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Engineering Superficial Zone Features in Tissue Engineered Cartilage

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Abstract

A major challenge in cartilage tissue engineering is the need to recreate the native tissue's anisotropic extracellular matrix structure. This anisotropy has important mechanical and biological consequences and could be crucial for integrative repair. Here we report that hydrodynamic conditions that mimic the motion-induced flow fields in between the articular surfaces in the synovial joint induce the formation of a distinct superficial layer in tissue engineered cartilage hydrogels, with enhanced production of cartilage matrix proteoglycan and type II collagen. Moreover, the flow stimulation at the surface induces the production of the surface zone protein Proteoglycan 4 (aka PRG4 or lubricin). Analysis of second harmonic generation signature of collagen in this superficial layer reveals a highly aligned fibrillar matrix that resembles the alignment pattern in native tissue's surface zone, suggesting that mimicking synovial fluid flow at the cartilage surface in hydrodynamic bioreactors could be key to creating engineered cartilage with superficial zone features.

Keywords

Tissue Engineering; Bioreactors; Cartilage; Chondrocyte; Interstitial flow; Shear Stress; Two Photon Microscopy

INTRODUCTION

Articular cartilage, the soft tissue that lines the ends of articulating bones in diarthrodial joints, has unique composition and structure features that enable it to facilitate nearly frictionless motion, while supporting loads that exceed the body weight, many times over, for millions of cycles throughout life. The remarkable resilience of articular cartilage is attributed to its biphasic composition and anisotropic extracellular matrix (ECM) structure (Mow and Wang 1999), which give rise to spatially heterogeneous *in vivo* biomechanics and differentially affect the metabolism of the chondrocytes – a paradigm known as mechanobiology (Wong and Carter 2003). When injured, articular cartilage lacks the intrinsic repair capacity of vascularized tissues such as bone, and even with the current surgical repair options, it generally heals with an inferior fibrocartilage scar tissue. This outcome feeds a cycle of injury-degeneration that leads to accelerated osteoarthritis (OA) later in life due to altered joint mechanics (Guilak et al. 2001). OA is the most prevalent

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disease in the US, equal in numbers to the next top 4 disorders combined (heart, pulmonary, mental health and diabetic conditions) (CDC 2009), which is projected to affect >67M people in the US by 2020 (Hootman and Helmick 2006). Given that there are no treatment strategies for OA that can rejuvenate the articular cartilage, regenerative medicine approaches such as tissue engineering offer important strategies to repair focal cartilage defects and preempt OA.

Cartilage tissue engineering typically involves seeding a scaffold or hydrogel with chondrocytes or stem cells and cultivating the resulting construct in bioreactors. Some bioreactors are designed to enhance interstitial nutrient transport through direct perfusion (Sittinger et al. 1994) or enhanced mixing (Vunjak-Novakovic et al. 1999). Other bioreactors are designed to mimic *in vivo* biomechanics to stimulate the constructs using compressive deformations (Buschmann et al. 1995; Mauck et al. 2000) or sliding articular motion (Wimmer et al. 2004). While these bioreactors typically yield constructs with enhanced ECM composition and improved biomechanical properties over time, they generally do not regenerate the zonal anisotropy in the collagen framework that characterizes native cartilage. Failure to restore the structural anisotropy of collagen in engineered cartilage has been cited as a contributing factor to the oft-reported failure of integrative repair (Khan et al. 2008).

Articular cartilage has been classically described as a stratified tissue with three successive “zones” based on ECM structural and compositional features (Figure 1) (Jeffery et al. 1991; Mow and Wang 1999). Of particular interest to this investigation, the densely-packed collagen fibers in the *surface zone* of cartilage are oriented parallel to the articular surface in a prestressed state (Clark 1990; Mow and Lai 1974), effectively forming a permeability barrier to regulate fluid efflux and the tissue's swelling behavior under loading, providing the tissue with the resilience to resist tensile and shear stresses during articular motion, and contributing to the production of the surface zone protein Proteoglycan 4 (PRG4), which facilitates the nearly frictionless articular motion (Klein et al. 2006). The significance of the dynamic interactions between the synovial fluid (SF) and the surface zone of cartilage during sliding articulation has long been recognized in squeeze film joint lubrication (Hou et al. 1989) and in hydrodynamic instabilities that could lead to chondrodegeneration (Mow et al. 1974). However, the metabolic effects of these interactions and their implications for cartilage tissue engineering have to date been understated.

