ORIGINAL ARTICLE

Nutrient Channels Aid the Growth of Articular Surface-Sized Engineered Cartilage Constructs

Alexander D. Cigan, PhD,¹ Krista M. Durney, MS,¹ Robert J. Nims, MS,¹
Gordana Vunjak-Novakovic, PhD,^{1,2} Clark T. Hung, PhD,¹ and Gerard A. Ateshian, PhD^{1,3}

Symptomatic osteoarthritic lesions span large regions of joint surfaces and the ability to engineer cartilage constructs at clinically relevant sizes would be highly desirable. We previously demonstrated that nutrient transport limitations can be mitigated by the introduction of channels in 10 mm diameter cartilage constructs. In this study, we scaled up our previous system to cast and cultivate 40 mm diameter constructs (2.3 mm overall thickness); 4 mm diameter and channeled 10 mm diameter constructs were studied for comparison. Furthermore, to assess whether prior results using primary bovine cells are applicable for passaged cells—a more clinically realistic scenario—we cast constructs of each size with primary or twice-passaged cells. Constructs were assessed mechanically for equilibrium compressive Young's modulus (E_Y), dynamic modulus at 0.01 Hz (G^*), and friction coefficient (μ); they were also assessed biochemically, histologically, and immunohistochemically for glycosaminoglycan (GAG) and collagen contents. By maintaining open channels, we successfully cultured robust constructs the size of entire human articular cartilage layers (growing to ~52 mm in diameter, 4 mm thick, mass of 8 g by day 56), representing a 100-fold increase in scale over our 4 mm diameter constructs, without compromising their functional properties. Large constructs reached E_Y of up to 623 kPa and GAG contents up to 8.9%/ww (% of wet weight), both within native cartilage ranges, had $G^* > 2$ MPa, and up to 3.5%/ww collagen. Constructs also exhibited some of the lowest μ reported for engineered cartilage (0.06–0.11). Passaged cells produced tissue of lower quality, but still exhibited native E_Y and GAG content, similar to their smaller controls. The constructs produced in this study are, to our knowledge, the largest engineered cartilage constructs to date which possess native E_Y and GAG, and are a testament to the effectiveness of nutrient channels in overcoming transport limitations in cartilage tissue engineering.

Introduction

CARTILAGE TISSUE ENGINEERING (CTE) is a promising approach to cultivate cartilage constructs *in vitro* for replacing tissue lost from osteoarthritis (OA), involving the seeding of cells within porous scaffolds with the expectation that they will reproduce critical functional properties of native cartilage tissue. Cartilage was identified as an early target for tissue engineering due to its thinness, avascularity, and population by a single cell type.^{1,2} To date, however, obstacles remain which prevent the *in vitro* cultivation of replacement human cartilage, including the inability to produce functional cartilage at large enough scales for clinical relevance. The primary objective of this study was to address this challenge using channels to enhance nutrient supply in

articular surface-sized tissue constructs, mimicking the network of cartilage canals that provide this role during early cartilage development.^{3,4}

The field of CTE has seen numerous advancements and produced a broad array of *in vitro* techniques for the cultivation of neocartilage, yet the vast majority of studies focus on small (3–6 mm diameter) tissue constructs.^{5–12} Chondrocyte-agarose constructs have proven to be an efficient model system, particularly when cultured in ITS media with transforming growth factor (TGF)- β supplementation.^{6,13–18} Using this system, our group consistently reproduces native cartilage equilibrium compressive Young's modulus (E_Y) and glycosaminoglycan (GAG) content in Ø4 mm constructs using either primary or passaged bovine chondrocytes.^{19–24} However, by the time OA manifests itself symptomatically,

Departments of ¹Biomedical Engineering, ²Medicine and ³Mechanical Engineering, Columbia University, New York, New York.

A portion of these data were presented as: Cigan, A.D., Nims, R.J., Vunjak-Novakovic, G., Hung, C.T., and Ateshian, G.A. Human-scale engineered cartilage with nutrient channels reaches native mechanical and biochemical properties. Presented at the Orthopaedic Research Society 2016 Annual Meeting, Orlando, FL, 2016.

articular cartilage lesions typically reach ~25 mm in diameter.^{25,26} While studies with small constructs are instrumental to the advancement of the field, they ultimately must be scaled up to successfully treat OA.

