ARTICLE

Scaffold-free generation of non-contractile bioengineered cartilage to investigate the effects of inflammatory mediators on human chondrocytes

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ABSTRACT

Current animal models, particularly in rodents, offer insight into Osteoarthritis mechanisms but translational limitations, due to species specific differences with humans, exist. Human cell culture models can be used, however, cell culture on monolayer may not fully replicate the molecular context of native human chondrocytes within a cartilaginous matrix. To address these limitations, we sought to generate bioengineered human cartilage tissues using passaged chondrocytes. To achieve this, passaged human chondrocytes were seeded within three-dimensional (3D) agarose molds and stimulated to redifferentiate by exposure to TGF-β3. While TGF-β3 upregulates cartilage-specific matrix molecule aggrecan (ACAN) and type II collagen (COL2A1) mRNA levels, TGF-β3 also enhances the contractile phenotype of cells which led to tissue shrinkage. To prevent tissue shrinkage, we targeted actin stress fibers by supplementing media with actin polymerization inhibitor, latrunculin A, which prevented contraction for up to 10 days. To prevent contraction after 10 days, we treated cells with Rho-associated coiled-coil kinase (ROCK) pathway inhibitor, Y-27632. This combination effectively reduced passaged cell mediated contraction preserving tissue integrity. Next, to determine if cytokine treatment led to cartilage degradation in bioengineered constructs, we exposed tissues to the inflammatory cytokine, interleukin-1 beta (IL1β). IL-1β reduced chondrogenic molecule and increased expression of matrix-degrading enzyme mRNA levels. These mRNA level alterations were accompanied by diminished ACAN staining and increases in staining for matrix metalloproteases-13, indicating a shift toward a matrix catabolism. Non-contractile bioengineered cartilage may be suitable to investigate the response of human chondrocytes to inflammatory mediators to provide further insights into human OA progression.

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INTRODUCTION

Inflammatory mediators play a central role by regulating cartilage degradation in the pathogenesis of osteoarthritis (OA) (Berenbaum, 2013, Scanzello and Goldring, 2012, Rahmati et al., 2016). Small animal models, particularly rodents, have long been used to study OA pathogenesis, employing a variety of approaches including surgical, chemical and genetic models to investigate joint degeneration and inflammation (Glasson et al.,

KEYWORDS

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2007, Little and Hunter, 2013, Skioldebrand et al., 2018, Chan et al., 2023). While these models allow for the study of joint degeneration, the complexity of *in vivo* systems makes it difficult to study the direct effects of inflammatory mediators on chondrocytes alone due to other changes to the mechanical environment and cross-talk with other joint cell-types. In addition, *in vivo* models may not fully replicate human OA due to interspecies differences (Christiansen et al., 2015). Over 90% of potential OA drugs that succeed in animal models fail in human clinical trials (Marshall et al., 2023), suggesting perhaps that interspecies differences may contribute to the limited translational success of OA therapeutics.

To gain a greater understanding of the inflammatory mechanisms driving cartilage degradation in human OA, animal models could be complemented with studies involving human cells, ideally in cellular contexts that are similar to the native *in vivo* condition. It is possible to investigate the effect of inflammatory cytokines on chondrocytes by treatment of *ex vivo* cartilage explants or osteochondral cores. However, *ex vivo* tissue is often derived from aged or osteoarthritic donors, limiting their use in modeling complete disease pathogenesis. Moreover, tissue availability can pose additional challenges (Li et al., 2021, Geurts et al., 2018). Finally, cell viability, especially at the cartilage surface in the superficial zone, steadily declines for cultured osteochondral or cartilage tissue (Pallante et al., 2009).

While *in vitro* mechanistic studies are often conducted on two-dimensional (2D) monolayer cultures of chondrocytes, chondrocytes in culture undergo rapid dedifferentiation (Parreno et al., 2017a). The use of monolayer expanded (passaged) chondrocytes is problematic as chondrocytes dramatically shift toward a fibroblast-like phenotype in the process of chondrocyte dedifferentiation (Schofield et al., 2024, Rzepski et al., 2025, Benya and Shaffer, 1982). Chondrocyte dedifferentiation results in a dramatic alteration in chondrocyte morphology when removed from native matrix causing a transition from a rounded to a flattened, elongated shape, increased actin stress fiber formation (Parreno et al., 2014). This coincides with a loss of chondrogenic gene expression and an upregulation of fibroblast matrix and contractile molecule expression (McCoy, 2015, Dell'Accio et al., 2001, Parreno et al., 2017b). The response of dedifferentiated chondrocytes may differ from that of primary cells. Furthermore, while minimally cultured primary cells could be used, they are in 2D monolayer and establish cell polarity. The response of chondrocytes to inflammatory mediators is context-dependent—cytokine and growth factor effects vary depending on culture conditions such as 2D versus three-dimensional (3D) environments (Woods and Beier, 2006).

3D culture of chondrocytes in hydrogels (i.e. agarose, alginate, etc.) may better mimic the native cartilage 3D environment by suppressing dedifferentiation - maintaining chondrocyte morphology and limiting fibroblastic gene expression. Passaged human chondrocytes remain responsive to inflammatory cues such as interleukin-1 beta (IL-1β) when cultured in 3D systems, supporting their use for investigations on osteoarthritis (OA) mechanisms. Several studies have demonstrated that IL-1β treatment of scaffold or pellet cultures derived from passaged human articular or nasal chondrocytes induces glycosaminoglycan loss and upregulation of matrix metalloproteinases (MMP-1, MMP-2, and MMP-13), mimicking features of cartilage matrix degradation observed in OA (Scotti et al., 2012, Choi et al., 2004). Previous studies further confirmed the inflammatory responsiveness of passaged chondrocytes in alginate bead cultures, noting OA-like changes in response to cytokine challenge (Skioldebrand et al., 2018). A limitation of hydrogels is the surrounding matrix does not fully replicate native cartilage composition which is rich in type II collagen (COL2A1) and aggrecan (ACAN) (Benya and Shaffer, 1982). Additionally, hydrogels can influence the response to cytokines. For instance, it was demonstrated that sulfated alginate hydrogels attenuate IL-1β induced alteration in gene expression (Abbas et al., 2017).

A model system using human cells within a 3D cartilaginous matrix that more closely recapitulates native cartilage structure and composition may offer improved translational relevance for OA research. We previously demonstrated that culturing passaged bovine chondrocytes in 3D within an adherent agarose mold promotes redifferentiation, characterized by re-expression of cartilage-specific markers (COL2A1, ACAN), suppression of fibroblastic and contractile genes, and restoration of a rounded morphology (Davis et al., 2024, Parreno et al., 2018). Building on this work, our current study will employ this scaffold-free culture system to redifferentiate passaged human chondrocytes, enabling endogenous matrix production. Once adequate matrix is produced we intend to examine the cytokine-driven responses in the human-derived, physiologically relevant cartilaginous constructs. The matrix produced in these constructs is rich in COL2 and ACAN, key components of native cartilage ECM that may better support the physiological chondrocyte responses.

