Effects of Perfusion and Dynamic Loading on Human Neocartilage Formation in Alginate Hydrogels

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Dynamic loading and perfusion culture environments alone are known to enhance cartilage extracellular matrix (ECM) production in dedifferentiated articular chondrocytes. In this study, we explored whether a combination of these factors would enhance these processes over a free-swelling (FS) condition using adult human articular chondrocytes embedded in 2% alginate. The alginate constructs were placed into a bioreactor for perfusion (P) only (100 μ L/per minute) or perfusion and dynamic compressive loading (PL) culture (20% for 1 h, at 0.5 Hz), each day. Control FS alginate gels were maintained in six-well static culture. Gene expression analysis was conducted on days 7 and 14, while cell viability, immunostaining, and mechanical property testing were performed on day 14 only. Total glycosaminoglycan (GAG) content and GAG synthesis were assessed after 14 days. *Col2a1* mRNA expression levels were significantly higher (at least threefold; $p < 0.05$) in both bioreactor conditions compared with FS by days 7 and 14. For all gene studies, no significant differences were seen between P and PL treatments. *Aggrecan* mRNA levels were not significantly altered in any condition although both GAG/DNA and ³⁵S GAG incorporation studies indicated higher GAG retention and synthesis in the FS treatment. Collagen type II protein deposition was low in all samples, link protein distribution was more diffuse in FS condition, and aggrecan deposition was located in the outer regions of the alginate constructs in both bioreactor conditions, yet more uniformly in the FS condition. Catabolic gene expression (matrix metalloproteinase 3 [MMP3] and inducible nitric oxide synthase [*iNOS*]) was higher in bioreactor conditions compared with FS, although *iNOS* expression levels decreased to approximately fourfold less than the FS condition by day 14. Our data indicate that conditions created in the bioreactor enhanced both anabolic and catabolic responses, similar to other loading studies. Perfusion was sufficient alone to promote this dual response. PL increased the deposition of aggrecan surrounding cells compared with the other conditions; however, overall low GAG retention in the bioreactor system was likely due to both perfusion and catabolic conditions created. Optimal conditions, which permit appropriate anabolic and catabolic processes for accumulation of ECM and tissue remodeling for neocartilage development, specifically for humans, are needed.

Introduction

NUMEROUS BIOREACTOR SYSTEMS have been specifically developed to create conditions for studies of chondrogenesis and neocartilage formation, or toward the production of cartilage grafts for eventual implantation and repair of articular defects. Apart from creating desirable environments (pH, pO₂, and CO₂, temperature, and nutrient supply), most bioreactor systems for engineering cartilage are constructed to include the means to biophysically stimulate the developing tissue or provide means to enhance medium exchange through perfusion (see reviews in

Refs.^{1,2}). These latter two parameters are generally thought to be significant factors for the enhancement of neocartilage formation and engineered tissue quality.

In a majority of studies, mechanical stimulation, whether applied through direct mechanical compression^{3–11} or via hydrostatic means,^{12,13} has improved extracellular matrix (ECM) deposition (principally proteoglycans) and the mechanical properties of the engineered cartilage. While dynamic compressive load may stimulate ECM production, this stimulus may also reduce accumulation or increase loss of newly secreted ECM into the medium in comparison to free-swelling (FS) or unloaded conditions.^{14–16}

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Perfusion of medium has been used to enhance cell seeding of porous scaffolds,^{17–20} and, in comparison with static cultures, perfusion can increase glycosaminoglycan (GAG) synthesis and deposition by chondrocytes and mesenchymal stem cells.^{1,17,19,21–24} Similar to compressive load, reports of reduced ECM accumulation have been observed as a consequence of perfusion, which appears to move newly synthesized proteins into the medium.^{25,26}

Few studies examined the combined effects of loading and medium perfusion. Seidel *et al.*²⁷ used a perfused chamber combined with mechanical loading, which led to increased GAG deposition. However, they did not report on the individual effects of perfusion and loading on ECM formation. Davisson *et al.*²⁸ showed that perfusion alone was sufficient to increase stiffness of engineered neocartilage (bovine chondrocytes seeded on polyglycolic acid felts) and comparable with perfused and compressed (10% offset and $\pm 5\%$ dynamic strain oscillations at 0.001 Hz). Most recently, Tran *et al.*²⁹ utilized a commercially available bioreactor system capable of perfusion and loading. In comparison to the FS condition, the biomechanical and biochemical properties of the scaffold-free engineered porcine cartilage were enhanced. No difference was found between the perfused or jointly perfused and loaded neotissues.