As scaffold dimensions are increased, constructs experience transport limitations as nutrients necessary for cell survival and/or matrix synthesis are consumed by cells at the construct periphery before they reach the construct center by diffusion, producing local deficiencies within the construct interior.^{9,19,24,27–31} Even using bovine calf chondrocytes, which readily synthesize matrix and represent a “best-case-scenario,” previous attempts to engineer constructs at clinically relevant scales have experienced severe tissue heterogeneity and poor mechanical integrity.³² Using small constructs to repair large defects would require a mosaicplasty-like procedure, which may suffer from poor integration.^{32–34}

The addition of channels reduces transport distances, mitigating diffusional limitations and improving mechanical properties of the constructs.^{10,35} In our recent studies, the combination of suspension culture and sufficient glucose supply permitted Ø10 mm constructs with 3 or 12 evenly-spaced Ø1 mm channels (CH3 or CH12, respectively) to attain functional properties on par with those of Ø4 mm constructs, including native EY and GAG content.^{36,37}

Many tissue engineering studies using bovine chondrocytes opt for the use of primary cells.^{19,38–40} In this approach, numerous calf joints may be harvested and pooled together to obtain enough chondrocytes for large-scale studies. While this holds benefit for CTE feasibility studies, it does not represent a realistic clinical scenario since the isolation of sufficient primary chondrocytes to resurface entire human joints poses a significant logistical hurdle. Thus, other studies have used passaged bovine, canine, or human chondrocytes.^{34,41–49} A direct comparison of passaged versus primary bovine chondrocytes in agarose could elucidate whether results derived with primary bovine cells are applicable for passaged cells.

The objectives of this study were twofold: to overcome transport limitations by incorporating channels in articular surface-sized constructs and to assess whether results from large constructs seeded with primary chondrocytes can forecast successes with passaged cells. By scaling up our culture system for Ø10 mm constructs, we developed techniques for casting and cultivating Ø40 × 2.3 mm (overall diameter × thickness) chondrocyte-agarose constructs, large enough to resurface human joints (e.g., the knee).⁵⁰ The specific hypothesis of this study is that these constructs can

develop functional properties on par with those of channeled Ø10 mm and channel-free Ø4 mm constructs of similar thickness. In addition, we compared primary bovine versus passaged bovine chondrocytes in constructs of these sizes.

Materials and Methods

Overview

Two studies were performed on eight casts of constructs (Table 1), using chondrocytes harvested and pooled from multiple bovine wrists. Study 1 served to preliminarily identify culture conditions for Ø40 mm constructs which enhance homogeneous growth. By staggering these cultures over several weeks and examining growth, protocols for manufacturing and culturing large constructs were refined. The experimental design of Study 2 was established to address our specific aims and hypotheses, using conditions optimized in Study 1. The outcomes of Study 1 were valuable, warranting their dissemination along with those of Study 2. The description of methods for mechanical, biochemical, histological, and immunohistochemical evaluation is summarized in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea).

3D culture

To enhance nutrient flow through channels, we modified a previous system optimized for culturing Ø10 mm channeled constructs suspended on racks with orbital shaking.^{36,37} The system was scaled up such that each Ø40 mm construct was cast in a three-dimensional (3D) printed mold and held by a culture rack within a 473 mL polypropylene container with a polyethylene lid (Fig. 1). Before culture, the containers were autoclaved and the lids sterilized in 70% ethanol.

Constructs were cultured 56 days in ITS media with 0.8 Hz orbital shaking.¹⁹ Constructs were supplemented with 1 or 10 ng/mL TGF-β for the first 2 weeks of culture, after preliminary experiments demonstrated similar or better properties of Ø10 mm constructs with reduced TGF-β dosage.¹⁸ Initially each Ø40 mm construct was provided 200 mL media; as cultures progressed, constructs swelled substantially, requiring more media to maintain immersion; after day 28, the volume was increased by 25 mL/week, reaching 300 mL in the final week of culture. Media was changed thrice weekly.