During articular motion, the sliding of the mating cartilages and the pressure gradients they induce within the joint compartment drive SF flow over the surfaces of cartilage (McCarty et al. 2011). The solutions for the simplified problems of Poiseuille and Couette viscous flow over a biphasic cartilage layer, which represent simplified approximations of the kinematic conditions in the articulating joint, have been formulated by applying the appropriate pseudo-slip and kinematic boundary conditions at the SF-cartilage interface, and predict the induction of interstitial flow within a thin boundary region in the surface layer of cartilage (Hou et al. 1989). These interstitial flow fields likely affect collagen metabolism and alignment, as has been observed in fibroblast-seeded collagen matrices (Ng et al. 2005), and cultured chondrocytes in perfusion and parallel plate bioreactors (Gemmiti and Guldberg 2009; Pazzano et al. 2000).

We have recently experimentally quantified the interstitial flow fields in a boundary layer near the surface of engineered cartilage hydrogel under Poiseuille flow (Chen et al. 2012), which simulates SF-cartilage interactions during articulation. These flow fields are mostly unidirectional, parallel to the surface of hydrogel. We also observed that in addition to the hydrodynamic effects, this interstitial flow enhances convective mass transport in this boundary layer, and ECM production near the surface (Chen et al. 2012). Therefore, we

hypothesized that the induced interstitial flow boundary region has important mechanobiologic implications for engineering the anisotropy of the surface zone in tissue-engineered cartilage.

To test our hypothesis, we designed a modular multi-chamber bioreactor system to cultivate chondrocyte-seeded agarose (hereafter tissue engineered cartilage or TEC) hydrogels in a stirred-tank bioreactor under simulated Couette flow conditions (Fig. 2). The hydrodynamic flow conditions were generated by periodic rotation of the multi-chamber system holding the TEC hydrogels within the culture media in the stirred tank. Since it has been reported that hypoxia enhances chondrogenesis (Wang et al. 2005), the TEC hydrogels were cultured in 5% oxygen conditions either under surface flow conditions in the bioreactor or under control no flow conditions in a hypoxia chamber for 14 days. The TEC hydrogels were then evaluated using histology, immunohistochemistry (IHC), in situ hybridization (ISH), proteoglycan (PG) biochemistry, and collagen organization using 2-photon second harmonic generation (SHG).

METHODS

Bioreactor Design and Operation

A modular multi-chamber bioreactor system was custom designed to hold 4 TEC hydrogels and cultivate them in a stirred tank under simulated flow conditions (Fig 2). The multi-chamber bioreactor features 4 compartments with rectangular wells into which the TEC hydrogels are cast, and 4 channels over the TEC hydrogel wells. The assembled bioreactor compartments with the TEC hydrogels are mounted onto the agitator shaft of a Bioflo 110® stirred tank bioreactor (New Brunswick Scientific, Edison, NJ). Chondrocyte-seeded TEC hydrogels were cultivated in the stirred tank for 2 weeks in hypoxic conditions (5% O₂) under simulated flow conditions generated by motorized rotation of the modular bioreactor/agitator assembly in culture media. This results in hydrodynamic conditions in the flow channels that simulate Couette flow.

TEC Hydrogel Preparation and Cultivation

All reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Chondrocytes were aseptically isolated from the elbow joints of pigs within 24 hours after slaughter as previously described (Chen et al. 2012). Briefly, cartilage bits from the joint were minced and enzymatically digested using pronase (CalBioChem, San Diego CA) for 1.5 hours followed by type-2 collagenase (Worthington Biochemical, Lakewood NJ) for 16 hours. The digested tissue suspension was then strained through a 40µm cell strainer and the retrieved cells were suspended in complete media (DMEM/F-12, 10% FBS, 1% P/S) supplemented with 10 ng/mL rhTGF-β1 (R&D Systems, Minneapolis MN) and mixed with molten (~40 °C) type VII agarose (Sigma-Aldrich) solution in PBS to create a 40×10⁶ cells/mL in 2% agarose solution. The chondrocyte/agarose solution was cast in the rectangular wells of the 4 sliding bioreactor compartments and allowed to polymerize at room temperature for 20 minutes to form the TEC hydrogel, after which it was anchored posteriorly by a cell-free agarose layer. The modular compartments of the system were then assembled and mounted on the agitator shaft of the Bioflo 110® stirred tank. These TEC hydrogels were cultured in complete media supplemented with ascorbic acid and maintained at 37 °C, 5% O₂ and 7.4 pH. To simulate Couette flow at the surface of the TEC hydrogels, the bioreactor system mounted on the agitator shaft was rotated periodically in a half-square wave (0–25 rpm, 1 minute on followed by 1 minute off) for 40 minutes, followed by steady rotational speed of 25 rpm for the remainder of the day. Due to the non-circular geometry of the bioreactor compartments, computational fluid dynamics (CFD) simulation predicted that the start of rotation from rest effectively results in oscillatory shear stress (~2 Hz) on the

