar = 1 mm) on day 35. Young's modulus and GAG content (% ww) of OC constructs seeded at 60 million cell/ml over 28 day culture period (top right). * indicates significant difference ($p < 0.05$) from day 0 constructs. Each data point represents the mean and standard deviation of 5 samples.

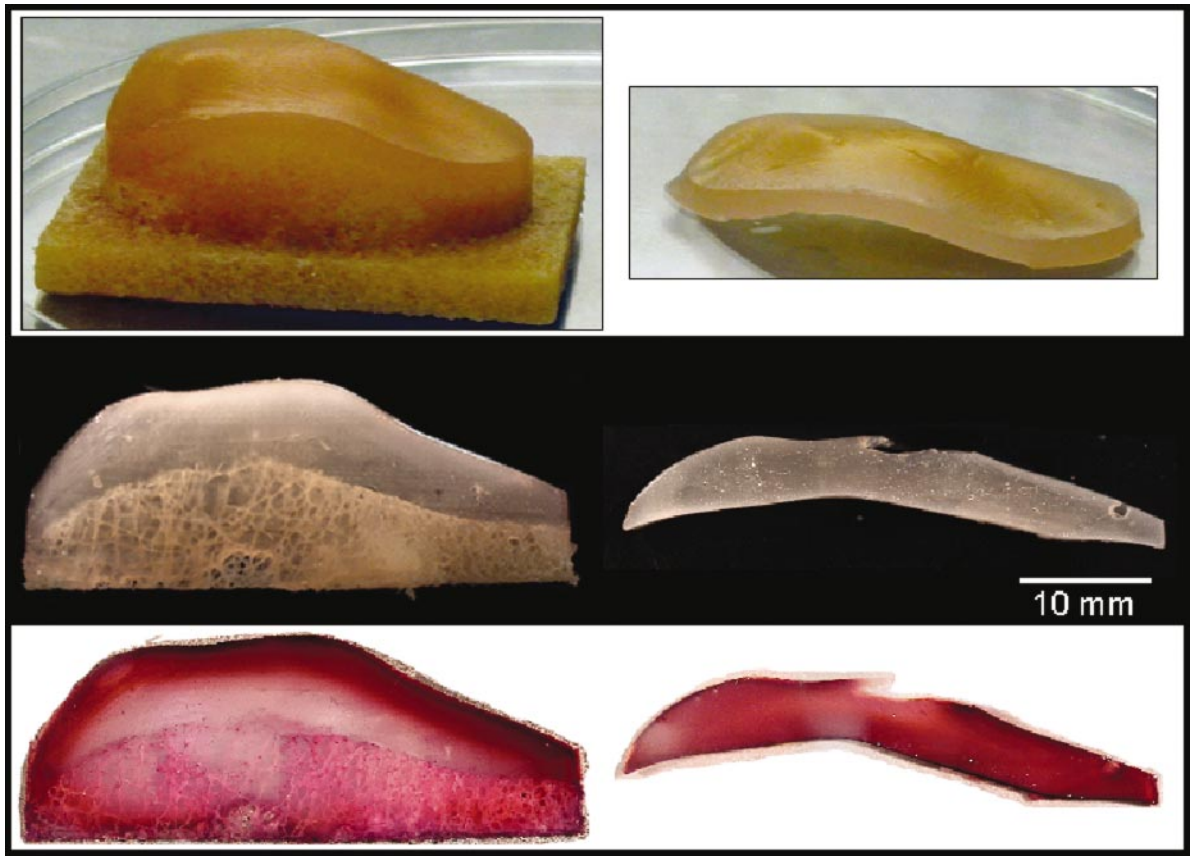

FIGURE 7. Gross appearance of osteochondral (left) and gel-alone (right) patellar constructs after 35 days of free-swelling culture (top). Cross-sectional slices (1.6 mm thick, middle) and Safranin-O stained sections (bottom). Scale bar = 10 mm.