Study 1. A series of preliminary studies were performed to fine-tune culture conditions for Ø40 mm constructs,

TABLE 1. SUMMARY OF CONDITIONS FOR Ø40 MM CASTED CONSTRUCTS

Cast	P	TGF-β (ng/mL)	CH3E/CH12E	CH open/fill	Controls, mm	Study
C1	P0	10	1/1	Filled	—	1
C2	P2	10	0/2	Filled	4	1
C3	P2	10	2/0	1 filled, 1 open	4	1
C4	P2	10	1/0	Open	4	1
C5	P2	1	1/1	Open	4	1
C6	P2	1	1/1 (2×CH)	Open	4	1
C7	P2	1	0/4 (2×CH)	Open	4, 10	2
C8	P0	1	0/3 (2×CH)	Open	4, 10	2

FIG. 1. Experimental setup: (A) sample assembled and disassembled Ø40 mm CH12E casting molds. (B) Culture system used to house Ø40 mm constructs, with a 2xCH12E construct pictured. Color images available online at www.liebertpub.com/tea

consisting of six casts (C1–C6) generating a total of $n = 11$ Ø40 mm constructs, each with simultaneously cast Ø4 mm constructs serving as controls ($n = 4$ per cast). All constructs had thicknesses of 2.3 mm. Culture conditions for each cast are summarized in Table 1. Constructs were varied by cell passage number (P0 or P2 bovine chondrocytes), TGF- β dosage (10 or 1 ng/mL), and channel arrangement. For C1–C5, Ø1 mm channels had CH3E or CH12E, respectively) based on Ø10 mm constructs from previous studies (Fig. 2).^{35–37} For C6, Ø1.4 mm channels (2x area of Ø1 mm) were spaced with similar edge-to-edge distances as CH3E and CH12E constructs, yielding 2xCH3E and 2xCH12E constructs, respectively.

In C1–C2 constructs, channels tended to fill in before day 56. This premature filling was suspected to be responsible for extreme heterogeneities observed in matrix deposition. Thus, channels in C3–C6 constructs were maintained open

throughout culture: during weeks 3 and 5, a Ø1 mm biopsy punch was passed through each channel to clear any occluding tissue. Only one of two C3 constructs was repunched, to compare resulting construct properties.

Study 2. Following Study 1, the most beneficial culture conditions were selected for Study 2: 2xCH12E constructs, supplemented with 1 ng/mL TGF- β , with channels repunched at weeks 3 and 5. In two casts, 2xCH12E constructs were cast with either P2 (C7, $n = 4$) or P0 cells (C8, $n = 3$). For each cast, CH12 Ø10 mm ($n = 4$) and Ø4 mm constructs ($n = 4$), of similar thickness (2.3 mm), served as controls.

Harvest and casting

Bovine articular chondrocytes were harvested from calf wrists (16 wrists per cast with primary cells, 6 wrists total

FIG. 2. Channel arrangements in Ø40 mm constructs: (A) CH3E, (B) CH12E, (C) 2xCH3E, and (D) 2xCH12E. Insets depict channel diameters and c-c spacings in mm.

for casts with passaged cells) and digested in collagenase solution as previously described.¹⁹ Released chondrocytes were either cast as primary cells (P0) or following monolayer culture. Cells cultured in monolayer were plated at 2×10^4 cells/cm² in expansion medium (high-glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% antibiotic-antimycotic, 10 ng/mL platelet-derived growth factor- $\beta\beta$, 5 ng/mL fibroblast growth factor-2, and 1 ng/mL TGF- β 3).^{45,48,51} After reaching confluence at second passage, P2 cells were trypsinized.

P0 or P2 cells were cast at nominal cell densities of 60×10^6 cells/mL in 2% agarose type VIIA (Sigma-Aldrich), either in one of the 3D-printed molds described above ($\varnothing 40 \times 2.3$ mm constructs) or as previously described to produce $\varnothing 4 \times 2.3$ mm and CH12 $\varnothing 10 \times 2.3$ mm constructs.^{19,36}

Mold design

Custom molds were produced to cast $\varnothing 40 \times 2.3$ mm cylindrical constructs with nutrient channels. The molds, designed in SolidWorks (Dassault Systèmes), consisted of three parts: (1) a top, featuring a pillar array, (2) a base piece, and (3) a collar (Fig. 1A). One set of molds had arrays of $\varnothing 1$ mm pillars (to produce channels) to produce CH3E or CH12E constructs, and a second set of molds had $\varnothing 1.4$ mm pillars to yield 2 $\times$ CH3E or 2 $\times$ CH12E constructs. The SolidWorks designs were 3D printed (Objet24; Stratasys, Eden Prairie, MN) from polyacrylate material (VeroWhitePlus RGD835), dried overnight under vacuum at 50 °C, and sterilized in 70% ethanol for 10 min.

