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Constrained Cage Culture Improves Engineered Cartilage Functional Properties by Enhancing Collagen Network Stability

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When cultured with sufficient nutrient supply, engineered cartilage synthesizes proteoglycans rapidly, producing an osmotic swelling pressure that destabilizes immature collagen and prevents the development of a robust collagen framework, a hallmark of native cartilage. We hypothesized that mechanically constraining the proteoglycan-induced tissue swelling would enhance construct functional properties through the development of a more stable collagen framework. To test this hypothesis, we developed a novel ''cage'' growth system to mechanically prevent tissue constructs from swelling while ensuring adequate nutrient supply to the growing construct. The effectiveness of constrained culture was examined by testing constructs embedded within two different scaffolds: agarose and cartilage-derived matrix hydrogel (CDMH). Constructs were seeded with immature bovine chondrocytes and cultured under free swelling (FS) conditions for 14 days with transforming growth factor-β before being placed into a constraining cage for the remainder of culture. Controls were cultured under FS conditions throughout. Agarose constructs cultured in cages did not expand after the day 14 caging while FS constructs expanded to 8 × their day 0 weight after 112 days of culture. In addition to the physical differences in growth, by day 56, caged constructs had higher equilibrium (agarose: 639 ± 179 kPa and CDMH: 608 ± 257 kPa) and dynamic compressive moduli (agarose: 3.4 ± 1.0 MPa and CDMH 2.8 ± 1.0 MPa) than FS constructs (agarose: 193 ± 74 kPa and 1.1 ± 0.5 MPa and CDMH: 317 ± 93 kPa and 1.8 ± 1.0 MPa for equilibrium and dynamic properties, respectively). Interestingly, when normalized to final day wet weight, cage and FS constructs did not exhibit differences in proteoglycan or collagen content. However, caged culture enhanced collagen maturation through the increased formation of pyridinoline crosslinks and improved collagen matrix stability as measured by α-chymotrypsin solubility. These findings demonstrate that physically constrained culture of engineered cartilage constructs improves functional properties through improved collagen network maturity and stability. We anticipate that constrained culture may benefit other reported engineered cartilage systems that exhibit a mismatch in proteoglycan and collagen synthesis.

Keywords: cartilage tissue engineering, constrained culture, collagen, proteoglycans, pyridinoline

Introduction

To date, most engineered cartilage culture techniques produce constructs lacking the comprehensive functional properties of native cartilage, such as equilibrium and dynamic moduli and failure properties in compression and tension, as well as frictional and wear properties. These characteristics of native cartilage are essential to its biomechanical function; therefore, functional cartilage tissue engineering aims to grow replacement tissues that are biomechanically similar to the native articular cartilage.[1] This limitation of growing functional replacement tissues for resurfacing osteoarthritic defects has hindered the clinical translation of cartilage tissue engineering.[2,3]

In particular, native cartilage has a dense type II collagen fibrillar network that provides the tissue with a high tensile modulus, and a negatively charged glycosaminoglycan (GAG) ground matrix that imparts a high fixed charge

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density and robust compressive properties to the tissue.[4–6] The interaction between collagen and GAG gives native cartilage unique compressive and tensile moduli as well as friction and wear properties ideally suited for its physiological function within the joint.[7,8] Current engineered cartilage culture protocols successfully develop native GAG content and compressive moduli[9]; however, achieving native collagen content and tensile properties remains elusive.[10]

Although the subphysiological functional properties of tissue-engineered cartilage constructs are often attributed to the low biosynthetic activity of chondrocytes, we have recently shown that chondrocytes seeded at the cell densities present in the developing joint can deposit native levels of collagen, at the expense of producing supraphysiological levels of GAG.[11,12] This rapid and excessive GAG synthesis and deposition results in a high degree of tissue growth and swelling as negatively charged GAG attracts ions and fluid from the surrounding media bath, increasing the osmotic swelling pressure within the construct. As the nascent collagen framework of engineered tissues provides only modest tensile stiffness,[10] constructs volumetrically expand under this swelling pressure. This expansion continues throughout culture as adequately nourished chondrocytes will continuously synthesize and deposit GAG.

In our earlier work, this process resulted in constructs volumetrically growing nearly 800% in wet weight (ww) over a 105 day culture.[12] This study was also our first promising evidence of constructs synthesizing native collagen contents, which reached 15% day 0 ww (%D0-ww). This deposition, however, was effectively diluted because of the profound tissue swelling ($\sim$1.75%ww). Moreover, collagen solubility, measured as the susceptibility of collagen to α-chymotrypsin digestion, increased during culture. Increases in collagen solubility are associated with collagen destabilization and remodeling phenomena, including collagen denaturation, immaturity, and damage, all of which may weaken the bulk mechanical properties.[13–15] Therefore, although both GAG and collagen are necessary for obtaining native cartilage functionality, high GAG deposition outcompetes and destabilizes the collagen network in developing cartilage constructs, hindering their functional properties.

Although the mismatch in GAG and collagen synthesis and deposition was originally observed in agarose scaffolds,[16–24] an open question is whether matrix deposition mismatches may be scaffold dependent. Differences in scaffold ultrastructure and pore size may lead to different rates of matrix deposition and retention. However, a review of engineered cartilage systems suggests that GAG deposition exceeds collagen deposition in the majority of systems and that matrix deposition ratios do not match mature cartilage in any system (Table 1)[9,25–33]; thus although the field has been able to successfully promote chondrocyte matrix synthesis, engineered tissues are biochemically distinct from native cartilage.