wet weight compared to 2.4% wet weight and 21.5% wet weight for the parent tissue, respectively. Interestingly, while swelling of dynamically loaded constructs was significantly smaller than free-swelling constructs, constructs exhibited similar biochemical content independent of loading. Together with the observation of a significant synergistic effect between applied loading and growth factors (245–277% modulus increase over either stimulus alone) in a low cell seeding density study (10×10^6 cells/ml),[84,87] as well as a 200% increase in the modulus (E_Y, G^*) of dynamically loaded constructs associated with an increase of cell-seeding density (from 10 to 60 $\times 10^6$ cells/ml),[92] we anticipate that tissue constructs possessing the functional properties of native articular cartilage can be cultivated successfully in the future with an optimal combination of deformational loading, growth factors, and cell-seeding density *in vitro*.

Similar to many cartilage tissue-engineering efforts, the PG content of our agarose constructs attains levels similar to native tissue whereas collagen levels are significantly lower.[24,34,110,142] While there appears to be a correlation between bulk biochemical constituents of PG and collagen and that of bulk material properties (H_A and E_Y) for our low seeding density chondrocyte-seeded agarose constructs,[86] this relationship is lost at later times in culture.[92] Analysis of the content, size, and connectivity of specific PGs[114] and collagens was not been performed in these studies, and may also explain this finding. Another possibility is that while similar amounts of PG and collagen are present, deformational loading may increase the distribution of these molecules (as can be seen in Fig. 3 (type II collagen) and Fig. 5 (picrosirius staining)), enhancing the formation of a functional tissue-spanning network. Alternatively, other ECM molecules (e.g., COMP,[27,28,45,90] Type IX Collagen,[37,140,148,149] link protein[33,57,116]) involved with ECM cross-linking and/or structural organization may also account for differences in material properties between free-swelling and loaded constructs.[90] Recent studies demonstrated that such molecules are differentially regulated (and deposited) with deformational loading.[45,90,91]

For cartilage biology and tissue-engineering applications, chondrocytes have been seeded and cultured in a variety of scaffold materials at concentrations ranging from 1 to 200×10^6 cells/ml.[19,26,112,143] For calf tissue, the cell density ranges from 240×10^6 cells/ml at the surface to 100×10^6 cells/ml in the deep zone.[64] The effect of initial cell-seeding density on tissue development in culture appears to be variable, and is dependent on the particular 3D culture (scaffold) environment.[26,42] A higher cell-seeding density expedites the development of construct properties, in free-swelling and dynamic loading conditions. In our studies, we have observed a cell density-dependent effect of applied dynamic loading on matrix elaboration and development of material properties,[92] when sufficient nutrients were available.[86]

In the studies presented, the equilibrium Young's modulus and dynamic modulus of growing constructs were measured.[92] The dynamic modulus of cartilage in unconfined compression is possibly the most important functional property to be reproduced in engineered cartilage tissue constructs, as it determines whether the tissue can sustain its physiological loading environment.[4,106] Indeed, a typical G^* would maintain the physiological tissue strain on the order of 20%,[106] which may be essential for maintaining chondrocyte viability. However, since G^* in unconfined compression is dependent on the equilibrium moduli in tension and compression, the tissue permeability, and the intrinsic solid matrix viscoelasticity, it becomes evident that functional properties of engineered tissue constructs would have to match those of native cartilage in all of these aspects in order to reproduce the same function. To date, most biomechanical analyses of engineered cartilage have focused on the determination of the equilibrium confined compression modulus and the hydraulic permeability.[99] Clearly these are valuable measures of the functionality of the tissue-engineered constructs; however, as progress is made in matching these properties with those of native cartilage, it becomes necessary to also investigate the tensile response of the engineered tissues. This can be achieved either by performing uniaxial tensile measurements of the engineered constructs, or dynamic unconfined compression tests (as adopted here).