However, bioengineering with human chondrocytes presents challenges—the production of a suitable cartilaginous matrix by passaged chondrocytes requires the use of growth factors (Parreno et al., 2018). Exposure of passaged chondrocytes to biochemical mediators such as transforming Growth Factor-β (TGF-β; specifically isoforms -1, -2, or -3) is essential for redifferentiation and matrix production in passaged human chondrocytes (Bianchi et al., 2017), but TGF-β isoform treatment also alters the actin cytoskeleton. TGF-β1 activates the RhoA/ROCK signaling pathway, which drives stress fiber formation and increases cytoskeletal tension (Massague, 2012, West et al., 2024, Parreno et al., 2008). Similar effects have been observed in chondrocytes, where TGF-β1 signaling enhances actin polymerization and increases cellular stiffness and contractile gene expression (Leipzig et al., 2006). While much of the literature focuses on the role of TGF-β1 in cytoskeletal remodeling and contraction, studies also indicate that TGF-β3 also modulates the actin cytoskeleton and cellular contractility, in part through activation of the RhoA/ROCK pathway (Kaartinen et al., 2002, Melchionna et al., 2021). This cytoskeletal contraction via the Rho/ROCK pathway leads to tissue compaction and deformation (Piersma et al., 2015, Parreno et al., 2008), which is not ideal for modeling OA. Contracted tissues have altered geometry and are dense limiting nutrient and drug diffusion (Lima et al., 2007). Cultures of contracted passaged chondrocytes in pellets can also lead to increased cellular death (Rayat et al., 2025). In addition, contraction itself may induce a catabolic response. Contraction has been shown to increase MMP1 and MMP3 expression in various cell types, including human osteoblastic MG-63 cells (Parreno et al., 2008, Parreno and Hart, 2009) and porcine fibroblasts (de Hemptinne et al., 2008). Contraction may confound the response of chondrocytes to inflammatory cytokines.

We hypothesize that non-contractile human tissue engineered scaffold free constructs can be used to model OA pathogenesis by induction of a catabolic phenotype with inflammatory cytokines, such as IL-1β, a cytokine known to induce matrix degradation (Goldring and Marcu, 2009). These systems offer a promising method to recapitulate specific molecular events in OA progression while overcoming the limitations of species differences, cellular dedifferentiation, and contraction.

METHODS

Cell Isolation and Culture

Primary human chondrocytes were obtained from StemBioSys (San Antonio, TX, USA). These chondrocytes were harvested from healthy cartilage of the knee from female and male patients ranging from the age of 19 to 28. The cells were expanded as previously described (Parreno et al., 2016). Briefly, the primary chondrocytes were seeded on plastic tissue culture flasks (Cat# 25–111; GenClone; El Cajon, CA, USA) at a concentration of 2.0×10^3 cells/cm^2. Cells were maintained in 'expansion media' which consist of high glucose Dulbecco's Modified Eagle's Medium (DMEM) (GenClone) supplemented with 20% Fetal Bovine Serum (GenClone) and 1x antibiotic-antimycotic (MilliporeSigma, Burlington, MA, USA). Media was replenished every 23 days. Once cells reached 70-80% confluency, they were released from culture flasks using 0.25% Trypsin (GenClone) and deemed Passage 1 (P1). P1 cells were repelleted and seeded at a concentration of 2.0×10^3 cells/cm^2 onto new culture vessels and grown 4 days past confluency. Once detached from culture vessels, using Trypsin, cells were considered Passage 2 (P2).

Primary bovine chondrocytes were used in preliminary experiments to investigate the general response of P2 chondrocytes to latrunculin A and Y-27632. For these experiments, knee joints from freshly slaughtered skeletally mature cows were obtained from a local slaughterhouse. Articular cartilage was collected from the femoropatellar groove with a sterile scalpel, washed in PBS containing antibiotics, minced into ~2 mm pieces, and digested overnight at 37 °C in growth medium with 1.5 mg/mL collagenase type II (Invitrogen/Gibco). The cell suspension was filtered (100 µm), centrifuged at 200 g for 5 min, washed with PBS, and resuspended in growth medium (Schofield et al., 2024).

Adherent agarose mold cultures (adAM) culture

Human P2 cells were redifferentiated and stimulated to form matrix in 3D adherent agarose mold (adAM) cultures as previously described (Parreno et al., 2018, Davis et al., 2024, Parreno et al., 2016). Briefly, 3ml of 2% agarose (Apex, Scottsdale, Arizona) in Ham's F12 (Sigma–Aldrich, St. Louis, MO) was added to 12-well plates (Falcon; Glendale, AZ, USA). Once the agarose cooled to form a gel, an 8mm biopsy punch (Precision Medical Devices; Northampton, PA, USA) was used to remove a core from the center of each well to create an agarose mold. Approximately $2x10^6$ human P2 cells were seeded within the agarose mold to achieve a seeding density of 3.98×10^4 cells/mm^2. Cells were maintained in DMEM (GenClone, 25-500) with 20% FBS (GenClone, 25550H) and 1% anti-mycotic/-biotic (Cytiva, Wilmington, DE, USA). After 5 days, media was replaced with redifferentiation media containing DMEM supplemented with 1× ITS+ (insulin transferrin-linoleic acid; BD Bioscience, MA), proline (40 mg/ml), pyruvate (110 mg/ml), dexamethasone (0.1 mM), and ascorbic acid (50 mg/ml) (DMEM + ITS). Cells were maintained for the first 2 days following seeding in expansion media containing 20% FBS with 0.25µM Latrunculin A (Cayman, Ann Arbor, Michigan, USA). After 2 days, media was replenished with expansion media in the absence of Latrunculin A. On day 5, cells were switched to redifferentiation media and simultaneously treated with TGF-β3 (R&D Systems, Minneapolis, MN, USA), which continued until day 15. Redifferentiation media was maintained throughout the remainder of the culture period. On day 8, 20µM Y-27632 (Tocris, Minneapolis, MN, USA) was added to prevent later contraction. The media conditions used in our study are shown in Table 1.

Culture of human cartilage constructs in inflammatory conditions

Stable cartilage constructs were generated by Day 15 using our protocol, prior to the addition of IL-1β. After 15 days of culture, media was replaced with fresh Redifferentiation media containing IL-1β (Millipore Sigma, SRP3083; Burlington, MA, USA) in the absence of TGFβ3 and contractile inhibitors (latrunculin A or Y27632). Two days following initial IL-1β treatment, the spent media was replaced with fresh media containing 5ng/mL IL-1β. On day 20 of culture (5 days of IL-1β treatment), tissues were harvested for RNA extraction and immunohistochemistry.

Table 1: Media conditions used in redifferentiation study

Culture Day	Media Type (%)		Supplement
	Expansion	Redifferentiation	
0	100		Latrunculin A
1			
2			
3	100		-
4			
5		100	TGF-β3
6			
7			
8		100	TGF-β3+Y-27632
9			
10		100	TGF-β3+Y-27632
11			
12		100	TGF-β3+Y-27632
13			
14			
15		100	IL-1β (5 ng/mL)
16			
17		100	IL-1β (5 ng/mL)
18			
19		100	IL-1β (5 ng/mL)
20			

Live/Dead Cell Viability Assay

Viability of bovine passaged chondrocytes cultured on 2D glass-bottom dishes following cytoskeletal inhibitor treatments was assessed at Day 2 using a Live/Dead assay (Figure S1). Cells were treated with 0.25 µM latrunculin A, 2.5 µM latrunculin A, 20 µM Y-27632, or kill control. Live cells were stained with Viafluor 488, nuclei with Hoechst, and dead cells with NucFix™ according to the manufacturer's (Biotium; Fremont, CA, USA) instructions. The kill control was generated by treating cells with methanol for 15 min prior to staining. Visualization of cells was performed on a Zeiss 880 laser scanning confocal microscopy (Zeiss, White Plains, New York, USA).