This trend seems to be present across scaffolds and cell types, importantly suggesting that clinical translational will remain limited if systems are not developed to address matrix synthesis mismatch. The field of cartilage tissue engineering has increasingly turned to extracellular matrix-based scaffolds to mimic the native cartilage environment[34–39]; of these, cartilage-derived and collagen-based scaffolds are widely used in both research and preclinical settings.[36,40]

Table 1. Glycosaminoglycan and Collagen Deposition Levels and Ratios from the Literature Using Chondrocytes and Mesenchymal Stem Cells

Study	*Cell type*	*Scaffold*	*Cell density (10^6 cell/mL)*	*GAG level (%ww)*	*COL level (%ww)*	*COL:GAG ratio*
Engineered cartilage: chondrocytes						
Vunjak-Novakovic et al.[25]	2–3 w.o. Bov	3% PGA	127	4.5	3.75	0.83:1
Makris et al.[30]	4–8 w.o. Bov	Self-assembled	117[a]	1.45[b]	1.75[b]	1.2:1
Mauck et al.[26]	3–6 m.o. Bov	2% agarose	60[c]	1.7	1.8	1.1:1
Ng et al.[28]	2–5 y.o. K9	2% agarose	30[c]	4.27[d]	1.06[d]	0.25:1
Mesallati et al.[32]	4 m.o. Por	2% agarose	23	1.8	0.96	0.55:1
Byers et al.[9]	4–6 m.o. Bov	2% agarose	6	6.6	3.3	0.5:1
Engineered cartilage: mesenchymal stem cells						
Bhumiratana et al.[31]	Human	Self-assembled	103	7.5[f]	9.5[e,f]	1.26:1
Erickson et al.[29]	Bov	1% MeHA	60[c]	4.8	4.8	1:1
Chung et al.[27]	Human	1:1 MeHA	15	2.5[f]	0.83[f]	0.32:1
Native cartilage: medial femoral condyle						
Williamson et al.[33]	1–3 w.o. Bov	N/A	66	2.1[e]	6.9[e]	3.2:1
Williamson et al.[33]	1–2 y.o. Bov	N/A	39	1.8[e]	7.9[e]	4.4:1

Data are arranged by cell type and density.
Native biochemical content in juvenile and adult bovine cartilage is listed in the two bottom rows.
[a]Based on an earlier study.
[b]Average between control groups of the two engineered cartilage studies. Cell densities were obtained either from the cell content reported, the DNA content reported and converted here based on a ratio of 7.7 pg DNA/cell, or, when experimental data were not reported, the nominal cell density; nominal cell densities are denoted with [c].
[d]Based on the day 28 mixed animal data.
[e]Assumes an OHP:collagen mass ratio of 1:7.6.
[f]Normalization reported in the study on a tissue ww (mg or g) basis and converted here to %ww assuming a density of 1 g/mL.
GAG, glycosaminoglycan; COL, collagen; Bov, bovine; K9, canine; Por, porcine; ww, wet weight; w.o., weeks old; y.o., years old; PGA, polyglycolic acid; MeHA, methacrylate hyaluronic acid; N/A, not applicable.

Although results are promising because of their high initial matrix content, these scaffolds are prone to initial contraction and it is unclear whether they can produce a functional collagen network.

We hypothesize that suppressing GAG-induced construct swelling and growth, while maintaining nutrient access and matrix synthesis, can enhance collagen integrity and thereby lead to enhanced functional properties. To this end, we developed a constraint-based culture system (''cage'') that physically confines osmotically induced tissue growth and swelling. Cages were designed to mechanically restrain construct growth within a fixed volume and shape, and provide sufficient nutrient access to the construct.

In Study 1, we test our hypothesis utilizing an agarose scaffold seeded with juvenile bovine chondrocytes, a system we have used successfully for more than a decade.[41] Based on the encouraging results of Study 1, we performed a pilot test (Study 2) of translating the cage system to a scaffold derived from native cartilage extracellular matrix (cartilage-derived matrix hydrogel, CDMH), which contracts significantly in the initial culture period, before swelling upon the synthesis and deposition of extracellular matrix. For both studies, constructs cultured within cages employed our previously reported tissue culture strategies for optimizing growth, namely the introduction of an optimal arrangement of nutrient channels,[42] which are perfused by orbital shaking of the culture system[43] at a sufficient media volume:tissue ratio that prevents glucose depletion.[44]

Methods

CDMH preparation

Hyaline cartilage was harvested from Yorkshire pigs weighing 40–50 kg immediately after euthanasia under a tissue-sharing protocol approved by the Columbia University Institutional Animal Care and Use Committee. Hyaline cartilage was cleaned of all connective tissue and debris, rinsed in cold sterile normal saline, and frozen at –80°C for a minimum of 24 h and a maximum of 2 weeks before isolation and processing of CDMH.

Cartilage was sectioned to 1 mm thickness and serially washed in an orbital shaker in hypertonic (2×) phosphate-buffered saline (PBS) for 15 min, 0.02% trypsin for 15 min, and 3% Tween-20 for 30 min. After each step, cartilage was washed in 2× PBS for 15 min. Cartilage sections were snap frozen in liquid nitrogen, milled into a fine powder, and lyophilized for 24 h. Powdered cartilage was washed in 4% sodium deoxycholate for 30 min, 2× PBS for 15 min, 0.2 mg/mL DNase I (Sigma D4527) for 24 h, 0.1% peracetic acid for 30 min, and sterile deionized water for 30 min. The cartilage matrix slurry was then snap frozen in liquid nitrogen, lyophilized for 24 h, and stored at room temperature.

Dry cartilage matrix powder was digested as previously described.[45] In brief, 1 g of lyophilized cartilage matrix powder was mixed with 0.1 g pepsin (Sigma P7012) in 0.01 M hydrochloric acid and digested for 18 h at room temperature (25°C) under constant stirring. Matrix digests were aliquoted and stored at –80°C until use. Cells were encapsulated within hydrogels by reconstituting the cartilage matrix digest with 0.1 M sodium hydroxide, 10× PBS, and chondrocytes suspended in culture media to yield a cartilage matrix hydrogel with a final concentration of 6 mg/mL.

Construct casting and culture

Chondrocytes were isolated from juvenile (4–8 weeks old) bovine carpometacarpal joints and passaged twice in monolayer culture, as previously described.[46] Cells were released with 0.05% trypsin and encapsulated in either agarose (Study 1) or CDMH (Study 2).

In Study 1, constructs were made as previously described[43]: chondrocytes (120×10^6 cells/mL) were mixed 1:1 with type VII-A agarose (Sigma) and poured into a glass and Teflon-backed mold with a pattern of prearranged pins, such that after punching ø10 mm × 2.34 mm constructs, each construct possessed 12 × ø1 mm nutrient channels.[43] In Study 2, chondrocytes (120×10^6 cells/mL) were mixed with CDMH and gelled at 37°C and 5% CO_2 before biopsy punching ø10 mm × 2.34 mm constructs. Final nominal cell densities were 60×10^6 cells/mL in the agarose constructs of Study 1 and 33×10^6 cells/mL in the CDMH constructs of Study 2 before scaffold contraction, as described hereunder.