surface of the TEC hydrogel, which was dampened as the rotation reached steady state within 1 minute (data not shown). Therefore, this regimen was used to simulate periods of loading and unloading during a moderate (*e.g.* walking at 1 to 2 Hz) activity, which has been shown in the literature to provide anabolic mechanical signals to chondrocytes in tissue engineered constructs (Mauck et al. 2000). Based on the dimensions of the bioreactor, this regimen effectively induced a range of nominal shear stresses of 0–0.12 Pa at the surface of the TEC hydrogel. This rotational regimen was repeated daily for 14 days. Alternatively, control TEC hydrogels in identical bioreactor wells were placed in a hypoxia chamber (Billups-Rothensberg, Del Mar, CA) maintained at 5% O₂, 5% CO₂, and 37 °C, and cultured for 14 days without simulated flow. After the 14 day culture period, the hydrogels were retrieved and cylindrical disks were sampled and either placed in 10% Neutral Buffered Formalin (NBF) for histology, ISH, and IHC processing, or frozen for biochemical analysis.

Histology and Immunohistochemistry

Samples were fixed in 10% NBF at room temperature for 24 hours. Samples were then removed from the NBF and dehydrated under vacuum through graded alcohol steps (30, 50, 70, 80, 95 and 100% ethanol) for one hour each, followed by two fifteen minute washes in Xylenes (Sigma-Aldrich, St. Louis MO). Samples were then infiltrated with 2 changes of paraffin at 60 °C for 2 hours each under vacuum before embedding. After samples were embedded in paraffin, 5 µm sections were cut and stained with Toluidine Blue and Picrosirius Red, or processed for immunohistochemistry. Briefly, sections were quenched of endogenous peroxidases using DAKO Dual Enzyme Block (DAKO, Glostrup Denmark). Antigen retrieval was performed using 4 mg/mL pepsin in 0.01 N HCl at 37 °C for 30 minutes. Sections were then blocked for an hour with 2% solution of Normal Rabbit Serum (Vector Laboratories, Burlingame CA) followed by a 1:100 dilution of mouse anti-Collagen II (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) or a mouse monoclonal anti-Collagen I (Cat #: ab90395; Abcam, Cambridge MA), in Normal Rabbit Serum. Biotinylated horse anti-mouse secondary antibody (Vector Laboratories) was then added for 30 minutes followed by addition of Pierce High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific Inc, Rockford IL) for 20 minutes. Romulin AEC (Invitrogen) was used to detect the labeled antigens. Lubricin immunofluorescence (green) staining was performed using a goat polyclonal antibody (Cat #: sc-50078; Santa Cruz Biotechnology, Inc., Santa Cruz CA). A FITC-conjugated anti-goat secondary was added for 30 minutes and then counterstained with DAPI (blue) for cell nuclei.

In Situ Hybridization

ISH assays were generally performed as previously described (Hilton et al. 2005), using ³⁵S-labeled riboprobes. Briefly, tissue sections were de-paraffinized in xylene, rehydrated through an alcohol gradient, postfixed in 4% paraformaldehyde, and rinsed in PBS. Sections were then treated with 10 µg/ml Proteinase K, 0.2 M HCl, and 0.1 M triethanolamine with acetic anhydride for 10 min each followed by PBS rinses. Sections were dehydrated in 100% alcohol and hybridized overnight with the appropriate antisense riboprobe at 55 °C. Riboprobes were generated from cDNA clones for *Agc1* and *Col2a1* available from Open Biosystems, Inc. Unbound riboprobes were removed from the tissue sections using a series of 2X and 0.2X SSC washes and dehydrated in alcohol before exposing the slides to Kodak NTB radiographic emulsion. Finally, the slides were developed using Kodak developer and fixer, counterstained with 0.5% Toluidine Blue, and imaged in both bright-field and dark-field using an Axioskop 40 microscope (Zeiss), a SPOT RT3 digital camera, and compatible software. Composite bright-field/dark-field images were constructed using Adobe Photoshop.

Biochemical Analysis

Samples were digested using 0.5 Units/mL of papain (Sigma-Aldrich) with 2 mM L-cysteine (Sigma-Aldrich) and 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) for 16 hours. The dissolved specimens were stored frozen at -80°C until assayed using 1,9-dimethylmethylene blue (DMMB) and Quant-iT™ PicoGreen assays for sulfated-glycosaminoglycan (s-GAG) and double-stranded DNA content, respectively, as previously described (Awad et al. 2004).