Of note, our preliminary studies, using bovine P2 chondrocytes determined that P2 chondrocytes are tolerant of short-term exposure to Latrunculin A up to 2uM (Figure S1). In addition, P2 chondrocytes are also tolerant to exposure of Y-27632. In a previous study, it was found that P2 chondrocytes treated with 50uM of Y-27632 were viable (Schofield et al., 2024).

RNA Extraction, Reverse Transcription, and Semi-Quantitative / Relative Polymerase Chain Reaction

RNA was extracted from cells using TRIzol (Sigma-Aldrich) followed by phase separation by adding chloroform. RNA was purified using Zymo Research RNA clean and concentrator Kit (Zymo Research; Irvine, CA, USA). Following RNA extraction, the RNA was then dissolved in molecular-grade water (Zymo Research) and reverse transcription into cDNA was performed using the UltraScript 2.0 cDNA Synthesis Kit according to manufacturer's directions (PCR Biosystems; Wayne, PA, USA).

Relative real-time RT-PCR was performed using 20ng per reaction of cDNA and qPCRBio Sygreen Blue Mix (PCR Biosystems) with gene-specific primers on a Cielo 3 real-time PCR machine (Azure, Dublin, CA, USA). The primers used were previously validated with primer sequences listed in Table below. mRNA levels were calculated using the ΔΔCT method (Schmittgen and Livak, 2008), with 18S serving as the normalization control.

Table 2: Primer sequences used for real-time RT-PCR

Gene	Species	Forward Primer (5'−3')	Reverse Primer (5'−3')	Accession Numbers
18S	Bovine, Human	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	NR_036642.1
ACAN	Human	TGGGACTGAAGTTCTTGGAGA	GCGAGTTGTCATGGTCTGAA	NM_001411096.1
COL1A1	Bovine, Human	CGGCTCCTGCTCCTCTTAG	CACACGTCTCGGTCATGGTA	NM_001034039.1
COL2A1	Human	GTGTCAGGGCCAGGATGTC	GCAGAGGACAGTCCCAGTGT	NM_001844.5
MMP2	Bovine, Human	GGTTACCCCAGACAGGTGAA	CCAGATCAGGTGTGTAGCCA	NM_174745.2
MMP13	Bovine, Human	CTTGTTGGTCTCTGCCCCTT	CTGCCAGTCACCTCTAAGCC	NM_002427.4

Immunocytochemistry

P2 human cells seeded on glass dishes (World Precision Instruments, Sarasota, FL, USA) at a density of 2×10^4 cells per dish. After an overnight incubation they were treated with TGF-β3 in 20% FBS expansion media. To visualize αSMA within the cytoplasm and within F-actin, cells seeded on glass dishes were fixed using 4% paraformaldehyde (PFA) in PBS. Cells were placed in permeabilization/blocking buffer (3% Goat serum, 3% BSA, and 0.3% Triton in PBS). After 15 minutes, cells were incubated in a primary antibody solution containing rabbit anti-αSMA (1:100, ab7817, Abcam) in permeabilization/blocking buffer at 4°C. After an overnight incubation, cells were washed three times in PBS for 5 minutes per wash. Cells were then incubated in secondary antibody solution which contained goat anti-rabbit CF488 (1:200, Biotium), Rhodamine phalloidin (1:50; Biotium) to stain F-actin, and Hoechst 33342 (1:500; Biotium) for visualization of nuclei. After 1 hour at room temperature, cells were washed three times in PBS for 5 minutes per wash.

Cells were mounted using Drop-n-stain Everbrite anti-fade reagent (Biotium) and imaged on a Zeiss LSM880 confocal microscope. Confocal images were processed using ZEN Blue.

Immunohistochemisty

3D tissues cultured to 20 days were fixed in 4% PFA overnight in the fridge. Following fixation, tissues were placed in sucrose overnight in the fridge. Tissues were then embedded into Optimal Cutting Temperature compound (OCT; Sakura, Tissue-Tek, Torrance, CA, USA) and frozen. Frozen blocks were sectioned to 10µm sections using a Leica CM3050 cryostat onto charged glass slides (Premiere; Rochester, NY, USA). Slides were then prepped for immunostaining by placing in PBS for 10 minutes to dissolve excess OCT. Sections were then incubated in permeabilization/blocking buffer (3% Goat serum, 3% BSA, and 0.3% Triton in PBS) for 30 minutes.

For visualization of specific proteins, primary antibodies (Table 2) diluted in permeabilization/blocking buffer overnight at 4°C After three washes in PBS, cells were incubated for 1 hour at room temperature in a secondary antibody solution containing either CF568conjugated anti-rabbit IgG (1:500; Biotium, Fremont, CA) or CF647-conjugated anti-mouse IgG (*1:500; Biotium), depending on the primary antibody species. Hoechst 33342 (1:500; Biotium) was includ*ed in the same solution for nuclear staining.

Table 3: Antibodies used in study.

Protein	Company	Catalog	Host Species	Concentration
COL2	Millipore	AB761	Rabbit	1:100
COL1	Abcam	NBP1-30054	Rabbit	1:100
ACAN	Abcam	ab34710	Mouse	1:500
MMP1	Proteintech	10371-2-AP	Rabbit	1:100
MMP3	Abcam	ab52915	Rabbit	1:100
MMP13	Abcam	ab84594	Rabbit	1:100

Image Analysis and Quantification
Images were acquired using a LSM880 confocal microscope on a 20x objective lens at 1.0x zoom. Laser excitation and detection settings were kept constant across all samples within a set to allow for comparative image quantification. Images were processed using ZEN 2.6 (Blue Edition, Carl Zeiss) software. Post-acquisition image adjustments included contrast enhancement applied uniformly across all images. Where appropriate, maximum intensity projections were generated from z-stacks. Quantification of fluorescence intensity, cell morphology, or marker expression was performed using FIJI ImageJ (National Institutes of Health (NIH), Bethesda, Maryland, USA).

Statistical Analysis
All experiments were repeated a minimum of three times using cells from different donors. Statistical analyses were conducted using GraphPad Prism 9 (San Diego, CA, USA). Data points pooled from multiple experiments were assessed for outliers using the ROUT method, with a maximum false discovery rate (Q) set to 1% (Motulsky and Brown, 2006). Comparisons between two groups were performed using unpaired t-tests. For comparisons involving three or more groups, an analysis of variance (ANOVA) was applied, followed by Dunnett's post hoc test to identify specific differences.

RESULTS

Human passaged chondrocytes exhibit a highly contractile phenotype that is enhanced by exposure to TGF-β3
Leveraging a previous protocol for scaffold-free bioengineering of cartilage-like tissues using three-dimensional cultures of bovine passaged cells within agarose molds (Parreno et al., 2018), we aimed to generate bioengineered cartilage-like tissues using human passaged chondrocytes. In contrast to bovine passaged cells, which can redifferentiated, in the absence of growth factors, it has been previously shown that TGF-β1 is required for matrix deposition by human passaged chondrocytes (Bianchi et al., 2017). Both TGF-β1 and TGF-β3 share similar signaling pathways and biological effects in chondrocytes, including the regulation of matrix production and cell contractility (Massague and Sheppard, 2023). Since it has been shown in other cell types that the contractile potential of cells is enhanced by exposure of cells to TGF-β1 (Weist et al., 2013), we investigated the effect of TGF-β3 on the contractile phenotype of human passaged cells. By exposing P2 human chondrocytes in 2D monolayer to TGF-β3, we determined that TGF-β3 treatment leads to cells that contain αSMA positive F-actin stress fibers (Figure 1A). Furthermore, when passaged human cells were cultured in 3D within agarose molds and stimulated to form tissue in redifferentiation media with TGF-β3, we observed tissues shrinkage within the first five days of culture (Figure 1B). This contrasted with control tissues that were grown in the absence of TGF-β3, which did not contract by 5 or 8 days of culture.