CDMH constructs were cast and initially punched without nutrient channels. Cellular contraction of the CDMH[47] reduced the ø10 mm × 2.34 mm constructs to approximately ø4 mm × 0.8 mm after 14 days of culture. In a preliminary study using CDMH, the scaffold contraction caused a large increase in cell density such that constructs exhibited severe matrix heterogeneities despite their small size (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Therefore, to enhance nutrient exchange in Study 2, a single ø1 mm channel was punched in the center of each ø4 mm × 0.8 mm CDMH construct on day 14; the large degree of scaffold contraction in the first 14 days of culture precluded our use of the channel molds from Study 1, as this contraction would have distorted any channel arrangement.

Constructs in both studies were cultured in standard chemically defined chondrogenic media under 0.8 Hz orbital shaking, and the large agarose constructs of Study 1 were placed vertically in culture racks to enhance nutrient access.[43] Constructs received 10 ng/mL transforming growth factor (TGF)-β3 supplementation (R&D Systems) for the first 14 days of culture.[48] As tissue growth and expansion typically become significant after the 14-day TGF-β priming, constructs were divided into free swelling (FS) and cage groups on day 14.[9,49] Caged constructs were cultured for the remainder of the study within the cage, whereas FS constructs continued their culture unconstrained. Constructs were assessed in Study 1 on days 0 ($n=2$), 14 ($n=4$), 56 ($n=6$), and 112 ($n=6$). As little differences were observed between days 56 and 112 in Study 1, Study 2 constructs were assessed on days 14 ($n=4$), 56 ($n=4$–6), and 85 ($n=6$) (Fig. 1A). Before scaffold contraction, CDMH constructs lacked mechanical integrity and were, therefore, not tested or sampled immediately after casting on day 0.

Cage components and manufacturing

Cages consisted of two faceplates abutting a single spacer and the assembly was secured with four pairs of stainless steel screws and nuts (Fig. 1B). Faceplates consisted of perforated stainless steel sheets (316, ø0.625 mm holes, center-to-center spacing 0.094 mm; McMaster-Carr) to allow media to flow to the construct while preventing axial swelling. Spacers were made from an impermeable

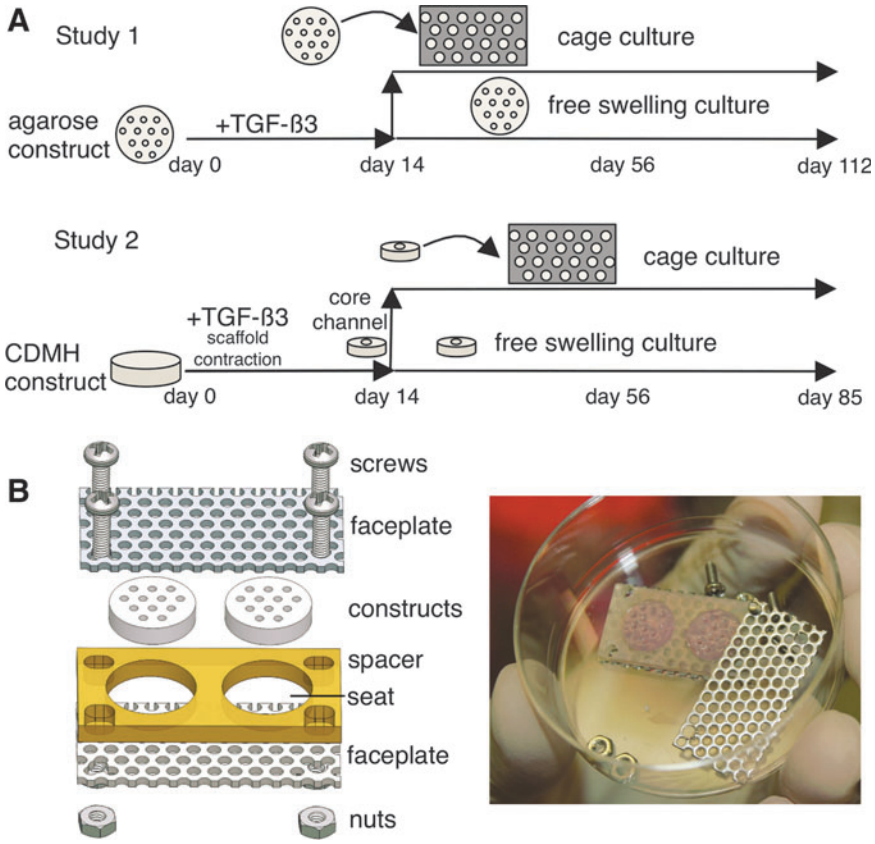


FIG. 1. **(A)** Study 1 (agarose) and Study 2 (CDMH) overview including channeling, transforming growth factor-β3 supplementation, caging, and construct time points. **(B)** Cage assembly diagram (*left*) and with construct (*right*).

biocompatible material (Study 1: polysulfone; Study 2: polytetrafluoroethylene) and construct seats were sized to fit day 14 construct dimensions (Study 1: ø10.5 mm × 2.34 mm; Study 2: ø4 mm × 0.8 mm) and prevent radial swelling. All materials were autoclaved before assembly.

Cages were assembled by placing constructs into the seats of the spacer and fastening faceplates to opposing sides of the spacer. To promote nutrient transport, channels were aligned with faceplate perforations and a ø1 mm biopsy punch was passed through the faceplates and construct to ensure clear media passage. Channels of the cage constructs became progressively occluded in Study 1 and were recored once (day 58). The supplementation of TGF-β3 (all constructs in Study 1 and Study 2) and recoring of channels (cage constructs, where channels were occluded, in Study 1) served to enhance nutrient transport (primarily glucose and TGF-β3) where we had evidence of nutrient limitation (large agarose constructs[46] and CDMH: Supplementary Fig. S1).