Second Harmonic Generation (SHG) Two-Photon Microscopy

Five microns thick paraffin histological sections were baked, deparaffinized in xylene and rehydrated through graded alcohols. The sections were then dehydrated and coverslipped. The sections were imaged using two-photon microscopy as previously described (Brown et al. 2003) at 810 nm excitation wavelength with a 20x/0.95NA objective and images were captured using identical imaging settings (laser power, photomultiplier tube voltage, etc.).

Fiber Alignment Algorithm

Alignment of collagen fibers was determined using a custom written Matlab (The Mathworks Inc, Natick MA) program based on a previously established algorithm (Chaudhuri et al. 1987). Regions of interest in the superficial layers of native porcine cartilage and TEC hydrogels (512×512 pixel) polarized light microscopy or SHG images were cropped. The cropped image was padded with the mean grayscale value of the image. A modified Blackman-Harris window was used to soften the edges of the padded image. The 2-D Fast Fourier Transform (FFT) of the processed images was applied. A cosine sector filter was used to isolate 15° bands of the FFT. The pixel intensity of the 15° band was then summed as a representation of the collagen fiber frequency within this orientation band, and the cosine sector process was repeated up to 180° in 15° increments or bands. A rose plot histogram of collagen fiber orientation was constructed and normalized to the angle band with the maximum frequency.

RESULTS

Hydrodynamic Bioreactor Culture Induces Heterogeneous Collagen ECM

We examined the effects of the hydrodynamic bioreactor stimulation on collagen metabolism and organization. *Col2a1* mRNA expression, assessed by ISH, was spatially uniform throughout the control TEC hydrogels (Fig. 3a), whereas the expression in the bioreactor-cultivated constructs was significantly increased at the surface (Fig. 3b). Consistent with these observations, IHC staining for type II collagen demonstrated the formation of a fibrous superficial layer in the bioreactor-cultivated constructs (Fig. 3d). These distinct features were lacking in the controls, in which no evidence of a mature, fibrous ECM formation could be observed (Fig. 3c). We also examined the distribution of type I collagen in our constructs. IHC staining against type I collagen was mostly negative in the ECM, except for faint and punctate intracellular staining, regardless of whether the constructs were grown in control conditions or in the bioreactor (Fig. 3e and f).

The degree of collagen alignment was then assessed by imaging the constructs using multiphoton laser-scanning microscopy (MPLSM) and analyzing the SHG phenomenon, which is caused primarily by collagen in connective tissues and tumors (Han et al. 2008b). SHG is sensitive to the structural characteristics of the collagen fibers, including their thickness and alignment. In control constructs, SHG indicates that fibrillar collagen is primarily found in a pericellular halo in the region typically described as the pericellular matrix (PCM) around the chondrocytes (Fig. 3g). In addition to the SHG signal in the PCM, the bioreactor-cultivated TEC hydrogels also showed a unique, robust SHG signal and a

higher order organization in the dense superficial layer stimulated by flow (Fig. 2*h*) that was not observed in control constructs. The distribution of collagen fiber orientations in the superficial layer of the TEC hydrogels were determined in MATLAB using a Fast-Fourier Transform (FFT)-based algorithm, and plotted as rose plot histograms. Wherein the control TEC hydrogels showed no preferred orientation or parallelism (Fig. 3*i*), the bioreactor-cultivated TEC hydrogels showed a higher degree of parallelism with preferred orientations within 0 ± 30 degrees bands (Fig. 3*j*), with remarkable qualitative resemblance to the orientation distribution and parallelism in the superficial zone of native cartilage tissue (Fig. 3*k*).

Hydrodynamic Bioreactor Culture Induces Heterogeneous Proteoglycan Production

Since PG represents the second major constituent of the solid ECM in cartilage, we also assessed the expression of aggrecan and the content and spatial distribution of sulfated glycosaminoglycans (GAG). Quantitatively, the GAG content in the bioreactor-cultivated hydrogels was significantly increased ($p < 0.05$) compared to the control hydrogels (Fig. 4*a*). As demonstrated by ISH, aggrecan (*Agc1*) mRNA was uniformly expressed by the chondrocytes throughout the depth of the TEC hydrogels cultured under control conditions (Fig. 4*b*). In contrast, the bioreactor-cultivated TEC hydrogels showed increased expression of *Agc* in the surface layer (Fig. 4*c*). Consistent with these observations, PG accumulation in the ECM of the control TEC hydrogels was diffuse but uniformly distributed, with more intense accumulation in the PCM region, as indicated by Toluidine Blue staining (Fig. 4*d*). In addition to these features, the bioreactor-cultivated TEC hydrogels exhibited a unique structurally mature superficial layer with dense accumulation of PGs (Fig. 4*e*) that was lacking in control constructs. Whereas the cells throughout the control TEC hydrogels had rounded morphology (Fig. 4*d*) as typically observed in these hydrogels (Mauck et al. 2000), the cells in the superficial layer in the bioreactor-cultivated TEC hydrogels were more flattened and aligned (Fig. 4*e*), resembling superficial zone chondrocytes in native articular cartilage (Klein et al. 2006).