In this current study, we employed the same bioreactor system used by Tran *et al.*²⁹ Mature human chondrocytes synthesize matrix components at lower rates than the young animal cells used in the majority of previous studies. Further, combined perfusion and dynamic compression might have a synergistic effect on scaffold-based tissue engineering. We therefore encapsulated human articular chondrocytes in alginate hydrogels and subjected them to FS culture conditions for comparison with encapsulated cells in perfusion only (P) or perfusion and dynamic loading (PL) cultures and characterized neocartilage tissues produced after 14 days. We assessed cellular response to perfusion and loading by measuring expression of anabolic and catabolic genes, biochemical assays, radioisotope uptake, immunohistochemistry (IHC), and mechanical properties. Our hypothesis was that a combination of perfusion and dynamic loading would increase the anabolic response relative to the catabolic response resulting in a net increase in synthesis of matrix components.

Methods

Tissues and cell culture

Human articular cartilage was obtained from tissue banks (approved by Scripps institutional review board). Chondrocytes were isolated via enzymatic digestion as described previously³⁰ and cultured in monolayer for one passage. Nine donors were utilized (age range: 14–55 years, mean age: 31.2 ± 14.0 years; two women and seven men). Cartilage was graded macroscopically according to the International Cartilage Repair Society map and using a modified Collins grading system.^{31,32} Full details of age, gender, and osteoarthritic grade for each donor are presented in Table 1.

Preparation of alginate

Alginate (PRONOVA UP LVG; Novamatrix, Sandvika, Norway) was weighed and dissolved in HBSS to create a 2%

TABLE 1. DONOR AGE, GENDER, AND OSTEOARTHRITIC GRADES

Donor	Age (years)	Gender	OA grade ^a
1	14	Male	Grade 0
2	19	Female	Grade 0
3	19	Male	Grade 0
4	27	Male	Grade 0
5	30	Male	Grade 1
6	30	Male	Grade 1
7	37	Male	Grade 0
8	50	Male	Grade 1
9	55	Female	Grade 3

^aOnly normal nonfibrillated cartilage surfaces were harvested. Osteoarthritic (OA) grades are based on the modified Collins grade.^{31,32}

mix, which was continuously stirred at 4°C for 2–4 h. The dissolved alginate was filter sterilized through a 0.2- μ m filter with filling bell (Mediakap-50; Spectrum Laboratories, Inc., Rancho Dominguez, CA) connected to a Masterflex L/S head pump (Cole-Parmer, Vernon Hills, IL).

Overview of bioreactor system

A bioreactor capable of perfusion and dynamic loading of the same sample (Fig. 1) was used in these studies (Dyna-Gen® Series; Tissue Growth Technologies [TGT], Minnetonka, NM). The system permits cyclic compressive strain stimulation and simultaneous culture medium perfusion in nine different wells. The bioreactor was outfitted with a load cell (FUTEK, Irvine, CA; Fig. 1A) that is connected to a control box (TGT) and laptop computer with purpose-built software program (TGT). Perfusion is achieved using a Masterflex peristaltic pump system (Masterflex L/S; Cole Parmer; Fig. 1A).

Cell preparation and bioreactor conditions used in this present study

Human chondrocytes were suspended in the 2% alginate solution at a density of 8×10^6 cells per mL. The alginate–cell suspension was pipetted into a polysulfone casting frame, 1 $\times$ 4 wide and 2.4-mm thick, sandwiched between Whatman 3-mm filter paper held in place by stainless steel mesh and clamps and subsequently placed into a beaker containing sterile CaCl₂ (120 mM; Sigma Chemical, St. Louis, MO) for at least 45 min to polymerize the alginate.³³ Alginate discs were created using 6-mm dermal biopsy punches and rinsed several times in culture media (Dulbecco's modified Eagle's medium [DMEM] with 10% calf serum, 1% penicillin/streptomycin, and 30 μ g/mL ascorbic acid). The discs were precultured for 24–48 h in DMEM before being transferred into chondrogenic medium (Fig. 1) used in the bioreactor and for FS conditions.