Mechanical testing

Mechanical testing included measurements of compressive unconfined equilibrium modulus at 10% compression (E_Y) and compressive unconfined dynamic modulus (G*) at 0.01 Hz and 1% strain amplitude, as previously described.[43]

Biochemical analyses

Biochemical analyses measured the matrix deposition of negatively charged GAG using the dimethylmethylene blue dye-binding assay[50] and collagen using the acid hydrolysis–orthohydroxyproline (OHP) assay and a collagen:OHP conversion ratio of 7.6:1.[51,52] Cell content was measured using the PicoGreen assay (Invitrogen) based on a ratio of 7.7 pg DNA/cell.[53] Cell and matrix concentrations were normalized to the current day ww (%ww) to provide a measure of the current matrix content and to the day 0 ww (%D0-ww) to provide a measure of total matrix deposition.[44]

Soluble collagen content was measured following the protocol of Bank *et al.*[13]: subpunched ø3 mm construct cores were desorbed of unbound collagen for 48 h at 4°C under continuous rotation in a 4 M guanidine HCl buffer solution (0.1 M TrisHCl, 10 µg/mL pepstatin-A, 1 mM iodoacetamide, 1 mM ethylenediaminetetraacetic acid, pH 7.3). Constructs were rinsed for 8 h in buffer at 4°C before adding a 0.5 mg/mL solution (0.5 mL per sample) of α-chymotrypsin (Sigma) and incubating for 16 h at 37°C. α-chymotrypsin separates collagens into soluble and intact (bound) fractions corresponding to their stability within the tissue. Aspiration of the incubation buffer isolates the soluble collagen fraction, leaving the intact collagen fraction within the tissue pellet. The aspirant and pellet were assayed with the OHP assay, and collagen solubility was calculated as (soluble collagen mass)/(soluble collagen mass + intact collagen mass). The collagen crosslink pyridinoline (PYD) was measured with a PYD enzyme linked immunosorbent assay (Quidel) using the construct acid hydrolysis digests.[49] PYD content is presented on a per tissue ww basis (nmol/mL) and a per collagen basis (mol PYD/mol COL), assuming a collagen molecular weight of 285 kDa.[54]

Histology

Samples were fixed for 24 h in acid–formalin–ethanol and placed into 70% ethanol before sectioning (5 µm slices).[46]

GAG was observed with 0.01% Safranin-O staining and collagen was observed with 0.1% Picrosirius Red staining.[55]

Statistics

Differences between treatments and time points of the mechanical and biochemical data were assessed with a two-way analysis of variance (ANOVA; $\alpha = 0.05$, computed in R, r-project.org) to determine the statistical influence of culture duration (day), culture treatment (FS vs. cage), and their interaction (duration × treatment). Tukey corrected *post hoc* comparisons were made between groups exhibiting significant differences ($p \leq 0.05$) according to the ANOVA. The significance of each factor (day, group, interaction) for each measure is listed in the Supplementary Table S1 and when differences between the specific factor levels (day or treatment) or individual groups (interaction) were significant ($p < 0.05$), the significance was displayed within the corresponding figure (Figs. 2–5). Data reported here are presented as mean ± standard deviation.

Results

Study 1: agarose constructs

FS agarose constructs grew to 8× their original size after 112 days of culture, whereas caged constructs swelled significantly less (2×, $p < 0.001$), essentially maintaining the size they had achieved before being placed in the cage on day 14 (Fig. 2). FS constructs had dense Safranin-O and Picrosirius Red staining in the periphery, but interior voids were present on days 56 and 112 (Fig. 2). Caged constructs also exhibited rich Safranin-O and Picrosirius Red staining and maintained tissue integrity on day 56, but by day 112, they appeared less organized and interior tears were evident.

Polarized light microscopy images showed no directed tissue ultrastructure in caged constructs, but showed highly aligned collagen ultrastructure between the interior tissue void and construct periphery of FS tissues, reminiscent of native cartilage (Supplementary Fig. S2).

Construct functional properties (Fig. 3A, B) improved with culture duration before plateauing after day 56 ($p > 0.40$ for E_Y and G* between days 56 and 112), with both E_Y and G* higher in caged constructs than in FS constructs ($p < 0.001$). GAG and collagen %ww contents followed similar temporal trends as the mechanical properties and plateaued after day 56 ($p < 0.11$ vs. day 112, Fig. 3C, D). GAG content was significantly lower in caged constructs ($p < 0.004$); meanwhile collagen content was similar between the groups ($p = 0.17$). In contrast, both GAG and collagen matrix deposition (%D0-ww) increased throughout culture and was significantly higher in FS constructs ($p < 0.001$, Fig. 3E, F). By day 14, constructs had deposited 4.8 ± 0.2%D0-ww GAG and 1.7 ± 0.05%D0-ww collagen, after which time FS constructs continued to deposit matrix, amounting to 66.6 ± 3.1%D0-ww GAG and 14.8 ± 1.7%D0-ww collagen by day 112 ($p < 0.001$). Caged constructs deposited less matrix once constrained, and matrix deposition did not change after day 56 ($p > 0.98$), holding at 14.7 ± 2.6%D0-ww GAG and 3.4 ± 0.3%D0-ww collagen.

By day 112, cell densities did not change significantly from day 0 levels (84 ± 14 million cells/mL) in caged constructs (74 ± 8 million cells/mL, $p = 0.56$), but significantly decreased in FS constructs (44 ± 2 million cells/mL, $p < 0.001$) (Fig. 3G). Conversely, total cell content (Fig. 3H) was higher in FS constructs ($p < 0.001$) and increased throughout culture ($p = 0.002$). Total cell content in caged constructs did not change with time ($p = 0.52$).

PYD content was higher in cage culture than in FS for both measures of PYD content ($p < 0.001$) and PYD normalized to collagen ($p < 0.001$) (Fig. 3I, J). PYD content did not significantly change after day 56 ($p > 0.34$), similar to collagen content. In particular, cage constructs exhibited 70% more PYD normalized to collagen and 67% more PYD content than FS constructs on day 112. Collagen solubility (Fig. 3K) increased between days 14 and 56 ($p = 0.003$) and was higher in FS constructs ($p = 0.03$), suggesting that collagen was more stable in cage constructs.