Targeting the Actin Cytoskeleton Mitigates Tissue Contraction

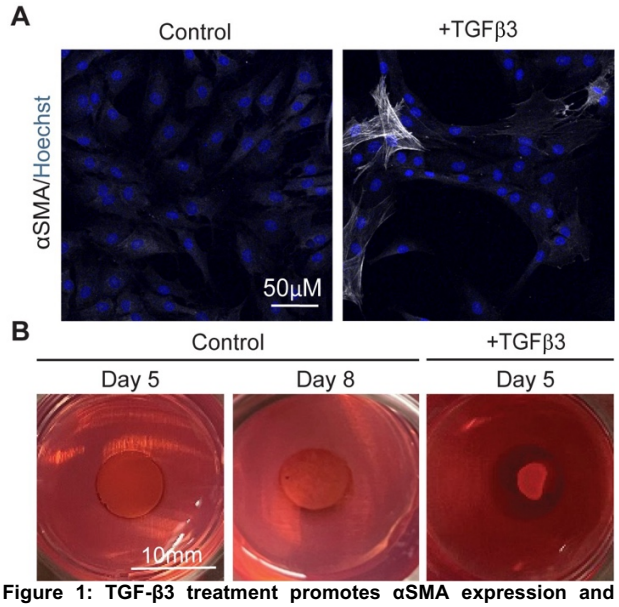

Figure 1: TGF-β3 treatment promotes αSMA expression and enhances tissue contraction. (A) Immunofluorescence staining of αSMA (grayscale) and nuclei (Hoechst, blue) in human chondrocytes cultured in 2D. TGF-β3–treated cells (right) exhibit increased αSMA-positive stress fiber formation compared to untreated controls (left). **(B)** Representative images of scaffold-free 3D constructs showing visible tissue contraction within the first five days of culture following TGF-β3 stimulation.

Due to our findings that human passaged chondrocytes exhibit a highly contractile phenotype when treated with TGF-β3, we next sought to explore strategies to mitigate tissue contraction by targeting actin directly. Latrunculin binds to globular actin preventing actin polymerization (Calaghan et al., 2004) and causes elevated G/F-actin in passaged chondrocytes (Schofield et al., 2024). It was previously shown that exposure of chondrocytes to latrunculin not only prevents contraction but also reduces contractile molecule expression in passaged chondrocytes (Parreno et al., 2017b). Therefore, we targeted actin polymerization using latrunculin A. We found that exposing human P2 chondrocytes in a 3D culture model to latrunculin A at seeding reduces the formation of F-actin stress fibers after one day of treatment (Figure 2A). The one bout of latrunculin A treatment effectively suppressed tissue contraction up to eight days of culture. Consistent with this, latrunculin A treatment also prevents the formation of contractile αSMA-positive F-actin stress fibers, as demonstrated by αSMA and F-actin staining of bovine passaged chondrocytes (Figure S2). Latrunculin represses the formation of stress fibers containing αSMA. The effects of latrunculin A on tissue contraction, however, wore off by 10 days where we found evidence of contraction (Figure 2B).

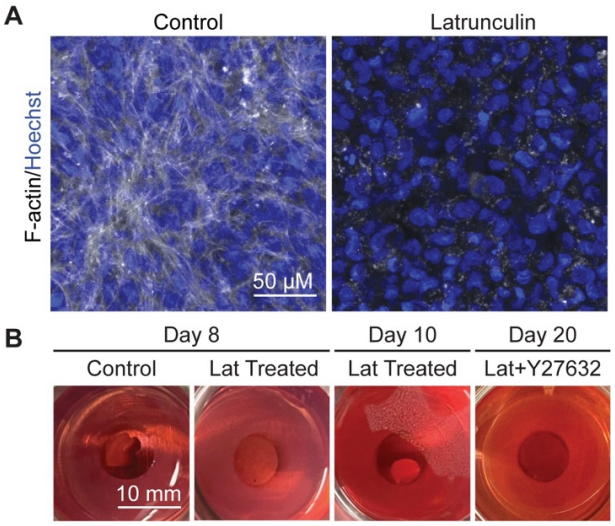

Figure 2: Inhibition of actin polymerization reduces stress fiber formation and delays tissue contraction. (A) Immunofluorescence images of cells in 3D culture after 1 day of growth with or without 0.25 mM latrunculin A (Lat) treatment. Lat-treated cells exhibit reduced phalloidin staining (grayscale) and appear more rounded compared to controls. Nuclei are stained with Hoechst (blue). **(B)** Representative images of 3D constructs over time. Control constructs show tissue contraction by day 8 (left), while Lat-treated constructs remain uncontracted through day 8. Contraction reappears by day 10 after Lat removal. Continuous treatment with 10mM Y-27632 from days 8–15 following Lat treatment prevents contraction through day 20. Latrunculin was applied for the first 48 hours of culture.

Prolonged exposure to latrunculin A can disrupt cortical actin and impair critical cellular functions such as protein export (Spector et al., 1989, Yamazaki et al., 2005). Therefore, we limited the use of latrunculin A to the early, contraction-prone phase of culture. Our preliminary studies indicate that short-term treatment with 0.25 μM or 2.5 μM latrunculin A, or 20 μM Y-27632, did not affect cell viability at Day 2 (Figure S1). To maintain tissue integrity beyond this point, we targeted ROCK signaling, a downstream effector of RhoA known to regulate actomyosin contractility through phosphorylation of myosin light chain (MLC). ROCK inhibition has been shown to reduce cellular tension and suppress stress fiber formation (Schofield et al., 2024). We exposed constructs beginning on day 8 to the ROCK inhibitor Y27632. We found that supplementing media with Y27632 from days 8-15 prevented contraction up to 20 days (Figure 2B).

TGF-β3 Enhances Tissue Formation in Human Chondrocyte Cultures

Next, we investigated the tissue formation ability of chondrocytes in cultures up to 20 days. While bovine chondrocytes formed tissues without TGF-β3, human chondrocytes require TGF-β3 for successful tissue formation (Bianchi et al., 2017). We found that in the absence of TGF-β3, tissues are thin and showed limited matrix formation (Figure 3A). Tissues were not stable, and when removed from agarose molds using forceps, they did not hold form. In contrast, TGF-β3 treatment resulted in thicker tissues. When tissues exposed to TGF-β3 were removed from agarose molds, they can be easily held by forceps and maintain their form (Figure 3A). RT-PCR analysis revealed that TGF-β3 treatment significantly upregulates cartilage matrix molecules, COL2A1 and ACAN, mRNA levels (Figure 3B). Immunostaining demonstrated that TGF-β3 increases COL2A1 and ACAN deposition at days 15 and 20 compared to untreated controls (Figure 3C). While the related isoform, TGF-β1, is known to increase COL1 expression in fibroblast, myoblast and cardiac fibroblast cells (Hillege et al., 2020, Pan et al., 2013), we did not see evidence for substantial COL1 staining in either untreated or TGF-β3-treated tissues.