A minimum of 12 alginate disks were made per experiment. Nine disks were added to the bioreactor, where three to four gels were placed into wells for perfusion only (loading platens above the appropriate wells were removed) and five to six gels were placed in the well for both perfusion and loading. The remaining three disks were maintained in 7 mL chondrogenic medium (Fig. 1) to represent the “free-swelling” control condition. The continuous perfusion flow rate in each bioreactor well was controlled at a rate of 0.1 mL

FIG. 1. DynaGen® bioreactor system. **(A)** Whole system setup with loading cell and chamber, peristaltic pump, and medium reservoir. **(B)** Open culture chamber with nine wells and removable loading platens. **(C)** Captured image of software to track and control loading parameters. Color images available online at www.liebertpub.com/tea

per minute and was applied during the whole duration of the experiment of 7 or 14 days, respectively. This rate was chosen based on cell viability assessments where higher rates of perfusion lead to decreased cell viability (data not shown). The total media volume used for the bioreactor was 70 mL and was changed once a week. The FS disc medium was changed every 3–4 days.

Measuring gel heights for calculation of 20% loading was performed using a set of digital calipers. Gels for loading were chosen to be within 0.05 mm of each other to ensure a relatively uniform loading among all gels within the bioreactor chambers. The loading regime consisted of 20% dynamic compressive loading (of the gel height) using a sine wave at 0.5 Hz for 1 h per day. Motor movement, platen displacement, as well as the applied force were monitored (Fig. 1C).

The chondrogenic medium used consisted of DMEM (Cellgro, Manassas, VA), ITS⁺ (Sigma; i.e., 10 mg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 0.5 mg/mL bovine serum albumin, and 4.7 mg/mL linoleic acid), 1.25 mg/mL human serum albumin (Bayer, Leverkusen, Germany), 10^{-7} M dexamethasone (Sigma), 0.1 mM ascorbic acid 2-phosphate (Sigma), and penicillin/streptomycin/gentamycin (Gibco, Carlsbad, CA).³⁴ This mix was filter sterilized through a 0.22- μ m filter. An end concentration of 10 ng/mL of TGF β 1 (PeproTech, Rocky Hill, NJ) was added to the medium immediately prior to use for all conditions.

Viability assessment

At least two half gels were selected from each condition for viability assessment. The sections were transferred into phosphate-buffered saline (PBS) containing calcein AM and ethidium homodimer-1 (Invitrogen, Carlsbad, CA), incubated at 37°C for 30 min as previously described for cartilage sections.³⁵ The fluorescence generated from each reagent was assessed on a confocal microscope (LSM-510; Zeiss, Jena, Germany). Viability was assessed using a script written in MATLAB software (MathWorks, Natick, MA), configured to enumerate green (live) and red (dead) cell signals. Viability is reported as a percentage of live cells.

Quantitative real-time polymerase chain reaction

Following culture, alginate-embedded chondrocytes were washed once in PBS and dissolved with 500 μ L of 50 mM

ethylenediaminetetraacetic acid (EDTA) in 2.5% trypsin-EDTA solution at room temperature for 10 min. The released cells were collected by centrifugation at 1500 rpm for 3 min. The pelleted cells were washed in PBS before lysis in RLT buffer used in the RNeasy® mini kit (Qiagen, Hilden, Germany) for total RNA extraction.

First-strand cDNA synthesis was performed using total RNA as a template according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using prevalidated TaqMan® gene expression reagents (Applied Biosystems) for the detection of *Col1a1*, *Col2a1*, *Col6a1*, *Col10a1*, *aggrecan*, proteoglycan (PRG4), chemokine (C-C motif) ligand 20 (*CCL20*), matrix metalloproteinase 3 (*MMP3*), inducible nitric oxide synthase (*iNOS*), interleukin-1 β (*IL-1 β*), and tumor necrosis factor α (*TNF α*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Expression levels were normalized to *GAPDH* using the Δ Ct method as previously reported.³⁶

Biochemical analyses

Total GAG was assessed according to a previously reported protocol adapted for assessing alginate samples with the 1,9-dimethylmethylene blue (DMMB) method.³⁷ Briefly, the dissolved alginate-cell suspension was incubated in digestion buffer (55 mM sodium citrate, 150 mM sodium chloride, 5 mM cysteine hydrochloride, 5 mM EDTA, and 0.56 units/mL papain; Sigma) at 60°C overnight. DMMB was used at pH 1.5 to avoid unspecific interaction between the dye and alginate (seen at higher pH levels³⁷), and the samples were measured at a wavelength of 595 nm. The standard curve was prepared with shark chondroitin 6-sulfate (Sigma). Total GAG was calculated relative to DNA content from the same lysate using the CyQUANT® assay (Invitrogen).