Fluid volume fractions (Supplementary Fig. S3) changed throughout culture and were higher in cage constructs ($p = 0.011$).

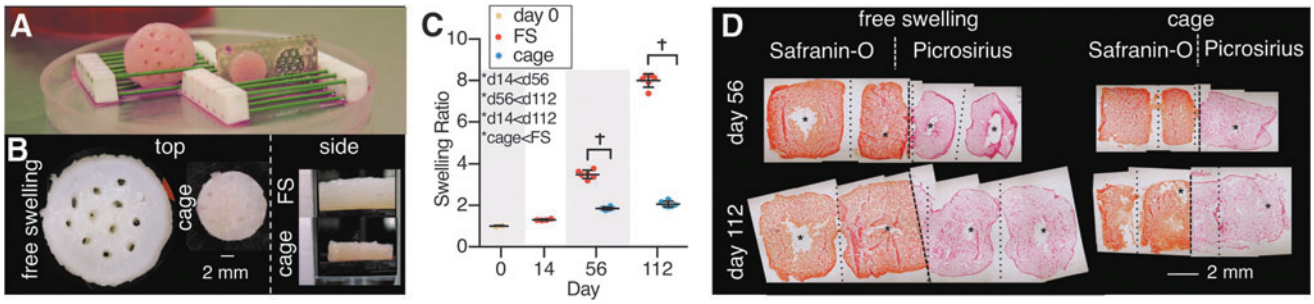
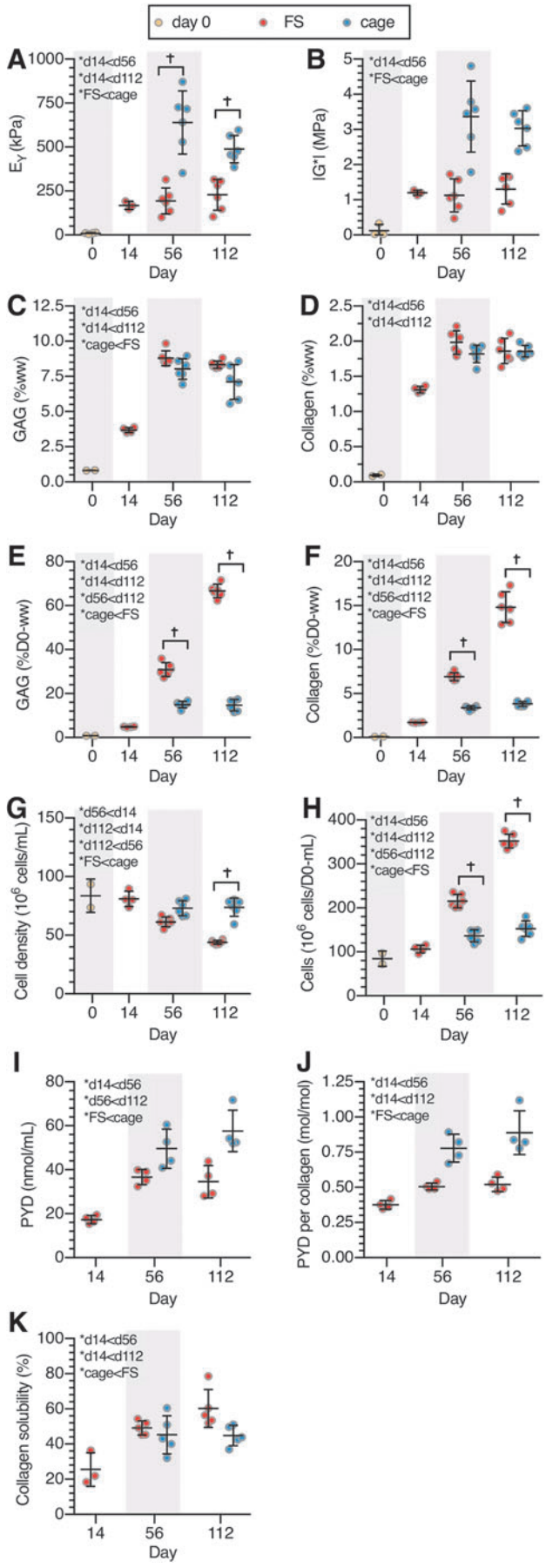


FIG. 2. Study 1, agarose constructs: **(A)** Image of construct differences from FS (*left*) and cage (*right*; front faceplate removed for construct viewing) on day 112. **(B)** Morphological differences in cage growth from *top* and *side* perspectives (FS *side* view only shows middle of construct). **(C)** Swelling ratio differences between FS and caged constructs. *Denotes significant ($p < 0.05$) differences between factor levels. †Denotes significant ($p < 0.05$) differences between individual groups at the same time point. X-axis spacing is not temporally representative. **(D)** Histological images (*side* view) of constructs (Safranin-O displays GAG, Picrosirius displays collagen). *Dashed lines* represent construct midlines and separates Safranin-O and Picrosirius Red images; *dotted lines* represent construct channels or preexisting channels; * are voids or tears in construct. GAG, glycosaminoglycan; FS, free swelling.



Study 2: CDMH constructs

CDMH constructs grew significantly over the 85-day culture (Fig. 4A), with FS constructs swelling significantly more (3.8×, $p<0.001$) than cage constructs (1.9×swelling) when normalized to their day 14 (postcontraction) ww (Fig. 4B). Adding a channel aided in nutrient availability and the large interior voids seen in our preliminary work (Supplementary Fig. S1) were not present in Study 2; with channels, robust Picrosirius Red and Safranin-O staining was observed throughout the CDMH constructs (Fig. 4C). In particular, constructs grown in cages exhibited more uniform staining than FS CDMH, and caged CDMH constructs displayed richer Picrosirius Red staining than FS CDMH.

Caged growth enhanced E_Y over FS culture ($p=0.007$) (Fig. 5A), whereas G* was similar between the groups, peaking on day 56 ($p=0.001$) before returning to day 14 levels by day 85 ($p=0.89$) (Fig. 5B). GAG content was also similar between the culture conditions ($p=0.52$) and increased with culture duration ($p=0.005$) (Fig. 5C). Collagen content trended higher in caged constructs, although not significantly so ($p=0.07$) (Fig. 5D).