Construct processing

At day 56, each $\varnothing 40$ mm construct was bathed in sterile phosphate-buffered saline for 45 min to elute culture media. The diameter, thickness, and mass were recorded. Constructs were photographed before being subdivided for subsequent analyses (Fig. 3). For histological analysis, narrow strips were taken along the diameter of each construct. For mechanical and biochemical analyses, a $\varnothing 6$ mm

biopsy punch was used to harvest cylindrical cores ($n=4$) from constructs, taking care to avoid construct peripheries. For friction testing, two rectangular strips ($\sim 20 \times 10$ mm) were cut from each construct.

Statistical analyses

For Study 1, two-way ANOVA was performed for C1–C6 constructs combined, with independent variables of either (1) channel spacing, open/filled channels, and their interaction or (2) channel spacing, TGF- β dosage, and their interaction. E_Y , G^* , GAG, collagen, or percent collagen damage served as dependent variables ($\alpha=0.05$); Tukey's HSD *post hoc* testing of the means was implemented upon determination of significance ($p<0.05$). Although $\varnothing 4$ mm controls provided points of reference for $\varnothing 40$ mm properties, statistical comparisons were not feasible due to low n (1–2 $\varnothing 40$ mm constructs per cast).

In Study 2, two-way ANOVAs were performed, with cell passage number (P0 or P2) and construct size ($\varnothing 4$, $\varnothing 10$, or $\varnothing 40$ mm) as independent factors. Data for both studies are presented as mean $\pm$ standard deviation.

Results

Study 1

Morphology and histology. After 56 days, constructs displayed cartilage-like opacity and appearance (Fig. 4A, B). Constructs exhibited marked swelling, reaching diameters of 49.3 ± 1.5 mm and thicknesses of 4.3 ± 0.4 mm. Channels tended to fill in (Fig. 4A), unless they were repunched (Fig. 4B). Histology revealed large matrix-devoid regions at the centers of constructs with filled-in channels; this deficiency was partially mitigated by repunching channels (Fig. 4C–H). Closer-packed channels (CH12E), when repunched, stained richly for GAG and collagen throughout the construct thicknesses (Fig. 4I, J).

Functional properties. E_Y was found to be more than twice as high in CH12E constructs compared to CH3E (236 ± 158 kPa vs. 110 ± 45 kPa) and was markedly improved in 1 ng/mL TGF- β constructs versus 10 ng/mL (263 ± 167 kPa vs. 113 ± 44 kPa); E_Y was similar for constructs with filled or repunched channels (173 ± 128 kPa, Fig. 5A). GAG content was only significantly improved by 1 ng/mL TGF- β compared with 10 ng/mL ($5.7 \pm 0.9\%$ /ww vs. $4.7 \pm 0.7\%$ /ww, Fig. 5B). Collagen contents were significantly higher in constructs with CH12E configurations ($1.8 \pm 0.8\%$ /ww), repunched channels ($1.7 \pm 0.3\%$ /ww), and 1 ng/mL TGF- β ($2.1 \pm 0.5\%$ /ww) than they were in constructs with CH3E configurations ($1.4 \pm 0.2\%$ /ww), filled-in channels ($1.2 \pm 0.1\%$ /ww), and 10 ng/mL TGF- β supplemented ($1.3 \pm 0.1\%$ /ww, Fig. 5C).

Control constructs ($\varnothing 4$ mm) from C2–C6 ($n=16$) possessed E_Y of 449 ± 77 kPa, $6.9 \pm 1.1\%$ /ww GAG, and $2.3 \pm 0.4\%$ /ww collagen. Although statistical comparisons were not made between $\varnothing 4$ and $\varnothing 40$ mm constructs due to low n , $\varnothing 4$ mm constructs generally had far better functional properties, excepting a C6 $\varnothing 40$ mm construct with a 2 $\times$ CH12E channel arrangement, repunched channels, and 1 ng/mL TGF- β ($E_Y=490$ kPa, 6.8% /ww GAG, 2.9% /ww collagen),

FIG. 3. Construct sampling: subdivision of $\varnothing 40$ mm construct (2 $\times$ CH12E construct pictured) into $\varnothing 6$ mm cores for E_Y and biochemical analyses, strips for friction testing, and diametric slices for histology.