Histology

For assessment of GAG produced, paraffin-embedded sections were prepared for acriflavine staining.³⁸ IHC analyses were conducted to examine protein deposition of collagen type II (II-II6B3), link protein (9/30/8-A-4), and aggrecan (12/21/1-C-6). All three antibodies were obtained from the Hybridoma Bank, University of Iowa. Species-matching isotype controls were also used in parallel.

Mechanical testing

Testing of mechanical properties of the gels was conducted by using a custom-built device consisting of two miniature brushless servo actuators (SMAC, Carlsbad, CA), one 50 g load cell (FUTEK) with steel plunger having a flat surface for compression, and LabVIEW (National Instruments, Austin, TX) software for movement control and data acquisition on a laptop. The gels were placed between two 100- μ m-thick cover slips and loaded into the test chamber. The gel height was measured using the internal linear encoder of the SMAC (1 μ m accuracy). A 5% of original height step compression was applied to the gel subsequently and the force was monitored and recorded. The gel was allowed to equilibrate for 2 min and then another 5% step compression was applied. The step compression was applied a total of four times, resulting in a net compression of 20%. Young's modulus was calculated from a linear fit to the equilibrium force at each 5% compression level.³⁹

Statistical analysis

Cycle numbers for the expression of each gene (in triplicate) obtained from RT-PCR were corrected to *GAPDH* levels

and used in a repeated measures analysis using R (R Development Core Team, www.r-project.org) to assess statistical significance between the gene expression levels for each treatment group. *p*-Values <0.05 were considered significant. We anticipated an experimental threshold of measuring a 1.5- to 2-fold difference in normalized gene expression between groups. Given the standard deviations in gene expression (of *Col2a1*, *Aggrecan*, *Col6a1*, *PRG4*, and *CCL20*) at 14 days, a sample size of nine would be therefore sufficient to detect a twofold or greater difference in gene expression with a *p*-value of 0.05 and a power of >80%.

Results

No significant differences in gel stiffness were detected between any of the treatment groups after 14 days of culture. *Col2a1* mRNA expression levels were at least threefold higher (*p*<0.05) in both bioreactor conditions compared with FS by days 7 and 14 (Fig. 2A). For all genes studied, no significant differences were seen between P only and PL samples. *Aggrecan* mRNA levels were not significantly altered in any condition (Fig. 2B), although both GAG/DNA and ³⁵S GAG incorporation studies indicated higher GAG

FIG. 2. Anabolic gene expression profiles of human articular chondrocytes embedded in alginate for 7 and 14 days in perfusion (P) and perfusion/loaded (PL) conditions relative to free swelling controls (FS; dotted line). Total mRNA expression levels for (A) *Col2a1*, (B) *Aggrecan*, (C) *Col6a1*, (D) *PRG4*, (E) *Col10a1* (note: for most samples, *Col10a1* was not detectable or only after >31 cycles), and (F) *Col1a1*.

retention and synthesis in the FS treatment (Fig. 3A and B). A trend of higher *Col6a1* expression in the P group compared with PL was observed (Fig. 2C). *PRG4* RNA levels were higher in the bioreactor conditions compared with FS by day 7, but no significant difference was noted by day 14 (Fig. 2D). On day 7, PL treatment led to higher *PRG4* expression levels (~12-fold) compared with approximately fourfold in P. For most samples, *Col10a1* was not detectable, or only detectable after >31 cycles; however, *Col10a1* gene expression levels in bioreactor samples were between 5- and 12-fold lower compared with FS (Fig. 2E). *Col1a1* expression levels were reduced twofold in the PL condition at day 7, but no significant difference was found after 14 days (Fig. 2F).

The response by the chondrocytes to the conditions studied here after 14 days of culture appears not to be age but rather donor dependent. The expression levels of *Col2a1*, *Aggrecan*, and *Col6a1* after 14 days in P or PL condition (relative to FS) in five donors spanning 27–55 years of age are presented in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/tea).

Catabolic gene expression levels show that *MMP3* expression was fourfold higher in both P and PL compared with FS by day 7, which increased ten-fold by day 14 (Fig. 4A). Compared with FS, *iNOS* expression levels were four- to sevenfold higher in P and PL by day 7, respectively. By day 14, *iNOS* levels had decreased to approximately twofold less than that of the FS condition (Fig. 4B). On day 7, *CCL20* mRNA levels were higher in the bioreactor conditions (Fig. 4C). Also, a greater than fourfold increase in *CCL20* expression was seen in P compared with twofold in PL (Fig. 4C). By day 14, *CCL20* mRNA levels were similar in all conditions. Both *IL-1 β* and *TNF α* were not detected in any samples in all conditions tested in this study.