GAG deposition in caged CDMH was lower than in FS CDMH constructs ($p<0.006$), as was collagen deposition ($p=0.01$). Overall, deposition of GAG and collagen increased from days 14 to 85 for both groups (cage $p<0.001$, FS $p<0.001$) (Fig. 5E, F).

Owing to the initial scaffold contraction over the first 14 days of culture, cell density in CDMH constructs (342±150 million cells/mL; Fig. 5G) was markedly higher than the seeding density used in Study 1 (84 million cells/mL). Total cell content (cells normalized to day 14 ww) did not change over the culture duration in CDMH constructs ($p=0.15$) and was not influenced by the cage treatment ($p=0.25$; Fig. 5H). However, when accounting for construct swelling, cell density within CDMH constructs decreased from day 14 to day 85 in both cage and FS groups ($p<0.03$; Fig. 5G).

Similar to the agarose constructs of Study 1, cages produced increased PYD content within CDMH constructs (Fig. 5I), although when normalized to collagen content, cage and FS had a similarly elevated level by day 85 (Fig. 5K; ~1 mol PYD/mol collagen). The collagen solubility assay did not detect any differences with treatment or culture duration in CDMH constructs (Fig. 5K).

Discussion

Constrained cage growth of engineered cartilage reduced swelling (Figs. 2C and 4B) and improved construct functional properties in both the agarose and CDMH constructs

FIG. 3. Study 1, agarose constructs: **(A)** equilibrium compressive modulus, E_Y, **(B)** dynamic modulus, G*, **(C)** GAG (%ww), **(D)** collagen (%ww), **(E)** GAG (%D0-ww), **(F)** collagen (%D0-ww), **(G)** cell density, **(H)** total cell content, **(I)** PYD (nmol/mL), **(J)** PYD/collagen (mol/mol), **(K)** collagen solubility. *Denotes significant ($p<0.05$) differences between factor levels. †Denotes significant ($p<0.05$) differences in individual groups at the same time point. X-axis spacing is not temporally representative. PYD, pyridinoline; ww, wet weight.

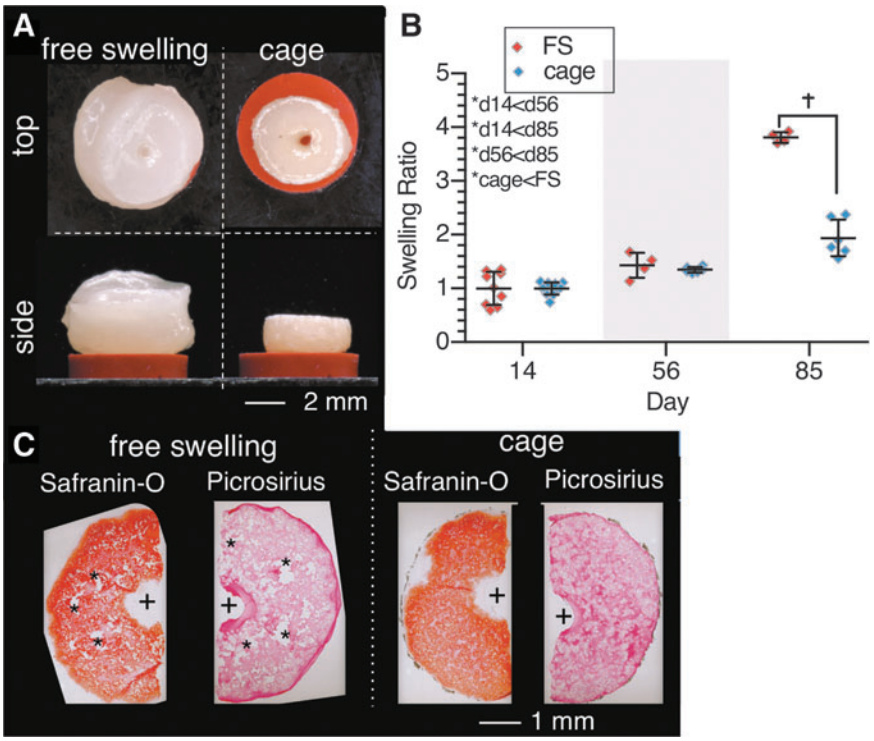


FIG. 4. Study 2, CDMH constructs: **(A)** Morphological differences (*top* and *side* profiles) of FS and cage groups on day 85. **(B)** Swelling ratio differences between FS and caged constructs. *Denotes significant ($p < 0.05$) differences between factor levels. †Denotes significant ($p < 0.05$) differences between individual groups at the same time point. X-axis spacing is not temporally representative. **(C)** Histological (*top* view) images of constructs (Safranin-O displays GAG, Picrosirius displays collagen) on day 56. + represents channel location, * represent voids or tears in construct. CDMH, cartilage-derived matrix hydrogel.

(Figs. 3A, B and 5A, B), supporting our hypothesis that osmotically induced swelling from rapid GAG deposition can hinder the properties of developing engineered cartilage constructs. We developed this strategy based on our prior observation that although cartilage constructs can synthesize GAG and collagen to match native levels, chondrocytes synthesize GAG more rapidly than collagen.[12]