Use of scaffold free human cultures to investigate inflammatory effects on cartilage homeostasis

By day 15, the tissues formed by the human passaged chondrocytes are cartilaginous. To investigate the effect of inflammatory cytokines on our human bioengineered cartilage system, we exposed cells within our bioengineered constructs to 5ng/mL IL-1β between days 15-20. Using RT-PCR, we observed that IL-1β decreases the expression of the cartilage matrix molecules *COL2A1* and *ACAN*. While increasing the expression of matrix-degrading enzymes, *MMP-2* and *-13* (Figure 4A). Immunofluorescent analysis of cartilage sections reveals a reduction in ACAN with little effect on staining for COL2A1 (Figure 4B). Immunostaining for MMP-13, reveals greater staining in IL-1β–treated tissues. In addition to MMP-13, we also stained sections for MMP-1 and MMP-3. As compared to MMP-13 staining, MMP-1 and MMP-3 staining were less impacted by treatment with IL-1β (Figure S3). In general, these findings suggest a catabolic shift in chondrocyte phenotype within bioengineered construct, impacting the expression of specific matrix and matrix degrading enzymes.

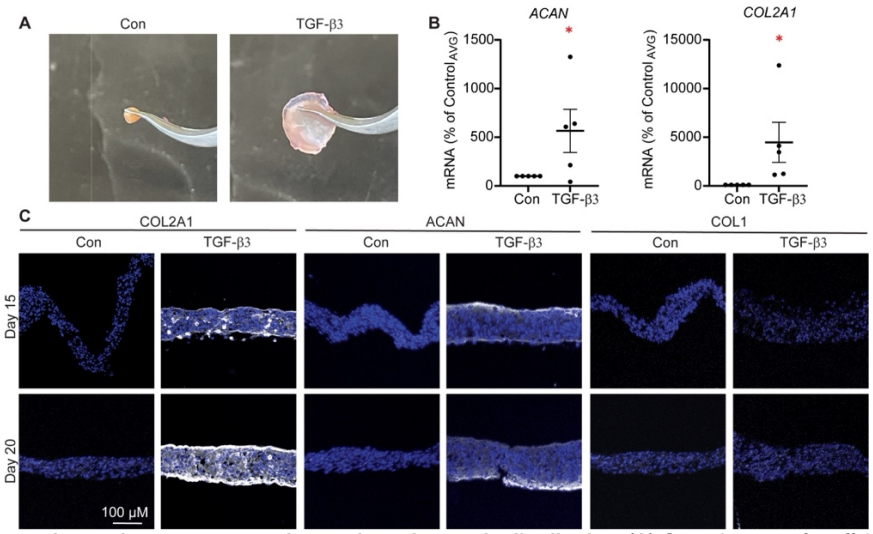

Figure 3: TGF-β3 influences tissue size, gene expression, and matrix protein distribution. (A) Gross images of scaffold-free 3D constructs after 20 days of culture. Constructs without TGF-β3 (left) appear smaller than those cultured with TGF-β3 (right). **(B)** RT-qPCR analysis showing relative expression levels of *ACAN* and *COL2A1* in control and TGF-β3–treated tissues. Statistical significance was determined using one-way ANOVA. $p < 0.05$ is indicated by *. (C) Immunofluorescence staining of tissue sections collected on days 15 and 20 for type II collagen (COL21A), aggrecan (ACAN), and type I collagen (COL1). All matrix markers are shown in grayscale; nuclei are counterstained with Hoechst (blue).

DISCUSSION

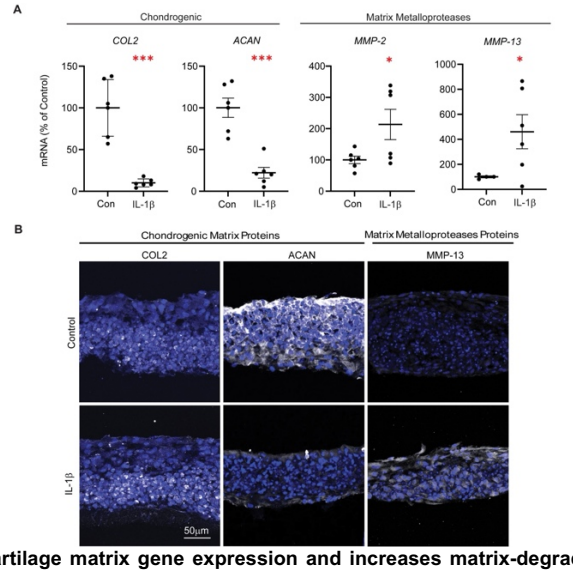

Figure 4: IL-1β treatment decreases cartilage matrix gene expression and increases matrix-degrading enzyme expression. (A) RT-qPCR analysis of *COL2A1*, *ACAN*, *MMP-2*, and *MMP-13* expression in 3D constructs with or without IL-1β treatment. IL-1β significantly reduces *COL2A1* and *ACAN* expression while increasing *MMP-2* and *MMP-13*. Statistical significance was determined using one-way ANOVA. $p < 0.05$ is indicated by *, and $p < 0.001$ is indicated by ***. **(B)** Immunofluorescence staining of tissue sections for COL2A1, ACAN, and MMP-13. All matrix and enzyme markers are shown in grayscale; nuclei are counterstained with Hoechst (blue).

In this study, we developed a methodology to generate stable cartilaginous tissue from passaged human chondrocytes for evaluating the effects of inflammatory cytokines. To achieve this, we first targeted cell-mediated contraction of tissue by sequential application of actin targeting pharmacological agents, latrunculin A and Y27632. By targeting the actin cytoskeleton, we enabled TGF-β3-driven redifferentiation and matrix production from passaged human cells, resulting in engineered tissues that respond to the inflammatory cytokines IL-1β resulting in a catabolic phenotype.

Transforming Growth Factor-β (TGF-β) was required for matrix formation in our human chondrocyte model, consistent with previous studies, which used TGF-β1 (Bianchi et al., 2017) showing its critical role in promoting redifferentiation and cartilage matrix synthesis. In contrast, previous studies using bovine chondrocytes have reported matrix formation in the absence of TGFβ3 and -β1, likely due to species or age-related differences. The bovine chondrocytes used in our previous study were harvested from young animals (6–9 months), whereas our human donors ranged from 18–28 years old. In addition to its role in matrix synthesis, TGF-β1 is known to enhance the contractile potential of cells by activating RhoA/ROCK signaling, which promotes stress fiber formation and may contribute to construct deformation in engineered tissues (Hsieh et al., 2016).

To mitigate contraction, we targeted the actin cytoskeleton. Actin has also been targeted for the generation of bioengineered cartilage using passaged sheep chondrocytes, where cells cultured within non-adherent agarose molds were exposed to 2 μM cytochalasin D for 3 days (Huang et al., 2018). In conjunction with the application of a deadweight onto the constructs, the passaged sheep cells produced stable cartilage tissue. In our preliminary experiments, we found that cytochalasin D was not effective in preventing contraction (Figure S4). A 2-day treatment was insufficient to prevent contraction under our conditions. This discrepancy may be due to differences in our culturing protocols. First, our protocol did not use a deadweight, which may confer additional benefit in preventing contraction. Second, our use of human chondrocytes instead of sheep chondrocytes may indicate species-specific differences in contractility (Huang et al., 2018). Finally, in the previous studies (Huang et al., 2018), the passaged cells were predifferentiated in 3D aggregates prior to seeding into agarose molds. We suspect that culturing the passaged cells in 3D aggregates suppresses contraction. It was previously have shown that 3D culture of passaged chondrocytes reduces the contractile phenotype of cells by reducing stress fibers and the expression of contractile molecules αSMA and TAGLN (Parreno et al., 2018, Parreno et al., 2017a, Parreno et al., 2014).