Hydrodynamic Bioreactor Culture Induces Surface Zone Protein PRG4

Since proteoglycan 4 (PRG4) is a component of synovial fluid secreted by superficial zone chondrocytes and synoviocytes, and contributes to lubricating the mating cartilage surfaces in the joint (Jones et al. 2009), we performed IHC probing for PRG4 synthesis in our TEC hydrogels. Consistent with published observations on the role of surface shear in stimulating *Prg4* gene expression (Wimmer et al. 2004), we found a significant upregulation of PRG4 in our bioreactor-cultivated TEC hydrogels (Fig. 4*g*), compared to control constructs (Fig. 4*f*).

DISCUSSION

Several bioreactor systems have been developed to optimize growth of tissue engineered cartilage subject to hydrodynamic conditions, including bioreactors utilizing continuous perfusion of the culture medium (Bujia et al. 1995; Neves et al. 2002; Raimondi et al. 2006; Sittinger et al. 1994), hydrodynamic shear and continuous mixing in spinner flasks (Bueno et al. 2009; Gooch et al. 2001; Vunjak-Novakovic et al. 1999), and concentric rotating cylinders (Saini and Wick 2003; Williams et al. 2002). In addition, dynamic compressive deformations that mimic in vivo joint loads in tissue engineering bioreactors (Buschmann et al. 1995; Mauck et al. 2000) have been shown to stimulate the biosynthetic activity of chondrocytes or stem cells and improve the biochemical composition and biomechanical properties of TEC constructs (*e.g.* agarose hydrogels) over time (Hung et al. 2004). In the latter, the observed enhancement in PG biosynthetic activity on the periphery of the constructs in unconfined compressive loading has been correlated with increased interstitial fluid flow, rather than hydrostatic pressure or compressive strains in these peripheral

regions, suggesting an important mechanotransduction role for interstitial fluid flow (Buschmann et al. 1995; Buschmann et al. 1999). Despite improvements in the growth characteristics of engineered cartilage constructs, these bioreactor systems typically have not yielded engineered tissue with stratified structural organization or mechanical properties similar to native cartilage. As such, the provision of a physiologically relevant hydrodynamic environment and the associated interstitial flow fields might be required for stimulating distinct compositional and structural features in the superficial region of engineered cartilage.

The hydrodynamic bioreactor system we used in this study was designed to simulate aspects of SF-cartilage interfacial fluid flow during *in vivo* joint articulation. Squeeze or thin-film lubrication is a primary mode of lubrication that facilitates the nearly frictionless articulation of cartilage in synovial joints. In this mode of lubrication, which involves cartilage-SF-cartilage relative motion (Hou et al. 1992), SF cannot instantaneously be squeezed out from the gap as the bearing surfaces approach each other. A pressure is therefore built up as a result of the viscous resistance generated by SF as it is being slowly squeezed from the gap. The pressure field in the fluid film can support large loads for short periods of 1 or 2 seconds before the film is depleted. With articular motion, pressure gradients force SF flow in the thin film between the articulating cartilage layers. This tribology problem of flow of SF over a biphasic cartilage layer has been described by Hou and Mow (1989) (Hou et al. 1989) and approximated to the classical problems of Poiseuille and Couette flow over a hydrated porous solid (Beavers and Joseph 1967). Inspired by this approximation, we have previously conceptualized a bioreactor chamber in which Poiseuille flow can be introduced to stimulate TEC hydrogels (Chen et al. 2012). We demonstrated through direct experimental measurements that this hydrodynamic stimulation induces interstitial fluid flow gradients within a boundary region near the TEC hydrogel surface. We also observed that PG and type II collagen synthesis is enhanced in that superficial region in association with enhanced convective mass transport (Chen et al. 2012). However, while these results were promising, the TEC hydrogels lacked many features of native articular cartilage. Thus, we undertook to evaluate the effects of hydrodynamic stimulation in the rotating bioreactor system on the evolution of surface zone features in TEC hydrogels. The bioreactor-cultivated hydrogels were subjected to simulated surface flow conditions generating nominal shear stress range of 0–0.12 Pa at the surface of the TEC hydrogel, which was selected based on previous reports (Gemmiti and Guldberg 2009) demonstrating anabolic effects in engineered cartilage under surface flow stimulation in this range of shear stress. While not directly measured in our current system, we believe that the simulated Couette flow in the rotating bioreactor induces interstitial flow and convective transport fields in the TEC hydrogels similar to those measured experimentally (Chen et al. 2012), which could explain the experimentally observed heterogeneity in composition of the ECM, qualitatively manifested by increased gene expression and ECM accumulation of PG and type II collagen, respectively, in the superficial layer. Future studies should attempt to quantify the PG and type II collagen accumulation in the superficial layer and correlate their levels with surface shear stress and convective transport changes. However, it was noted that *Col2a1* expression was suppressed in the region immediately beneath the newly formed superficial layer. The mechanism of this suppression is not clear but one explanation might be that the *de novo* accumulated ECM in the superficial layer might create a transport barrier that was not predicted in our experimental or computational analysis of the day 0 TEC hydrogels (Chen et al. 2012). Modulating the hydrodynamic conditions at the surface to account for permeability and transport barrier changes might be necessary to overcome this limitation. This warrants further examination in future studies.