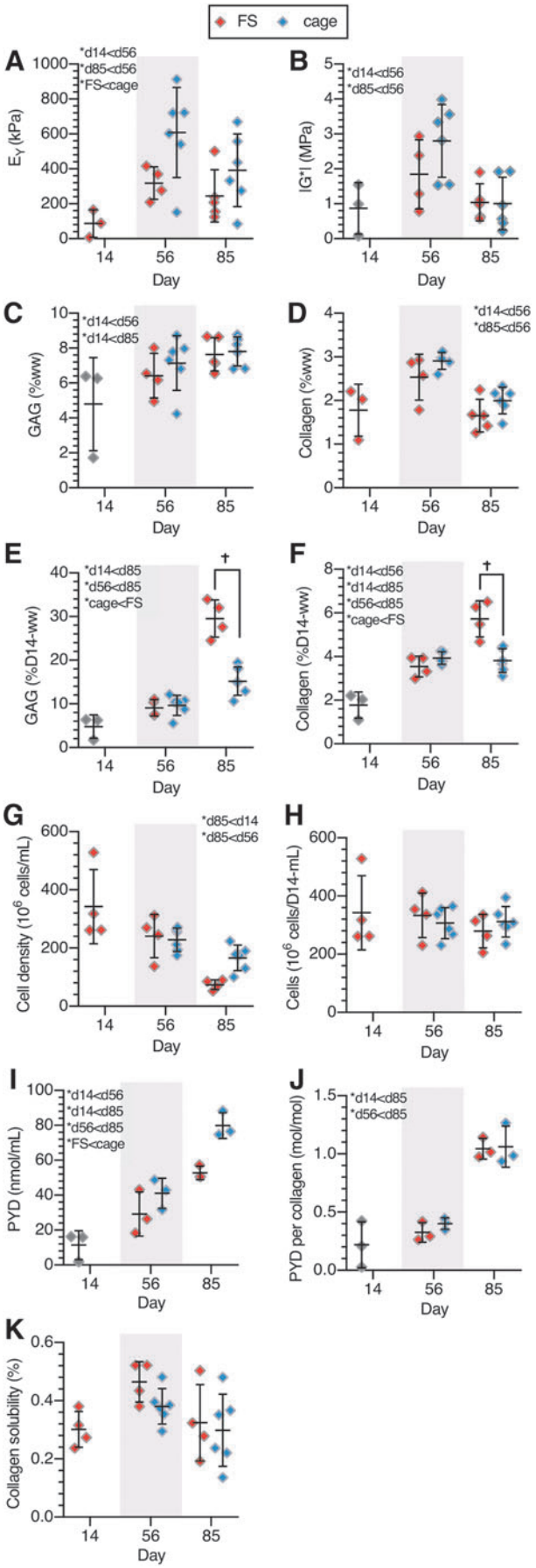
Here, the mismatch in matrix deposition in FS constructs resulted in extreme tissue swelling and growth [8×in agarose constructs (Fig. 2C) and 3.8×in CDMH (Fig. 4B)] and disrupted maturation of an intact collagen network (Fig. 3I–K for agarose), thereby weakening the tissue[56] (Fig. 3A, B for agarose; Fig. 5A, B for CDMH). Cage culture constrained tissue swelling and excessive deposition of GAG, improving functional properties in constructs with both agarose (E_Y and G*, Fig. 3A, B) and CDMH (E_Y, Fig. 5A) scaffolds.

Therefore, mechanical constraints against swelling can enhance functional properties by preventing excessive proteoglycan deposition (agarose: Fig. 3E; CDMH: Fig. 5E), a widespread issue across engineered cartilage tissues (Table 1). However, cages also constrained total collagen deposition (agarose: Fig. 3F; CDMH: Fig. 5F), so that concentrations of GAG and collagen were similar in FS and caged constructs when accounting for the tissue swelling disparities (agarose: Fig. 3C, D; CDMH: Fig. 5C, D).

The best mechanical properties achieved in this study were observed in caged agarose (Fig. 3A, B) and caged CDMH (Fig. 5A, B) constructs on day 56. The equilibrium Young's modulus in unconfined compression was $E_Y = 639 \pm 179$ kPa for agarose constructs and $E_Y = 608 \pm 257$ kPa for CDMH constructs and the dynamic unconfined compression modulus at 0.01 Hz was $G^* = 3.4 \pm 1.0$ MPa for agarose constructs and $G^* = 2.8 \pm 1.0$ MPa for CDMH constructs. For comparison purposes, we have previously measured $E_Y = 660 \pm 230$ kPa and $G^* = 6.5 \pm 2.1$ MPa in native immature bovine cartilage explants.[57] Therefore, E_Y in the best constructs matches the native level, whereas G* is half the native value. These findings are consistent with the observation that GAG levels in this group of constructs (agarose: 8.0 ± 0.7%ww; CDMH: 7.7 ± 0.8%ww) are higher than native cartilage levels (3.3 ± 0.8%ww), whereas collagen content (agarose: 1.8 ± 0.1%ww; CDMH: 2.9 ± 0.2%ww) is lower than native levels (9.5 ± 2.1%ww).[58] Proteoglycans are known to enhance the compressive properties,[59,60] as manifested in E_Y, whereas collagen enhances the tensile properties[61] and thus the dynamic unconfined compression modulus G*.[57]

Despite the increase in GAG content from days 56 to 85, the functional properties of all CDMH groups declined between these time points as did collagen content (Fig. 5A–D), whereas the functional properties in agarose constructs were similar on days 56 and 112. Unlike the cage agarose constructs, cage CDMH swelling ratio increased between days 56 and 85, suggesting that swelling was still possible within the cages likely because of heterogeneous construct sizing after cellular contraction, resulting in an imperfect fit within the confining cage (Fig. 4B). Despite the variability in CDMH cell density on day 14, 342 ± 150 million cells/mL (Fig. 5G), constructs had similar total cell contents, 2.44 ± 0.51 million cells per construct, suggesting that scaffolds were still transiently contracting at this time point. The low variance of cell density at later time points supports this idea: as initial scaffold contraction subsides, a reversal occurs and the tissue swells from matrix deposition, leading to more consistent biochemical measures. Future studies will characterize the role of cell seeding density on scaffold contraction magnitude and duration.

Although matrix deposition was nearly constant in FS constructs throughout culture (nearly linear rise in %D0-ww



for FS agarose constructs shown in Fig. 3E, F starting on day 14 and CDMH constructs shown in Fig. 5E, F), net deposition ceased in caged constructs after day 56. However, despite this dramatic difference in net matrix deposition, both FS and caged constructs had similar matrix concentrations (agarose: ~8.0%ww GAG and ~1.9%ww collagen; CDMH ~7.3%ww GAG and ~2.4%ww collagen). These two observations suggest a signaling role of the extracellular matrix to control chondrocyte matrix synthesis. In FS constructs, negative feedback cues may be continually diluted by the swelling effect, promoting continuous matrix synthesis. Conversely, when construct swelling is prevented, these signals concentrate and inhibit matrix synthesis. Although the nature of this signaling mechanism was not identified here, recent studies have implicated TRPV-4 channels in the transduction of extracellular environment signals to matrix synthesis modulation.[62] TRPV-4 is responsive to the osmotic environment that is regulated by the GAG %ww concentration; thus, the observed plateau in GAG %ww for both groups (Fig. 3C) would be consistent with this type of signaling mechanism.