FIG. 4. Histomorphology: (A) day 56 Ø40 mm CH12E construct, with filled-in channels, beside an acellular agarose gel; (B) day 56 Ø40 mm CH12E construct with repunched channels. Stains for Safranin O (GAG; C, E, G, I) and Picrosirius Red (collagen; D, F, H, J). Bottom panels (K–R) represent higher magnifications corresponding to the regions denoted by lower case letters (k–r). * Filled channels, ▼ open channels, and + regions of little matrix deposition. High-magnification scale bar (K) = 200 µm. GAG, glycosaminoglycan. Color images available online at www.liebertpub.com/tea

which had similar properties to its Ø4 mm controls ($E_Y = 464 \pm 86$ kPa, $6.5 \pm 0.7\%$ /ww GAG, $2.8 \pm 0.4\%$ /ww collagen; $p > 0.5$) and the highest functional properties among all Ø40 mm constructs. This combination of culture parameters was thus implemented for all constructs in Study 2.

FIG. 5. Construct compositions and mechanical properties: (A) E_Y , (B) GAG, and (C) collagen contents of Ø40 mm constructs in Study 1, grouped by culture parameters. CH3E and CH12E contain constructs with 2×CH3E and 2×CH12E channel configurations, respectively. * $p < 0.05$ between groups. Approximate native cartilage values are indicated by shaded chart regions.

Study 2

Morphology, histology, and immunohistochemistry. All Study 2 constructs grew substantially in size; Ø40 mm constructs had all exceeded Ø50 mm by day 56 (Fig. 6A, B). At this size, constructs exceeded the dimensions of articular surfaces of the human knee (Fig. 6C, D). Due to manual repunching most channels remained open, except in P2 Ø10 mm constructs which had nearly all filled, as shown in Figure 6B.

Constructs generally showed stronger histological staining for GAG and collagen than in Study 1 (Figs. 4C–J and 7). Matrix-devoid regions were observed at the centers of Ø4 mm constructs, of Ø10 mm constructs with filled-in channels, and in regions outside the channel arrays of Ø40 mm constructs. Notably, at either end of Ø40 mm diametrical cross sections, regions appeared which were analogous to the centers of Ø4 mm constructs (Fig. 7).

Immunostaining showed type II collagen distributed throughout construct centers, with darker staining localized to construct peripheries (Fig. 8). Type II stained darker for P0 cells in larger constructs (Ø10, Ø40 mm) compared to P2, although in Ø4 mm constructs P0 staining for type II was lighter. Type I collagen staining was generally consistent throughout construct centers and peripheries, with the exception of P2 Ø4 mm and P0 Ø10 mm constructs, in which construct edges were stained more darkly. P2 Ø10 mm constructs possessed the darkest type I staining, while Ø40 mm constructs exhibited less type I staining than other construct sizes. Due to disparate binding affinities and concentrations of the collagen I and II primary antibodies, we cannot make qualitative assessments of the relative contents of collagen types based on stain intensity. Quantitative measures of collagen II relative to total collagen content are reported below.

FIG. 6. Construct appearance: day 56 Ø40 mm (2×CH12E), Ø10 mm, and Ø4 mm constructs using (A) P0 or (B) P2 cells; (C) scale of a P0 Ø40 mm 2×CH12E construct contrasted with an adult human knee's tibial and patellar cartilages; and (D) eclipsing the lateral tibial plateau articular surface. Color images available online at www.liebertpub.com/tea

Functional properties. On average, Ø40 mm constructs possessed $E_Y = 450 \pm 114$ kPa and $8.1 \pm 0.7\%$ /ww GAG, reaching or exceeded native cartilage values, and select constructs reached E_Y of 623 kPa, G^* of 2.48 MPa, 8.9% /ww GAG, and 3.5% /ww collagen. Deposited collagen was $51.3\% \pm 5.9\%$ denatured in all samples, with no significant effects of construct size or cell passage number ($p > 0.05$). Collagen type as determined by ELISA was predominantly type II, with abundances of $60.3\% \pm 6.1\%$ and $62.1\% \pm 1.6\%$ relative to total collagen content in P0 and P2 Ø40 mm constructs, respectively, compared to $81.6\% \pm 10.1\%$ measured in bovine cartilage.