Previous reports examining the growth of TEC constructs in concentric wall (Saini and Wick 2003) and wavy wall (Bueno et al. 2005) mixed bioreactors have demonstrated the

formation of a fibrous type I collagen capsule on the periphery of engineered cartilage disks. However, our control and bioreactor culture conditions resulted in constructs that were mostly devoid of type I collagen. Indeed, the gene expression and IHC staining data suggest that the fibrillar ECM in the superficial region of our bioreactor-cultivated TEC hydrogels is predominantly comprised of type II collagen, the phenotypic collagen in healthy, native articular cartilage. Furthermore, the enhanced collagen alignment of this distinct superficial layer bears qualitative resemblance to the surface zone of articular cartilage. While we cannot definitively explain the differences in these results, one possibility might be related to the high oxygen (hyperoxia or 21% O₂) concentration used in these previous studies. Since it has been reported that hypoxia enhances chondrogenesis (Markway et al. 2010; Wang et al. 2005), our TEC hydrogels were cultured in 5% O₂ conditions either under surface flow conditions in the bioreactor or under control no flow conditions in a hypoxia chamber. Indeed, previous studies demonstrate that hypoxia and hypoxia-inducible factor 1- α (HIF-1 α) not only enhance gene expression of type II collagen and aggrecan, but also downregulate the gene expression of types I and III collagen (Duval et al. 2009). Interestingly, oxygen transport through tissue-engineered cartilage in a parallel plate (Poiseuille) flow bioreactor has been modeled, and while demonstrating higher tissue oxygenation with bioreactor flow compared to controls, the model also suggested that the flow induces a time- and depth-dependent gradients in oxygen concentration in the engineered cartilage (Pierre et al. 2008). By extrapolating these model results to our system, one can assume that the surface region of our TEC hydrogels was maintained at the hypoxic 5% O₂ concentration, whereas the deeper regions might be at significantly more hypoxic conditions (<<5%), although this has yet to be mathematically or experimentally determined. The hydrodynamic conditions at the surface of the TEC hydrogels could also differentially affect the ECM at the surface of the TEC constructs. For example, it has been demonstrated through computational fluid dynamics (CFD) modeling that turbulent eddies at the surfaces of TEC disk in concentric wall bioreactors create high shear stress states that could contribute to the formation of the observed fibrous capsule (Williams et al. 2002). Future work will attempt to use CFD and multiphysics models to map the interstitial flow fields and quantify the hydrodynamic (shear stress) and transport effects in our experimental bioreactor. Furthermore, it is possible that the biomaterial used in engineered cartilage constructs could modulate the ECM produced by the cells. Indeed, while previous studies utilizing poly-lactic acid (PLA) or poly-glycolic acid (PGA) scaffolds reported the formation of a fibrous type I collagen capsule, we didn't observe this phenomenon in our agarose TEC hydrogels. It is tempting to hypothesize that the acidic environment resulting from the degradation of the PLA and PGA scaffolds might have contributed to the formation of the collagenous capsule. While it has been reported that collagen synthesis and cell viability of chondrocytes in agarose hydrogels is not pH-dependent within a pH range of 7.3 to 6.6 (Wu et al. 2007), it is still possible that lower, more acidic pH microenvironments might be detrimental to collagen synthesis or perhaps more catalytic to the activity of MMP3, a cartilage acid metalloprotease (Wilhelm et al. 1993), which could affect the collagen ECM indirectly.