Despite cessation of net matrix deposition, caged constructs developed higher PYD content and better functional properties. In effect, the 54% increase in PYD between FS and caged agarose constructs (Fig. 3I, J) correlated with a threefold increase in E_Y and G* on day 56 (Fig. 3A, B) and a 10% decrease in solubility (Fig. 3K). This finding suggests that enhancing collagen maturity is critical to developing functional engineered cartilage.[63,64] PYD and other intermolecular collagen crosslinks form spontaneously between enzymatically (lysyl oxidase, LOX) formed residues on adjacent collagen molecules.[65,66] The finding that collagen maturation is promoted by constrained growth is strong evidence that the physical expansion of a growing engineered tissue hinders the formation of PYD and prevents collagen maturation because of collagen content dilution.

Makris *et al.* demonstrated that mechanical properties can be increased with increase in PYD content by treating self-assembled cartilage constructs with exogenous LOX.[30] However, even the highest LOX dose did not develop tissue matching the functionality of native cartilage. In comparison with their study, the constrained culture approach of this study increased PYD content more than exogenous LOX supplementation alone. These results suggest that a proximity between collagen molecules needs to be maintained to facilitate PYD formation in engineered cartilage. Furthermore, the considerable enhancement in E_Y and G* between FS and caged groups may not have resulted entirely from the increased PYD levels, because other types of crosslinks may also exist in cartilage[67] that are not characterized here.

FIG. 5. Study 2, CDMH constructs: **(A)** equilibrium compressive modulus, E_Y, **(B)** dynamic modulus, G*, **(C)** GAG (%ww), **(D)** collagen (%ww), **(E)** GAG (%D0-ww), **(F)** collagen (%D0-ww), **(G)** cell density, **(H)** total cell content, **(I)** PYD (nmol/mL), **(J)** PYD/collagen (mol/mol), **(K)** collagen solubility. *Denotes significant ($p < 0.05$) differences between factor levels. †Denotes significant ($p < 0.05$) differences between individual groups at same time point. X-axis spacing is not temporally representative.

The constraint strategy employed here increased collagen stability in agarose constructs and maintained cellular content but did not improve collagen levels, even though PYD of caged constructs matched native levels in adult bovine cartilage (0.74–0.97 mol PYD/mol COL)[33] when normalized to collagen. This deficiency in collagen content likely explains why tissue construct G* remains less than that of native cartilage. In previous work, our laboratory and others digested existing GAG using the enzyme chondroitinase ABC to temporarily increase collagen content relative to GAG. Although initially promising, these treatments proved deleterious to cellular health and viability, stymieing further matrix synthesis.[68–70] Therefore, although the constrained culture presented here will likely prove critical in enhancing tissue functionality and collagen stability, the field of cartilage tissue engineering still requires strategies to enhance collagen content.

The nonintuitive strategy of constrained growth became evident after our extensive work to enhance and optimize growth conditions utilizing native cell densities in young cartilage with sufficient nutrient availability.[12,52] Elevated cell densities, approaching those found in developing cartilage, were seeded in both agarose (89 million cells/mL) and collagen (342 million cells/mL) scaffolds.[71] Physiological cell densities, effective for engineered cartilage growth with juvenile bovine and adult human chondrocytes, require adequate nutrition to the developing tissue. Tissue constructs of either high cell density or anatomical dimensions, or both, are prone to nutrient scarcities and subsequent heterogeneous matrix deposition and growth.[3,72,73] Supplementing nutrient channels within engineered cartilage constructs has provided a simple means of ameliorating nutrient scarcities.[74–77] Although the need for channels was previously demonstrated in an agarose scaffold system, this study suggests that channels can also improve contracted collagen-based scaffold culture where the cell densities reach very high levels and may prove beneficial across other tissue engineering systems. In particular, scaffoldless systems, which exhibit high cell densities, often display heterogeneous matrix deposition and growth despite their small size, and may benefit from nutrient channel placement and a caging strategy, similar to the CDMH constructs here.[31,73,78]

Cages were specifically designed to allow media transport through the nutrient channels. The steel faceplates used here were adequate for this study, but the selection of cage materials will be dependent on the tissue culture system. In this study, we placed the constructs in cages only after 14 days of FS culture, coinciding with the termination of TGF-β supplementation. We have previously shown that steel has a high binding affinity for active TGF-β, suggesting that culture systems requiring continuous TGF-β supplementation will require different materials.[79,80]

The species of cells and CDMH scaffold presented here (bovine and porcine, respectively) were selected based on availability and past reports and experience. Future studies will involve validating the cage strategy using mature chondrocytes (canine and human) with agarose and CDMH scaffolds. With translation in mind, we will continue to use the porcine CDMH scaffolds, because sourcing canine and human cartilage matrix for the scaffolds would be limited because of availability. Mature canine and human chondrocytes exhibit the GAG–collagen matrix deposition mismatch as previously reported in immature chondrocytes and mesenchymal stem cells.[27,28,52,81] Our recent findings demonstrate that after 56 days, mature human chondrocytes can create cartilage tissue with biochemical and mechanical properties matching tissue grown using immature bovine cells.[52] The results of this work show promise for the translation of the caging strategy across multiple scaffold platforms and, in conjunction with our recent study,[52] demonstrate the potential for translation to improve tissues grown from adult human chondrocytes.

This study demonstrated that cages may be used effectively for constraining the swelling of engineered constructs resulting from high levels of matrix deposition. The primary benefits of this cage system were to enhance mature crosslink formation and to maintain the desired construct dimensions, leading to better functional properties than FS controls. This approach was shown to be effective using agarose as well as cartilage-derived collagen scaffolds. Therefore, this novel constrained culture approach holds promise for improving functional properties of large anatomically sized constructs in a variety of tissue engineering systems.[46]

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Disclosure Statement

No competing financial interest exist.

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