Cell viability >80% was maintained in all conditions (Fig. 5A–C). IHC indicated an overall low deposition of collagen type II in all conditions (Fig. 5D–F), although some groups of cells in the bioreactor condition were clearly positive, supporting gene expression observations (Fig. 2). Link protein IHC showed a more intense stain surrounding the cells in all conditions (Fig. 5G–I), although a greater distribution in the intercellular region was seen in the FS gels (Fig. 5G). Aggrecan staining appeared most intense surrounding most cells in the PL treatment (Fig. 5I) and a slightly lower frequency of cells in the P condition (Fig. 5K). However, for many samples, aggrecan staining was mainly detected in cells on the peripheral regions of gels in both P and PL

FIG. 3. Biochemical assessments for each culture condition. (A) Glycosaminoglycan (GAG)/DNA ($\mu\text{g}/\mu\text{g}$) ratios; (B) total ^{35}S Sulfate GAG incorporation.

FIG. 4. Catabolic gene expression levels of (A) matrix metalloproteinase 3 (*MMP3*), (B) inducible nitric oxide synthase (*iNOS*), and (C) chemokine (C-C motif) ligand 20 (*CCL20*) by chondrocytes embedded in alginate for 7 and 14 days in P and PL conditions relative to FS controls (FS).

conditions (Supplementary Fig. S2). Chondrocytes in the FS condition showed positive aggrecan staining throughout the gel, although less intense compared with positive cells in the P and PL conditions (Fig. 5J). The more uniform deposition of aggrecan observed throughout the gel, together with the link protein staining patterns, supports the higher GAG levels detected in the biochemical and ^{35}S assessments (Fig. 3). In our hands, alcian blue, safranin O/fast green, and toluidine blue all stained alginate in a nonspecific or false positive manner (tested also on alginate gels without cells). Acridine staining (Fig. 5M–O) appeared similar to both aggrecan and link protein immunostaining patterns.

Discussion

Balancing anabolic and catabolic processes is crucial in maintaining cartilage homeostasis⁴⁰ and it is beginning to be recognized that these processes are important in

FIG. 5. Viability assessment, immunohistochemistry (IHC), and acriflavine staining of 14-day-old alginate gels containing human articular chondrocytes in FS, bioreactor P, and PL conditions. Live-dead cell viability images (10 $\times$) using confocal microscopy (a–c). IHC staining for collagen type II (d–f), link protein (g–i), and aggrecan (j–l). Acriflavine staining (m–o). (Magnification of IHC and acriflavine images: 40 $\times$). Color images available online at www.liebertpub.com/tea

regenerating functional tissue. An appropriate application of dynamic mechanical stimulation is one way to initiate both processes.⁴¹ Co-induction of anabolic and catabolic responses in our study is consistent with other studies examining dynamic loading of cartilage explants⁴² and engineered tissues.^{41,43,44} Interestingly, our data indicate that perfusion alone can induce both anabolic and catabolic responses in human chondrocytes, similar to observations by Tran *et al.*²⁹ employing porcine chondrocytes. This effect forces us to re-examine the requirement of dynamic stimulation. However, from the IHC data in the present study, greater aggrecan protein deposition was seen in the PL treatments.

This current study highlights the complexity of identifying optimal conditions that enhance neocartilage production in human cells. It is clear from our study that gene expression profiles and the final distribution of protein deposition do not correlate in a predictable manner. Aggrecan mRNA levels were not significantly altered in any condition, yet higher GAG and ³⁵S incorporation were noted in the FS condition. Conversely, IHC staining shows significant deposition of aggrecan immediately surrounding the cells in the PL treatments, although more restricted to the peripheral regions in many samples. Increased MMP3 gene expression levels in the bioreactor treatments (P and PL) suggest increased MMP3 enzymatic activity. Since aggrecan and collagen types II, IX, X, and XI are substrates for MMP3,^{45,46} it is possible that the GAGs produced in the bioreactor conditions were both degraded and then removed under perfusion conditions.^{25,47} De Croos *et al.*⁴ reported increased MMP3 and MMP13 following loading. Aggrecan staining in FS indicates that most chondrocytes throughout the gel deposit aggrecan compared with chondrocytes in the P and PL conditions where many cells on the outer regions of the gel produced high-interterritorial levels of aggrecan. These differences in aggrecan distribution may be related to discrepancies seen between the gene expression and biochemical analyses.

