Title

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Journal

Osteoarthritis and Cartilage, 24(12)

ISSN

1063-4584

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Publication Date

2016-12-01

DOI

10.1016/j.joca.2016.07.006

Peer reviewed

Osteoarthritis and Cartilage 24 (2016) 2126–2134

Osteoarthritis and Cartilage

Tendon and ligament as novel cell sources for engineering the knee meniscus

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ARTICLE INFO

Article history:
Received 15 March 2016
Accepted 20 July 2016

Keywords:
Tissue engineering
Primary cell sources
Meniscus fibrocartilage
Self-assembling process
Knee surgery

SUMMARY

Objective: The application of cell-based therapies in regenerative medicine is hindered by the difficulty of acquiring adequate numbers of competent cells. For the knee meniscus in particular, this may be solved by harvesting tissue from neighboring tendons and ligaments. In this study, we have investigated the potential of cells from tendon and ligament, as compared to meniscus cells, to engineer scaffold-free self-assembling fibrocartilage.

Method: Self-assembling meniscus-shaped constructs engineered from a co-culture of articular chondrocytes and either meniscus, tendon, or ligament cells were cultured for 4 weeks with TGF-β1 in serum-free media. After culture, constructs were assessed for their mechanical properties, histological staining, gross appearance, and biochemical composition including cross-link content. Correlations were performed to evaluate relationships between biochemical content and mechanical properties.

Results: In terms of mechanical properties as well as biochemical content, constructs engineered using tenocytes and ligament fibrocytes were found to be equivalent or superior to constructs engineered using meniscus cells. Furthermore, cross-link content was found to be correlated with engineered tissue tensile properties.

Conclusion: Tenocytes and ligament fibrocytes represent viable cell sources for engineering meniscus fibrocartilage using the self-assembling process. Due to greater cross-link content, fibrocartilage engineered with tenocytes and ligament fibrocytes may maintain greater tensile properties than fibrocartilage engineered with meniscus cells.

Introduction

Meniscus pathology plays an important role in the development of knee osteoarthritis. Increasing evidence suggests a prominent role for the meniscus in load transmission as well as lubrication and nutrition of articular cartilage[1]. Accordingly, a meniscus tear results in an unequal load distribution within the knee joint and abnormal loading of underlying articular cartilage. Further, meniscus injuries are commonly accompanied with catabolic events that initiate inflammatory processes leading to articular cartilage degeneration[2,3]. Unfortunately, the common treatment of meniscectomy increases contact forces on articular cartilage by up to 350%, and often leads to osteoarthritis[1,4]. Thus, total meniscectomy has been abandoned, and partial meniscectomy is also losing favor compared to meniscus repair and replacement[5–7]. Treatment strategies that restore the functional role of the meniscus within the knee are the most promising approaches toward effective management of a meniscus tear and knee osteoarthritis in the future.

Among the options that ensure restoration of meniscus function, tissue engineering stands out as having high potential despite numerous technical challenges. Engineering a meniscus *de novo* has gained increased interest over the last decade[1,8]. Promising results have been reported with both cellular and acellular approaches, as well as with synthetic and natural scaffolds[9,10]. However, tissue engineering of the meniscus faces many hurdles, such as acquiring large numbers of allogeneic or autologous cells, matching scaffold degradation and tissue formation to one another, the poor mechanical properties of many engineered tissues, and generating mechanical anisotropy that mimics the organization of native tissues[1,11].

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http://dx.doi.org/10.1016/j.joca.2016.07.006
1063-4584/

To address these challenges, a scaffold-free tissue engineering approach that uses a co-culture of meniscus cells together with articular chondrocytes has been proposed[12,13]. Termed the self-assembling process[12,14], this technique has demonstrated success in creating scaffold-free constructs that can replicate the geometry and biochemical composition of native tissue[15,16]. In particular, prior work has shown that a 1:1 ratio of articular chondrocytes and meniscus cells produces appropriately shaped meniscus constructs with greater matrix content than constructs engineered with meniscus cells alone[13,15]. Self-assembling meniscus constructs with mechanical properties approaching native tissue values have been reported[17]. Additionally, two different cell types have been used in co-culture toward creating tissue heterogeneity[18]. Yet, in terms of tensile properties, self-assembling tissues have not yet achieved the values that the native meniscus exhibits, which has been reported as having a Young's modulus of 50–150 MPa[1]. Additionally, this technique requires a large number of cells, likely from an autologous source or an allogeneic donor. Additional efforts toward improving the tensile properties and the clinical translatability of self-assembling engineered menisci could represent a key advance in meniscus tissue engineering.