In contrast to cytochalasin D, we found in our culture system that latrunculin A effectively suppressed contraction. Latrunculin A may have been more effective than cytochalasin D at suppressing contraction, as it not only ablates stress fibers but also reduces contractile molecule mRNA levels (Parreno et al., 2017b). Cytochalasin D is a known activator of the MyocardinRelated Transcription Factor (MRTF) pathway and has been shown to increase contractile gene expression (Olson and Nordheim, 2010, Miralles et al., 2003, Parreno et al., 2017b). It binds to the barbed ends of F-actin, a region where MRTF-A also interacts with G-actin, and competitively displaces MRTF-A, allowing its nuclear translocation and activation of target genes. In contrast, latrunculin A sequesters G-actin monomers without competing at the MRTF-A binding site, allowing MRTF-A to remain bound and cytoplasmic, thereby attenuating its transcriptional activity (Sotiropoulos et al., 1999).

In addition to treatment with latrunculin, subsequent treatment with ROCK inhibitor Y27632, between days 8 to 15, prevented later stage contraction of tissues. Latrunculin A is a potent agent that disrupts both stress fibers and cortical actin, which can reduce cell viability during prolonged exposure (Konishi et al., 2009) and prevent the proper export of proteins that rely on an intact actin network (Williams and Rousseau, 2022). In addition, extended exposure can impair protein trafficking and matrix secretion, indirectly compromising cell viability (Egea et al., 2006). For these reasons, we limited its use to the initial contraction-prone early phase of redifferentiation culture. After eight days, we used Y-27632 to prevent contraction at later stages of culture. Y-27632 acts by inhibiting Rho-associated kinase, a downstream effector of RhoA, thereby reducing downstream cytoskeletal tension by inhibiting myosin light chain phosphorylation via ROCK inhibition. In bovine P2 chondrocytes, ROCK inhibition led to a dosedependent increase in the G/F-actin ratio, indicating disassembly of filamentous actin structures (Schofield et al., 2024, Rzepski et al., 2025).

This was accompanied by a significant reduction in the expression of contractile genes, consistent with suppression of the dedifferentiated, fibroblastlike phenotype (Schofield et al., 2024). These effects support our use of Y-27632 to attenuate contractility and prevent cell-mediate contraction of our cartilaginous constructs. In preliminary studies, we also tested the inhibition of another RhoGTPase, CDC42 by inhibiting with pharmacological inhibitor ML141, but we did not see the reduction of contraction that we saw with Y-27632.

To determine whether ROCK inhibition alone (as well as CDC42 inhibition alone) could prevent contraction from the start of culture, we applied Y-27632 beginning on Day 1 without latrunculin A. This approach was insufficient to prevent early contraction (Figure S5), demonstrating that early disruption of actin polymerization by latrunculin A is essential to prevent early tissue contraction. Latrunculin treatment was only required for the first two days of culture and later exposure to ROCK inhibitor was adequate to prevent further contraction throughout the remainder of culture.

Both latrunculin A and Y-27632 were used specifically to inhibit cell-mediated contraction and enable the formation of stable bioengineered cartilage constructs. These agents were removed prior to IL-1β stimulation experiments, so we did not directly evaluate the impact of contraction on inflammatory signaling. However, previous studies suggest that cytoskeletal tension can influence inflammatory pathways in chondrocytes (Pepper et al., 2025), indicating that future work should investigate how contraction modulation affects chondrocyte responses during inflammation.

Although TGF-β1 is well documented to induce contraction and a myofibroblast-like phenotype through RhoA/ROCK signaling—including increased αSMA expression—the effect of TGF-β3 on contractility is less understood (Desmouliere et al., 2005). In our study, we observed notable contraction in response to TGF-β3, likely reflecting dedifferentiation and culture-induced changes in chondrocyte behavior. Thus, pharmacological suppression of contraction was employed to stabilize tissue geometry and enhance tissue health, consistency, and physiological relevance.

Our non-contractile cartilaginous human tissues allow for insight into the regulation of chondrocyte catabolism by IL-1β. Contraction of bioengineered tissues may complicate the assessment of the effect of IL-1β on human cartilage tissues. Previously it has been shown that contraction increases expression of matrix metalloproteinases (MMPs), including MMP-1, MMP-3, and MMP-13 in cells (Huang et al., 2018) (Parreno et al., 2008) (Parreno and Hart, 2009, de Hemptinne et al., 2008). In addition, contracted tissues lead to denser tissues that could impair nutrient and drug diffusion and promoting necrotic core formation (Lima et al., 2007, von der Mark et al., 1977). Furthermore, the generation of stable tissues may enable future studies on the functional mechanical effect of IL-1β on tissues (Huang et al., 2016). Therefore, preventing contraction to allow for the generation of stable, flat tissues may serve as a more ideal model to study the inflammatory response in human cartilaginous tissues. It is noteworthy that contraction may be achieved by other cell culture means.

Contraction is also influenced by inadequate adhesion of constructs to the culture surface. Previous studies have used type II collagen-coated plates to improve cell adhesion to stabilize cartilage formation (Bianchi et al., 2017).

In the present study, we evaluated the effect of five day exposure to IL-1β on chondrocyte homeostasis. It would be valuable to gain an understanding on the longer-term effects of IL-1β. While IL-1β treatment significantly decreased COL2A1 mRNA levels (Fig. 4), immunohistochemical assessment showed no corresponding decrease in COL2A1 protein. In a previous study that examined the redifferentiation of bovine cells in adherent agarose molds (Parreno et al., 2018), COL2A1 mRNA levels peak at day 10 and steadily reduced by day 20. In the present study, we evaluated COL2A1 on day 20. Therefore, it is possible that the reduction in COL2A1 mRNA levels may only reflect a small proportion of COL2A1 expressed in cartilage tissue. It would be of interest in future studies to examine if extended IL-1β exposure would lead to a reduction in COL2A1 staining as a result of continued reduction in COL2A1 expression and MMP activity. Our model system will also allow for thorough future investigation on the regulation of expression for other matrix molecules and matrix degradation enzymes by IL-1β on human cells using -omic and sequencing approaches.

This system offers improvements over existing *in vitro* models used to study inflammatory mediators. Unlike traditional hydrogel systems, our scaffold-free engineered cartilage forms cartilaginous matrix consisting of COL2 and ACAN, providing a more physiological context for investigating the effects of cytokines. While more physiologically relevant than hydrogel-based models, the use of bioengineered cartilage alone lacks the complex mechanical and cellular interactions present in native joint tissue, including contributions from synovium, subchondral bone, and immune cells. Therefore, while we are able to examine the isolated effects of cytokines on chondrocytes, the interaction and cross-talk between multiple tissue types cannot be assessed.

This study demonstrates that stable, scaffold-free cartilage constructs can be engineered from passaged human chondrocytes and retain responsiveness to inflammatory stimuli. This study provides a human-relevant model system to study OA pathogenesis. Future studies should explore long-term stimulation conditions, inclusion of mechanical loading, and co-culture with synovial or immune cells to better capture the joint environment. The use of stable, bioengineered cartilage to examine the effects of inflammatory cytokines on chondrocytes may provide novel insights that could be used to develop therapeutics against human OA.