E_Y was significantly influenced by cell passage number ($p < 0.005$) and construct size ($p < 0.005$), but not by their interaction ($p = 0.7$; Fig. 9A). Primary cells yielded higher E_Y than passaged cells (P0: 415 ± 136 kPa, P2: 282 ± 90 kPa; $p < 0.005$). The largest constructs were significantly stiffer (450 ± 114 kPa) than Ø4 mm (289 ± 68 kPa; $p = 0.005$) and Ø10 mm (312 ± 145 kPa; $p < 0.05$) controls. G^* was significantly affected by construct size ($p < 0.05$; Fig. 9B), with Ø10 mm constructs exhibiting higher G^* (1.99 ± 1.19 MPa) than Ø4 mm constructs (1.00 ± 0.35 MPa, $p < 0.05$); Ø40 mm constructs (1.70 ± 0.60 MPa) were not different from Ø10 mm constructs ($p = 0.77$) and showed a trend toward higher G^* than Ø4 mm constructs ($p = 0.067$). Passage number had a strong effect on G^* , ($p < 0.005$), with primary cells (2.05 ± 1.03 MPa) outperforming passaged cells (1.10 ± 0.35 MPa).

Primary cells yielded significantly higher matrix contents ($9.5 \pm 1.3\%$ /ww GAG, $3.6 \pm 0.7\%$ /ww collagen) than passaged cells ($8.5 \pm 0.7\%$ /ww GAG, $p < 0.05$; $2.4 \pm 0.4\%$ /ww collagen, $p < 0.0001$; Fig. 9C, D). Construct size influenced GAG content (Ø4 mm: $9.5 \pm 1.0\%$ /ww, Ø10 mm: 9.2 ± 1.2 ,

Ø40 mm: 8.1 ± 0.7 ; $p < 0.05$); Ø4 mm constructs had more GAG than Ø40 mm constructs ($p < 0.05$), but construct size did not affect collagen ($p = 0.13$). The interaction of passage number and size was significant for collagen content ($p < 0.0005$), and P0 Ø10 mm constructs had significantly higher collagen than any other group ($4.4 \pm 0.8\%$ /ww, $p < 0.05$). Collagen was found to be damaged at a fraction of $51.3\% \pm 5.9\%$ of the bulk content and did not vary between groups ($p > 0.05$).

Swelling ratio (construct final/initial ww) depended on passage number ($p < 0.0001$), size ($p < 0.0005$), and their interaction ($p < 0.001$, Fig. 9E). Swelling ratios by construct size were statistically distinct ($p < 0.05$); the most swelling was observed in Ø40 mm constructs (3.5 ± 0.2), followed by Ø4 mm (2.9 ± 1.3) and Ø10 mm (2.4 ± 1.0). P2 constructs swelled more (3.6 ± 0.5) than P0 constructs (2.1 ± 0.9).

Constructs exhibited favorable frictional behavior (Supplementary Movie S1), possessing μ_0 , μ_f , and μ_{avg} of 0.064 ± 0.008 , 0.110 ± 0.018 , and 0.090 ± 0.010 , respectively. Large (Ø40 mm) constructs were robust to manipulation and handling, as illustrated in Supplementary Movie S2. In comparison, 2% agarose gels representative of day 0 constructs are fragile and may easily break upon similar manipulation and handling, as illustrated in Supplementary Movie S3.

Discussion

The primary objective of this study was to engineer articular surface-sized cartilage constructs, using channels to enhance nutrient supply, with functional properties and compositions comparable to those of much smaller-sized constructs. Our secondary objective was to assess whether results

FIG. 7. Histological stains: (A, B) for total collagen, by Picrosirius Red or (C, D) GAG by Safranin O in Ø4, Ø10, and Ø40 mm 2×CH12E constructs. Lower panels (E–P) represent higher magnifications corresponding to the regions denoted by lower case letters (e–p). Dotted lines indicate cut lines, + regions of little matrix deposition, * filled channels, and ▼ open channels. High-magnification scale bar (E) = 100 μ m. Localizations of types I and II collagen are delineated by immunohistochemical stains in Figure 8. Color images available online at www.liebertpub.com/tea

from large constructs seeded with primary chondrocytes can forecast successes with passaged cells.

Results summarized in Figure 9 demonstrate that both of these objectives were achieved, producing large engineered cartilage constructs whose size is comparable to that of articular layers in lower extremity joints (Fig. 6). Furthermore, Young's modulus and GAG levels achieved in these constructs (Fig. 9A, C) fall within or exceed the range of native levels for immature bovine articular cartilage (E_Y : 250–730 kPa, GAG: 1–5%/ww).⁵² The dynamic moduli and collagen levels (Fig. 9B, D) are robust, but remain below native values (G^* : 5–10 MPa at 0.01 Hz, collagen: 10–15%/ww); they are on par with engineered cartilage from previous studies.^{52–60}

On average by day 56, large constructs had reached sizes of Ø52 × 4.3 mm, weighed 8.1 g, had E_Y of 450 kPa, and had 8.1%/ww GAG and 2.6%/ww collagen. The most robust Ø40 mm construct was cast with P0 cells and reached E_Y = 623 kPa, 8.9%/ww GAG, and 3.5%/ww collagen. The size of these constructs afforded us a unique opportunity to test entire apposing strips of engineered cartilage in friction.