Within musculoskeletal tissues, articular cartilage represents the end of a continuum, demonstrating a hyaline cartilaginous profile rich in glycosaminoglycan (GAG) molecules, while tendon and ligament occupy the other end of this continuum with a more fibrous profile rich in collagen[19]. The meniscus stands at the middle of this continuum, as it combines both hyaline and fibrous characteristics, and thus may be referred to as a fibrocartilaginous tissue. Due to the high collagen content and large number of pyridinoline cross-links (40–60 nmol/g of tissue for tendon and ligament, as compared to 15–30 nmol/g for hyaline cartilage[19]), the tensile properties of tendon and ligament are superior in comparison to other musculoskeletal tissues. The native cells residing in tendon and ligament, known as tenocytes and ligament fibrocytes, respectively, are responsible for maintaining tissue composition, and may potentially be explored in a co-culture model to create tissues with more fibrous biochemical profiles and greater tensile properties. Furthermore, increasing the number of cell types that menisci can be engineered from improves the feasibility of acquiring large numbers of cells and increases clinical translatability.

The objectives of this study were threefold: (1) to evaluate the feasibility of using tenocytes and ligament fibrocytes as compared to the gold standard of meniscus cells in a co-culture model for meniscus tissue engineering, (2) to study whether the incorporation of alternative cell sources in co-culture would result in improvements to the biochemical and biomechanical content of engineered menisci, and (3) to measure pyridinoline cross-links and correlate their presence with the mechanical properties of engineered tissues created from various co-cultures. Meniscus cells, tenocytes, and ligament fibrocytes were harvested from bovine tissues and co-cultured with articular chondrocytes for 4 weeks in self-assembling meniscus-shaped tissues. After culture, constructs were assessed with a range of assays to determine engineered tissue quality. We hypothesized that tenocytes and ligament fibrocytes would be applicable toward meniscus tissue engineering, that tenocytes and ligament fibrocytes would improve the functional properties of engineered tissue, and that pyridinoline content in the various co-cultures would be correlated with tensile properties.

Materials and methods

Media formulation

The serum-free chondrogenic media formulation used throughout the study consisted of Dulbecco's Modified Eagle Medium (DMEM) with 25 mM glucose/GlutaMAX™ (Life Technologies, Carlsbad, CA), 1% v/v penicillin/streptomycin/fungizone (Lonza, Basel, Switzerland), 1% v/v insulin/transferrin/selenium (BD Biosciences, San Jose, CA), 1% non-essential amino acids (Life Technologies, Carlsbad, CA), 100 μg/mL sodium pyruvate (Thermo Fischer Scientific, Waltham, MA), 50 μg/mL ascorbate-2-phosphate (Sigma, St. Louis, MO), 40 μg/mL L-proline (Sigma), 100 nM dexamethasone (Sigma), and 10 ng/mL TGF-β1 (Peprotech, Oak Park, CA).

Isolation of cells

Meniscus, tendon, and ligament tissues were harvested from bovine calf knee joints (Research 87). The inner four-fifths of the meniscus from both hind legs were taken to obtain meniscus cells. The midsections of the long digital extensor and semitendinosus tendons were harvested to obtain tenocytes, while the midsections of the posterior cruciate, patellar, and anterior cruciate ligament (ACL) were harvested to obtain ligament fibrocytes. Extreme care was taken to remove sheath, adipose, and synovial tissue from all specimens. Tissues were minced to pieces roughly 2–4 mm^3 in size, washed with phosphate-buffered saline (PBS), and digested with 0.25% w/v pronase at 7 units/mg (Sigma) for 1 h followed by 0.2% w/v collagenase type 2 at 250 units/mg (Worthington Biochemical, Lakewood, NJ) for 18 h at 37°C/5% CO_2. Digestion solutions contained DMEM, 3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 2% penicillin/streptomycin/fungizone. Digest solutions were strained through a 70 μm filter and washed three times. Cells were then counted and cryopreserved in chondrogenic media with 20% fetal bovine serum and 10% dimethyl sulfoxide (Sigma). Cell viability observed by trypan blue was >95%.