CONCLUSION

We established methodology to generate stable, scaffold-free cartilaginous tissue from passaged human chondrocytes. The generation of stable engineered cartilage derived from human chondrocytes provides a platform to investigate the effects of inflammatory mediators on cartilage degeneration. This system may enable new insights that could inform the development of targeted therapeutics for osteoarthritis.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

SRS, AWS, and JP conceived and designed the study, supervised the project, and contributed to manuscript review and editing. SRS, JP, and DR performed the experiments. SRS and JP analyzed the data and prepared the original draft of the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY INFORMATION

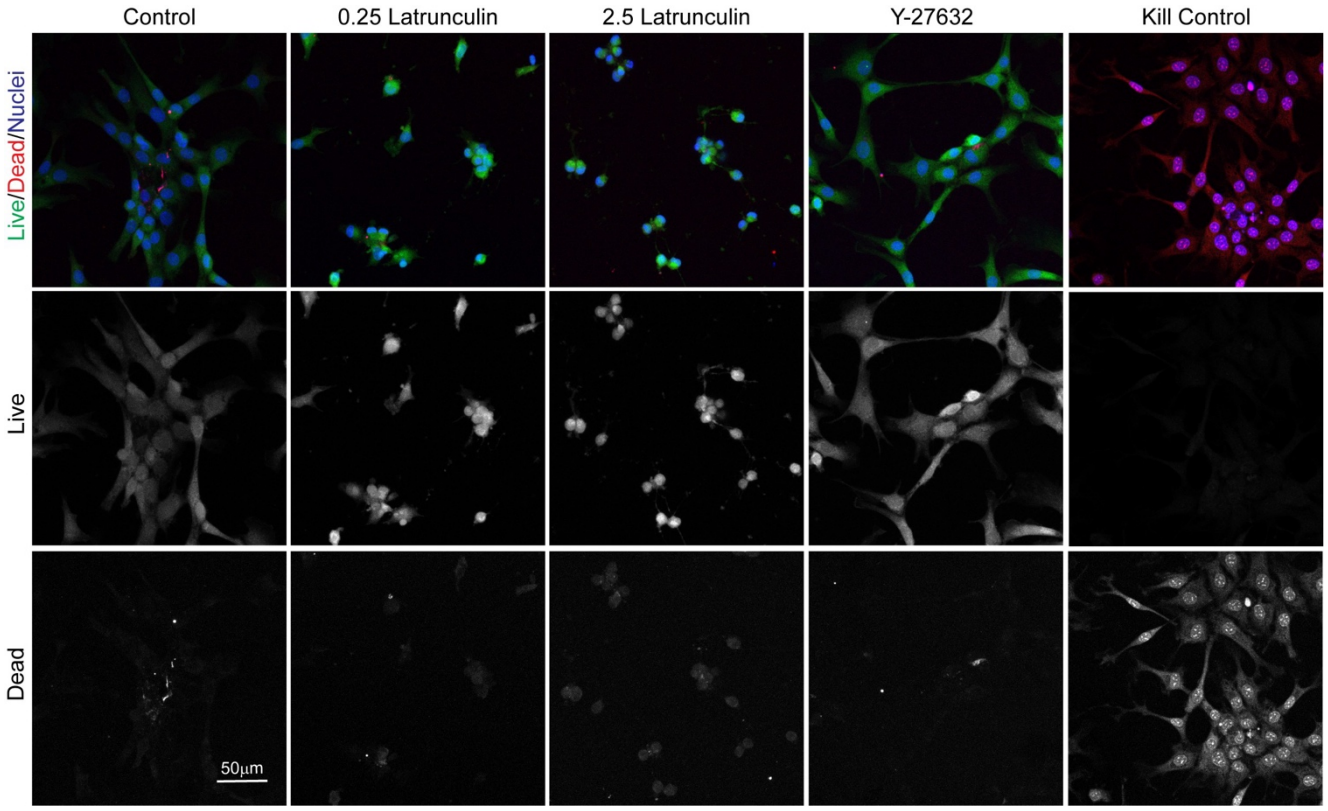

Figure S1: Live/Dead staining of bovine passaged chondrocytes on day 2 of culture following treatment with 0.25 μM latrunculin A, 2.5 μM latrunculin A, or 20 μM Y-27632. Live, dead, and the nuclei of all cells were stained using Viafluor-488 (Green; Biotium), NucFix (Red; Biotium), and Hoechst (Blue), respectively. The kill control cells were fixed in 100% methanol prior to incubating in live-dead stains. Live cells had greater staining for Viafluor488 and dead cell nuclei are positive for staining with NucFix. Note that there is non-specific, non-nuclear staining of live cells with NucFix. All treatments exhibited aside from whereas the kill control were mainly. Scale bar = 50 μm.