Our results also suggest that hydrodynamic stimulation of the TEC hydrogels enhanced PG synthesis, and induced a gradient in the PG distribution within the newly formed ECM. Interestingly, the dense accumulation of PGs in the superficial layer of the TEC hydrogel appears to be at odds with PG distribution within native adult tissue (Fig. 1a), in which the PGs are most abundant in the middle zone and least abundant in the surface zone (Ratcliffe et al. 1984). This is likely due to the lack of compressive and shears forces in our system, since it has been suggested that regions of cartilage that experience intermittent compressive *and* shear stresses in the joint are characterized by a highly parallel collagen network and relatively low proteoglycan content as a consequence of the loading pattern (Hytinen et al. 2009). It has also been shown that the combination of sliding (shear) and compressive

motion upregulates PRG4, which is a multidomain glycoprotein (also known as lubricin) product of the *Prg4* gene (Jones et al. 2009), in chondrocyte-seeded engineered scaffolds (Wimmer et al. 2004). Consistent with published observations on the role of surface shear in stimulating *Prg4* gene expression (Wimmer et al. 2004), we found a significant increase of PRG4 in our bioreactor-cultivated TEC hydrogels (Fig. 4g), compared to control constructs (Fig. 4f). This observation has important ramifications for engineering cartilage constructs with lubricious properties (Blewis et al. 2010). Surprisingly, the hydrodynamic stimulation-induced secretion of PRG4 was not restricted to the superficial layer, but was observed deeper within the TEC hydrogels. It is possible that paracrine signals from the superficial chondrocytes subjected to hydrodynamic stimulation might become available to deeper chondrocytes along transport gradients. This possibility remains to be verified. However, it is important to note that while PRG4 in native tissue is thought to be produced by the phenotypically distinct surface chondrocytes, it has been shown that chondrocytes harvested from middle/deep regions of cartilage secrete PRG4 in monolayer or in alginate hydrogels, albeit at significantly reduced levels compared to surface zone chondrocytes (Klein et al. 2006).

One limitation of this study is that we did not evaluate the effects of the hydrodynamic culture conditions, and the resulting heterogeneity in the collagen ECM on the biomechanical properties of the TEC hydrogels. It has been shown that similar hydrodynamic stimulation regimen in parallel plate bioreactors results in significant enhancements in the tensile properties of engineered cartilage (Gemmiti and Guldberg 2006; Gemmiti and Guldberg 2009). However, it has also been long understood that collagen interacts with other ECM molecules including PG and thus plays multiple roles in determining the shear and swelling properties of articular cartilage (Mow and Wang 1999), as well as the compressive biomechanical properties. Future studies will examine the effects of bioreactor cultivation on the biomechanical properties of the TEC hydrogels.

One of the major limitations of the current work is that the bioreactor-cultivated TEC hydrogels lacked other important features of articular cartilage, including the characteristic middle and deep zone architecture. This suggests that the biomimetic surface flow in our bioreactor is not sufficient to recapitulate the stratified zonal architecture of the native tissue. Other methods of creating stratified tissue have been reported, including exploiting the differential behavior of zonal chondrocytes to create anatomically-shaped, scaffold-free constructs with stratified layers for cartilage engineering applications (Han et al. 2008a), and the use of pre-aligned, mesenchymal stem cell-seeded electrospun fibers (Wise et al. 2009). These studies suggest that predisposing cells to a preferred alignment and morphology, perhaps even by a mechanical stimulus such as interstitial fluid flow, may provide necessary queues to create differential tissue alignment and anisotropy. We believe that our approach, perhaps in combination with other loading regimens and/or prealigned scaffold fabrication techniques, could lead to transformative advances in cartilage tissue engineering and merits future studies of efficacy in cartilage repair *in vivo*.

CONCLUSIONS

In conclusion, the hydrodynamic bioreactor culture that we have described enables the formation of TEC hydrogels with surface zone features. The *in vitro* results show that hydrodynamic stimulation of the surface of the TEC constructs with fluid flow enhances the metabolism of type II collagen and proteoglycans, including aggrecan and PRG4, and induces a distinct superficial layer with enhanced collagen alignment. This study emphasizes the importance of mimicking the flow conditions at SF-cartilage interface in providing appropriate mechanical signals and nutritious gradients to induce surface zone features in

engineered cartilage hydrogels. These results represent an exciting first step towards the creation of stratified engineered cartilage constructs.

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Figure 1. Zonal Anisotropy of Articular Cartilage

(a) Safranin O/Fast Green stained articular cartilage micrograph depicting the spatial distribution of proteoglycan (red) and non-proteoglycan (green) ECM through the chondral depth. (b) Polarized light microscopy image of articular cartilage depicting the depth-dependent anisotropy in the collagen fiber alignment. Light enhanced regions denote the presence of aligned structures while dark regions denote randomly aligned fibers. The anisotropy is verified with scanning electron microscopy (SEM) imaging of regions of interest spanning the 3 classical zones of cartilage. (c) The superficial tangential zone has collagen fibers aligned parallel to the cartilage surface. (d) The middle zone has randomly aligned fibers. (e) The deep zone has vertically aligned fibers. (SEM Scale bars represent 1 μm .)