Although the coefficients measured (μ_0 = 0.06, μ_f = 0.11) are above those characteristic of native cartilage under similar conditions (μ = 0.016), they are among the lowest recorded by engineered cartilage to date.^{46,61–66}

In this study, we report an extensive set of properties; nevertheless, an additional useful measure of mechanical integrity of constructs is their failure response.⁶⁷ Although we did not measure failure properties in the current study, we have thusly characterized chondrocyte-seeded agarose constructs in previous work.^{34,68} As we further improve culture conditions in this model system, we expect to characterize failure properties systematically before *in vivo* implantation of constructs.

When comparing Ø40 mm constructs with smaller constructs, collagen content and G^* remained comparable, GAG contents decreased statistically but were still within native ranges, and E_Y values were improved (Fig. 9A–D), supporting our hypothesis that similar functional properties could be achieved in scaled-up constructs. This scaling-up of conventionally sized (Ø4 mm) cartilage tissue constructs by 100-fold—as large as human knee cartilage layers

FIG. 8. Immunohistochemical staining for collagen types I and II in constructs of each size (Ø4, Ø10, Ø40 mm), at selected sites near centers or edges of constructs. Due to disparate binding affinities and concentrations of the collagen I and II primary antibodies, the relative contents of collagen types may not be compared based on stain intensity. Color images available online at www.liebertpub.com/tea

FIG. 9. Study 2 construct functional properties: (A) E_Y , (B) G^* , (C) GAG content, (D) collagen content, and (E) swelling ratio. * $p < 0.05$ for ANOVA effect of cell passing (P), construct size ($\emptyset$), or their interaction ($P \times \emptyset$); + $p < 0.05$ between specific groups. n is superimposed on bars. Approximate native cartilage values are indicated by shaded blue regions. Color images available online at www.liebertpub.com/tea

(Fig. 6)—without compromising their functionalities represents the major contribution by this study and is a testament to the capability of nutrient channels to promote growth in constructs of any size. The significantly higher compressive properties of the $\emptyset 40$ mm constructs may be attributed to the fact that cores taken for mechanical testing were from within the channel array and, therefore, suffered no edge effects that $\emptyset 4$ and $\emptyset 10$ mm constructs likely experienced.

Although P0 constructs exhibited better functional properties, those with P2 cells followed similar trends based on construct size, and P2 $\emptyset 40$ mm constructs were within native values of E_Y and GAG content for human knee cartilage (Fig. 9).⁵⁵ This result is encouraging, since it suggests that our past and present results using primary cells are applicable to passaged cells, which represent a more realistic clinical scenario.

Although adult cells were not used in this study, allogeneic implantation of juvenile human chondrocytes has previously shown clinical promise, and therefore, our results hold relevance for the treatment of adults.⁶⁹ The current study, in tandem with our recent and promising results from constructs seeded with young and adult human chondrocytes (ages 17 to 35), suggests that engineering articular surface-sized constructs with human cells is a feasible strategy for treating advanced OA.⁴⁹

Study 1 showed that premature channel filling was detrimental to constructs (Fig. 4C, D and 5C), whereas Study 2

constructs with repunched channels exhibited the highest functional properties (Fig. 9). These large channeled constructs displayed some of the most favorable compressive and frictional properties reported thus far in CTE literature. Furthermore, at least two commercial chondral allografts, Cartiform® (Arthrex, Inc., Naples, FL) and ProChondrix® (AlloSource, Centennial, CO), intentionally include similar pores. These pores aid in surgical implantation and host integration, serving as sites for suturing, gluing, and infiltration by stem cells from the subchondral bone. The shorter diffusion distances due to channels would also more effectively predispose the tissue for cryopreservation, as for Cartiform. Therefore, persistent channels in large tissue constructs may be advantageous.