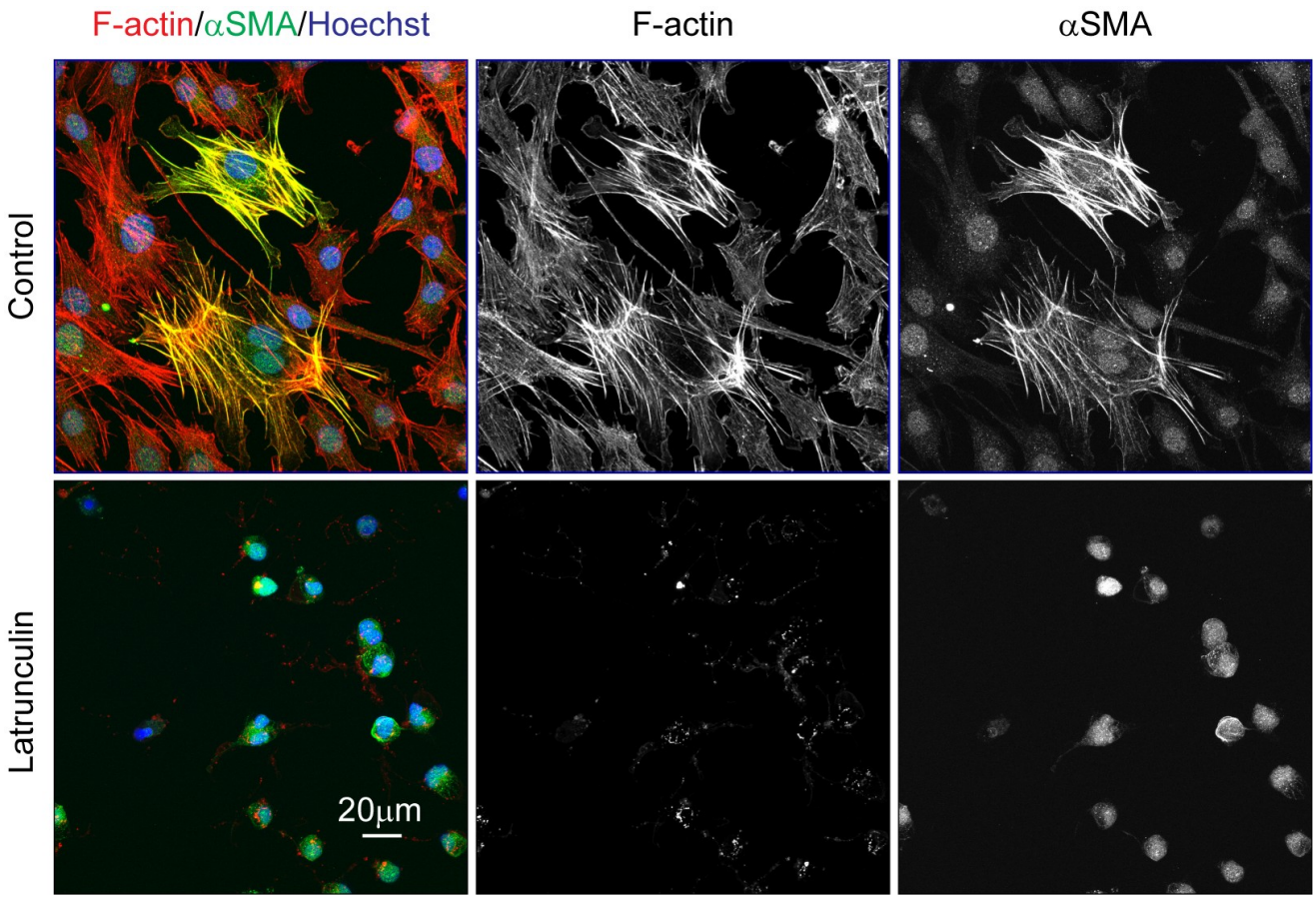

Figure S2: Latrunculin A treatment disrupts F-actin stress fiber organization and prevents formation of contractile αSMA-positive fibers in bovine passaged chondrocytes. P2 cells were seeded in monolayer culture at 2.5 × 10^5 cells per dish and treated with TGF-b3. After 1 day were treated with 0.25 μM latrunculin A. Cells were fixed in 4% PFA, then stained for F-actin (phalloidin; red), αSMA (Alexa Fluor 488; green), and nuclei (Hoechst; blue). Images show merged channels (F-actin, αSMA, nuclei) as well as individual αSMA and F-actin channels in grey scale. Control cells display well organized αSMA-positive F-actin stress fibers, whereas latrunculin A–treated cells fail to form αSMA positive stress fibers. Scale bar = 20 μm.

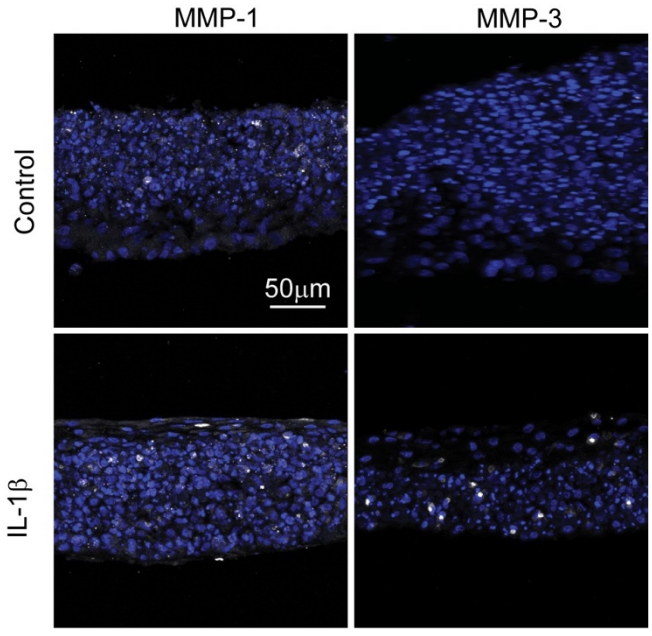

Figure S3: IL-1β treatment does not alter staining for matrix-degrading enzyme expression in engineered cartilage constructs. Immunofluorescence staining of tissue sections for MMP-1 and MMP-3 in constructs treated with or without IL-1β. Both MMP-1 and MMP-3 signals are shown in grayscale; nuclei are counterstained with Hoechst (blue)

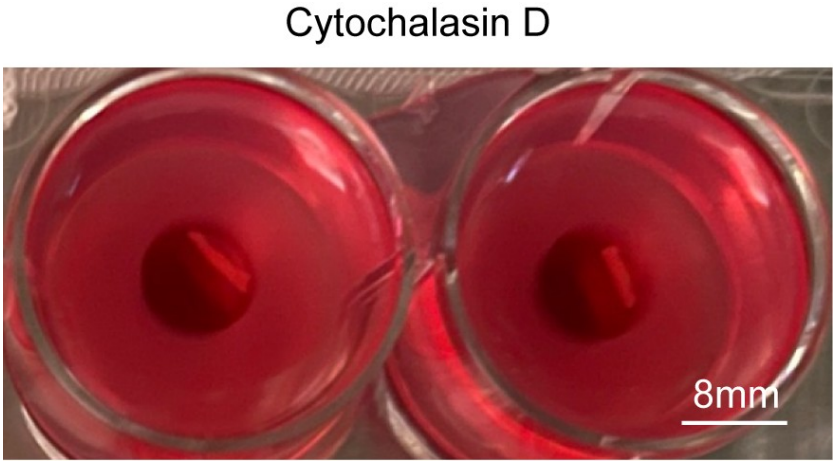

Figure S4: Gross images of bioengineered cartilage constructs treated with 10 µM cytochalasin D on days 0-2. Tissues are contracted by day 14.

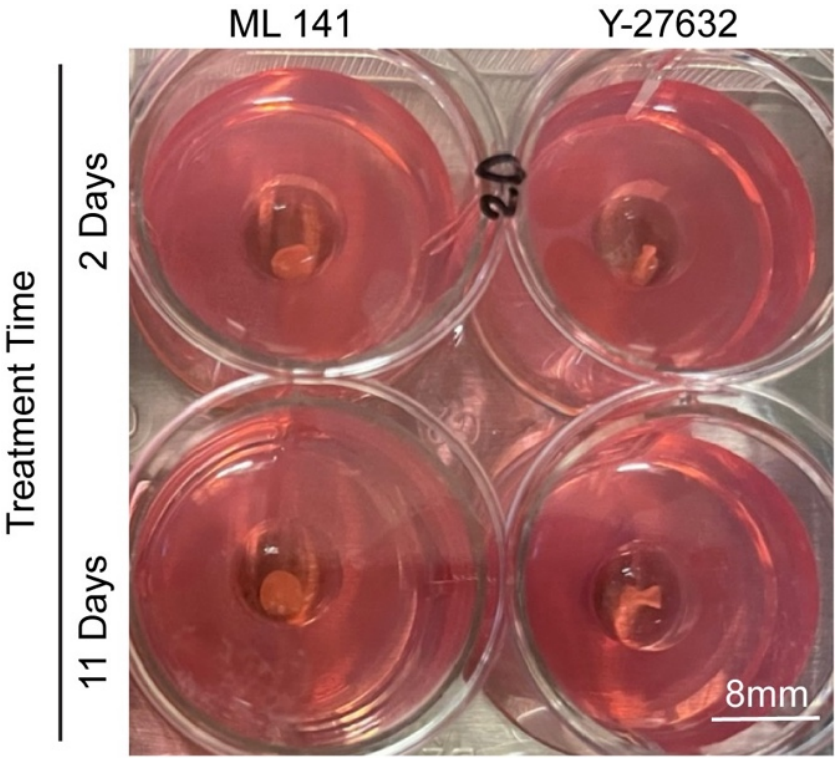

Figure S5: Gross images of human bioengineered cartilage constructs treated with 10mM CDC42 inhibitor (ML141;left) or 10µM ROCK inhibitor (Y-27632;right) at 10 µM. Treatments were for 2 days (top row) or continuously for 11 days (bottom row). All constructs exhibited contraction by 11 days. Scale bar = 8 mm (bottom right).