Col2a1 mRNA expression levels were higher in both bioreactor samples, yet the accumulation of collagen type II protein was conspicuously low, except for a few clusters of cells. As noted previously, suspected increased MMP3 activity may be partly responsible for collagen degradation, although other MMPs not examined in this study may have also been enhanced and released (e.g., MMP13).

The decrease in *iNOS* mRNA levels in the bioreactor (compared with FS) indicates that perfusion may aid in decreasing an inflammatory response, perhaps by removing normally accumulated factors that stimulate its expression like IL-1 β and TNF α ,⁴⁸ yet these cytokines were not detected at the mRNA level in this current study.

Both *iNOS* and *CCL20* have been previously identified as early phase mechanoresponsive genes.⁴⁹ We also see an increased expression by day 7, which indicates that the conditions created in the bioreactor were sufficient to generate a mechanoresponsive change. By 14 days, *iNOS* and *CCL20* expression levels were similar to FS gels. The reduction of *iNOS* is postulated earlier; however, the reduced *CCL20* may be related to its role in actin cytoskeletal rearrangements³³ and linked to changes in cell phenotype during culture (i.e., re-differentiation reflected by the increased *Col2a1* and decreased *Col1a1* expression). The mixed results here indicate a need to optimize timing of perfusion, loading, and FS con-

ditions. Intermittent loading regimes have significant effect on ECM production.^{41,50} In particular, Nicodemus and Bryant⁴¹ showed that loading and the timing of when loading is applied can dramatically influence the anabolic and catabolic activities of chondrocytes in polyethylene glycol gels. A delayed loading regime has less effect on stimulating anabolic and catabolic gene expression in comparison to using an immediate application of an intermittent loading regime. However, Khan *et al.*²¹ showed that following 2 weeks of continuous perfusion of bovine articular chondrocytes increased collagen and proteoglycan syntheses. Our FS condition showed a more uniform aggrecan and link-protein deposition; thus, a preculture approach should be tested with the system used in this current study as a possible means to improve ECM accumulation.

One feature of this current study, in relation to many previous studies, is the aspect of species and age. The literature is dominated by very promising results in young animal cells (bovine and porcine) that respond dramatically to growth factors, mechanical stimuli compression, and perfusion. In humans, however, the generally poor response in mature adult cells and the high variability make it difficult to translate animal results to patients. In the present study, we did not see a large variation in response to the conditions used based on age (see Supplementary Fig. S1), but rather a donor-specific response. This indicates that further modification of the system to aid in retaining ECM proteins is needed and may then delineate an age-related response, with respect to the passage used in the current study.

In the original seminal publication by Brittberg *et al.*⁵¹ and in a more recent study by Chubinskaya *et al.*,⁵² it is stated that cells being prepared for autologous chondrocyte transplantation or autologous chondrocyte implantation are cultured in monolayer for 7–21 days. The chondrocytes used in our current study are cultured for 1–2 weeks in P0 and then another 1–2 weeks at P1. Therefore, our cells are actually in culture for a comparable amount of time or even a little longer compared with the standard practice. This may reflect, in part, the more negative results observed. Thus, culturing the cells for comparably shorter times may be needed.

This study represents a first attempt to replicate positive responses to mechanical compression and perfusion that have been reported in animal cells. The negative results and high variability are in fact the challenges facing cartilage tissue engineering for clinical applications. Our observations with human cells are a reflection of the challenges inherent in cartilage tissue. Given the number of positive reports supporting mechanical compression and perfusion it is surprising that none of these have been clinically relevant. To our knowledge, no study has reported the effect of combined perfusion and dynamic compression in one system using human cells. Several studies have shown promising effects of mechanical compression and perfusion on young animal cells. However, the inability to extrapolate these results to human cells is a significant barrier for clinical translation.

In conclusion, it is clear that perfusion and perfusion with loading create conditions somewhat favorable to neocartilage formation, although the application of perfusion and dynamic stimulation requires further optimization for the accumulation of ECM proteins using human chondrocytes. The production of catabolic mediators, in particular MMPs, appears to be a normal and even required part of

neotissue formation, since during this dynamic process there will be remodeling before a final tissue equilibrium is reached. Of course an excess of enzymes may be detrimental. What really matters is the end product. In that regard, it is important to continue longer-term cultures and assess the effect of various loading schemes on functional tissue development—until a steady-state is reached.

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Disclosure Statement

No competing financial interests exist.

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