We propose that in clinical applications, large constructs may be cultivated *in vitro*, maintaining unobstructed nutrient channels, until reaching native cartilage mechanical and biochemical properties; any remaining diseased cartilage in the target joint may be debrided and the construct fixated onto the underlying bone by gluing, suturing, and/or performing concurrent microfracture surgery. In cases of OA involving extensive degradation of the subchondral bone, osteochondral constructs may be preferable. In support of this strategy, ongoing studies by our group seek to produce joint surface-sized channeled constructs with porous metal bases, which have shown success *in vitro* and in canine studies.^{44,45}

Future animal *in vivo* studies will allow us to investigate whether channels fill in sufficiently with cartilage-like matrix when exposed to blood supply from the microfracture procedure. Alternatively, we may allow channels to fill in *in vitro*, once the construct has achieved functional properties, to minimize the risk of structural failure due to stress concentration near channels.

A common criticism of our CTE strategy is our reliance on agarose as a scaffold. We have reviewed many arguments in favor of agarose in our previous studies.^{23,33,70} In particular, we have previously reported a canine study⁴⁵ where similar chondrocyte-seeded agarose constructs exhibited very good functional outcomes, excellent integration, and no evidence of rejection. In humans, as a component of Cartipatch® (Tissue Bank of France, Lyon, France), agarose has been used in a multicenter randomized controlled trial in France, comparing Cartipatch to mosaicplasty; although the long-term outcome of patients treated with mosaicplasty was superior to those treated with Cartipatch, there were no significant adverse events attributed to the agarose-alginate scaffold.⁷¹

In this study and previous experiments, large swelling effects force apart the agarose scaffold and dilute its overall concentration to below 1%/ww, rendering it a minor component of the final construct. Furthermore, a recent investigation reported that agarose implanted in rats does degrade as a result of phagocytosis, suggesting that lack of biodegradability may not be a significant concern.⁷²

This study represents a major advance in our effort to engineer functional, articular surface-sized cartilage constructs for the resurfacing of OA joints. From our perspective, the remaining challenges include (a) the need to reach native levels of collagen content, enhance interstitial fluid pressurization, and produce dynamic compressive moduli and friction coefficients that match native values;

and (b) translation of our promising results achieved with immature bovine chondrocytes to young adult human chondrocytes.

With regard to (a), high cell seeding densities, as used in this study, can produce native levels of collagen. Indeed, when normalizing the collagen content to day 0%/ww (combining results from Fig. 9D, E), we find that the collagen content in constructs ranges from 5.8% to 11.5%. These values are comparable to native levels showing that a sufficient amount of collagen was synthesized based on the original construct dimensions. However, the concomitant rapid synthesis of GAG caused swelling, which diluted this collagen content and resulted in the damage of ~51% of the newly deposited collagen matrix, a level consistent with those shown in our recent study for small constructs cultured 56 days at higher cell seeding densities.⁷³ Therefore, we are currently exploring strategies to delay GAG deposition until the elaboration of a strong collagen matrix that can resist swelling and damage. Once we overcome this challenge, we may focus on assessing the ultrastructural organization of the deposited collagen matrix relative to the native tissue, as other researchers have approached using a variety of techniques such as electrospinning, *in vitro* proteoglycan assembly, magnetic fibril alignment, and 3D bioprinting.^{12,74–77}

With regard to (b), we have recently observed that young adult human chondrocytes, isolated from expired osteochondral allografts from a tissue bank, exhibit chondrogenic potential nearly comparable to that of immature bovine chondrocytes.⁴⁹ Therefore, we plan to further investigate this source of human cells in our effort to translate our tissue engineering approach to the clinic.

This study demonstrated the efficacy of nutrient channels in articular layer-sized constructs comprised of chondrocyte-seeded agarose. However, all 3D culture strategies for CTE at large scales must contend with nutrient transport limitations, and due to the fundamental nature of incorporating channels to shorten nutrient diffusion distances, the general techniques presented in this study are not system-specific and could be applied for various cell and moldable scaffold types. Thus, these results hold a broad impact for large CTE strategies for the treatment of OA.

Acknowledgments

Research reported in this publication was supported by the National Institutes of Health under Award Numbers R01AR060361 and T32AR059038, R01 DE016525 and P41 EB002520. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Gerard A. Ateshian, PhD

Department of Mechanical Engineering

Columbia University

500 West 120th Street

Room 220

New York, NY 10027

E-mail: ateshian@columbia.edu

Received: May 4, 2016

Accepted: July 18, 2016

Online Publication Date: August 23, 2016