Figure 2. Bioreactor Design

A modular bioreactor was custom designed to hold 4 tissue engineered cartilage (TEC) hydrogels and cultivate them in a stirred tank under simulated flow conditions. (a) A partially exploded view of the modular bioreactor consisting of a base block (light grey), 4 sliding compartments (gold) with rectangular wells into which the TEC hydrogels are cast, and 4 lids (dark grey) that form channels over the TEC hydrogel compartments. (b) The modular compartments of the system were then assembled and mounted on the agitator shaft of the Bioflo 110® stirred tank. (c) Schematic representation of the Bioflo 110® stirred tank bioreactor (New Brunswick Scientific, Edison, NJ) consisting of a water-jacketed culture vessel with stackable control units and various probes and tubing (not shown) to regulate temperature, pH, dissolved gas(s) concentration, and the rotational speed of the agitator. (d) TEC hydrogels were cultured in complete media supplemented with ascorbic acid and maintained at 37°C, 5% O₂ and 7.4 pH for 14 days, and were subjected to simulated surface flow conditions generated by periodic rotation in the stirred tank filled with culture media in a half-square wave (0–25 rpm at 0.004 Hz) for 40 minutes, followed by steady rotational speed of 25 rpm for the remainder of the day, which resulted in a nominal shear stress of 0.12 Pa at the surface of the TEC hydrogel.

Figure 3. Effects of the bioreactor cultivation on collagen transcription, synthesis, and alignment in the chondrocyte-seeded TEC hydrogel after 14 days in culture

Spatial localization of type II collagen mRNA is visualized using *in situ* hybridization (ISH) for *Col2a1* in (a) control and (b) bioreactor-cultivated TEC hydrogels. Type II collagen accumulation and distribution in (c) control and (d) bioreactor-cultivated TEC hydrogels is visualized with immunohistochemistry (IHC). Type I collagen IHC staining in (e) control and (f) bioreactor-cultivated TEC hydrogels. Arrows in b, d, and f indicate the flow direction. Scale bars in a–f represent 250 microns. Two-photon microscopy imaging of second harmonic generation (SHG) of fibrillar collagen in the (g) control and (h) bioreactor-cultivated TEC hydrogels. Note the strong SHG signal depicting improved alignment in the hydrodynamically-stimulated superficial layer in bioreactor-cultivated TEC hydrogels, which is absent in controls. Note also the localization of the SHG signal to the pericellular matrix around the chondrocytes throughout the controls and in the deeper regions of bioreactor-cultivated TEC hydrogels. Fiber orientations in the superficial region of the hydrogels were determined in MATLAB using a Fast Fourier Transform-based algorithm, and binned in 15° intervals and plotted as normalized frequency histograms (rose plots) for (i) control and (j) bioreactor-cultivated hydrogels, as well as for (k) the superficial zone of articular cartilage. Note that the collagen fibers are predominately aligned along the direction of fluid flow in the hydrodynamically-stimulated surface regions of the TEC hydrogels (j), which bears striking resemblance to the native superficial zone alignment pattern (k).

Figure 4. Effects of the bioreactor cultivation on PGs expression, synthesis, and accumulation in the chondrocyte-seeded TEC hydrogel after 14 days in culture

(a) The proteoglycan content in control and bioreactor-cultivated TEC hydrogels were quantified biochemically using the DMMB assay. Data presented as box plots showing the mean, quartiles, and range of the PG content normalized by DNA content. Note that the origin of the vertical axis starts at 10 $\mu\text{g s-GAG}/\mu\text{g dsDNA}$. Asterisk indicates significant differences as determined by an unpaired t-test ($\alpha < 0.05$). Spatial localization of aggrecan mRNA is visualized using *in situ* hybridization (ISH) for *Agc1* in (b) control and (c) bioreactor-cultivated TEC hydrogels. Proteoglycan accumulation and distribution is visualized using Toluidine blue stained histological sections of (d) control and (e) bioreactor-cultivated TEC hydrogels. Representative immunofluorescence staining for PG4 (green fluorescence) at the surface of (f) control and (g) bioreactor-cultivated hydrogels counter stained with DAPI (blue) for cell nuclei. Scale bars in b–g represent 250 microns. Arrows in c, e, and g indicate the flow direction.