At early time points, agarose provides a neutral, homogeneous matrix for the study of chondrocyte mechanotransduction. With matrix elaboration over time, the cell environment becomes more complicated, with cell–matrix interactions and cell–cell interactions playing an increasing role in chondrocyte responses to physical signals. It has been demonstrated that chondrocytes seeded in agarose elaborate a local extracellular matrix within a week of culture that shields them from applied matrix deformation.[19,72] This finding stems from the stiffer properties of the local matrix compared to the agarose separating these islands of ECM.[51] Thus, while chondrocytes see cell deformation in early culture times, they may not experience significant deformation again until after an interterritorial matrix has been formed that is stiffer than the local matrix immediately adjacent to the cells, thereby permitting matrix deformations to be transmitted to the cell (as occurs in native cartilage).[100] Thus, the underlying stimulatory cues from long-term applied deformational loading of agarose constructs remain unclear. In our recent study,[81,83] we have found strong theoretical support for the finding that dynamic loading enhances the transport of large solutes, such as growth factors. Such enhanced transport, making nutrients more readily available to cells in the construct, may therefore serve as a mechanism underlying the beneficial effects of applied loading. This premise is consistent with our findings that TGF-β1 (25 kDa) and IGF-1 (7.6 kDa) act synergistically with deformational loading to increase both the construct

biochemical content and mechanical properties.[80,87,88] Additional support from the literature include reports of synergistic interaction between growth factors and deformational loading in cartilage explant studies[10,11] as well as growth factors and hydrodynamic bioreactor conditions for tissue-engineered constructs.[47]

Cylindrical bilayered constructs, resembling native tissue grafts for autologous or allograft cartilage transfer procedures or for repair of focal lesions,[55,56] were investigated under free-swelling culture conditions to assess the feasibility of using trabecular bone as an underlying substrate for chondrocyte-seeded agarose hydrogels.[89] The disparate material properties in the upper and lower regions of these constructs may present opportunities for applied deformational loading to impart construct inhomogeneity.[76] When loading gel–bone constructs, the loading condition becomes effectively a combination of unconfined and confined compression; with a top impermeable platen and a porous bottom platen (i.e., the bone) to which the gel is bonded. With dynamic compressive loading under such boundary conditions the uppermost regions (away from the bone) are free to expand radially with axial compression, while those regions close to the bone are confined from radial change (though fluid can still flow through both the radial edges and the bone/gel interface). Within the bone region, there is very little deformation (due to the much greater stiffness of bone) with some associated fluid flow. Such characteristic mechanically induced signals (and their variation with depth and axial position) may give rise to depth-dependent inhomogeneity, while at the same time increasing nutrient availability to cells within the construct.[76]

The rationale for extending the development of cylindrical osteochondral constructs to anatomically shaped constructs stems from our vision that the successful development of an anatomically shaped osteochondral construct may provide a new biological graft to address instances of traumatic injury or severe OA. In such cases, replacement of a substantial portion or entirety of the articular surface may be necessary.[63] The design of constructs having functional material properties[50] with complementary surface contour and thickness can provide tissue substitutes that recapitulate the normal contact geometry of the articulating surfaces and normal load distribution across the joint when implanted.[5,13,31,35,61] The latter may be an important contributing factor to the development of a successful tissue-engineered articular cartilage substitute, providing the proper stimulus to the chondrocytes.[44,95,100] For these larger constructs[62] (two-orders of magnitude greater in volume than the cylindrical gel constructs,[82,89]) applied deformational loading may be particularly important in overcoming nutrient diffusion limitations that lead to greater tissue development at the construct periphery.[82] The spatial uniformity of cells after seeding and their ability to maintain shape in culture are attributes of hydrogels which may further facilitate this effort.[14,26,89]

The studies described in this review have utilized cells from young tissue (with the greatest repair and growth capabilities[44,46,49]) and a biocompatible scaffold material with relatively low degradative properties.[9,115] These materials have been chosen so as to maximize the efficacy of applied loading in fostering the growth of a functional tissue *ex vivo*.[85–87,89,92] This bovine model system as well as agarose 3D culture have been widely used in the literature, and have yielded significant insights to cartilage biology and chondrocyte regulation to physical and chemical stimuli over four decades (as reviewed in Ref. 53). We anticipate that this effort will lead to functional chondrocyte-seeded agarose constructs that can be implanted in future animal studies, as well as provide an underlying framework from which an FTE approach[21] (using applied deformational loading) may be applied to general cell-scaffold systems adopted for cartilage tissue engineering.

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