Self-assembly of constructs

After thawing, cells were seeded in non-adherent, ring-shaped agarose molds based on the rabbit meniscus. Each mold was saturated with chondrogenic media for 48 h before seeding. Wells were 9.5 mm long and 7 mm wide, and seeded with 5×10^6 articular chondrocytes combined with either 5×10^6 meniscus cells, tenocytes, or ligament fibrocytes within a volume of 180 μL. For comparison, a group of constructs seeded with 10×10^6 articular chondrocytes was cultured in parallel. Constructs were left undisturbed for 4 h before feeding with chondrogenic media, and then fed each day thereafter. At 7 days of culture, constructs were removed from their agarose wells and kept in free-floating culture. Each ring-shaped construct could be cut into two halves to represent the two compartments of the native meniscus. Following 4 weeks of culture, constructs were portioned for histological staining, biochemical assays, and biomechanical testing.

Histology and immunohistochemistry

Tissue samples were cryoembedded in HistoPrep (Thermo Fisher Scientific) and sectioned to 12 μm. After formalin fixation, sections were stained with Safranin-O/Fast Green or Picrosirius Red. To stain collagens type I and II, Vectastain ABC kits (Vector Labs, Burlingame, CA) were used. Sections were incubated with mouse anti-collagen type I antibody diluted 1:1000 (Accurate, Westbury, NY) or rabbit anti-collagen II antibody diluted 1:300 (Cedarlane Labs, Burlington, NC). Bovine articular cartilage, meniscus, and tendon served as staining controls.

Biomechanical analysis

For tensile testing, dog bone-shaped samples were created with a scalpel and 3 mm biopsy punch. Samples were photographed, glued to paper tabs, and subjected to uniaxial strain using a 22 N load cell (TestResources, Shakopee, MN). Testing was performed in both the circumferential and radial directions of the ring-shaped constructs. A strain rate of 1% gauge length per second was used. Gauge lengths were 2.33 mm for circumferential samples and 1.33 mm for radial samples. Cross-sectional areas were calculated from images of samples using ImageJ software (NIH, Bethesda, MD). Young's modulus and ultimate tensile strength were obtained from the linear region and maximum stress of the strain curve, respectively.

For compressive testing, a 3 mm diameter tissue sample was subjected to unconfined incremental stress-relaxation testing in a PBS bath at room temperature. Sample heights were determined by lowering a platen until a 0.02 N load was detected. Samples were compressed to 10% strain at 1% of the sample height per second, held for 200 s, compressed to 20% strain, then held for 450 s. Instantaneous and relaxation modulus were determined by curve fitting the standard linear solid model under finite deformation with MATLAB software (MathWorks, Natick, MA).

Biochemical content and correlations

Tissue samples were measured to obtain wet and dry weights. Lyophilized samples were digested in papain for 18 h at 60°C. Collagen content was determined by measuring hydroxyproline using a chloramine T assay. GAG content was measured with the Blyscan sGAG kit (Biocolor, Carrickfergus, UK). DNA content was determined using PicoGreen dsDNA reagent (Invitrogen, Carlsbad, CA). Pyridinoline cross-links were measured as previously described[20]. Briefly, tissue samples were digested in 6 N HCl at 100°C for 18 h, dried, re-suspended in a solution containing pyridoxine and homoarginine (Sigma), then analyzed under high performance liquid chromatography. Linear regression was performed between biochemical data and mechanical properties to determine significant correlations.

Statistics

Cells harvested were obtained from 16 joints from eight bovine calves. Measurements were obtained with $n = 6$ samples for HPLC, biochemistry, and tension testing, and $n = 7$ samples for compression testing and gross morphology. HPLC data was repeated twice and averaged between repeats. Biochemistry measurements were taken in triplicate and averaged. Due to the destructive nature of testing, tensile and compressive data was measured once. Data sets were tested for homogeneity via Levene's test. Non-homogeneous data were normalized using a log transformation prior to ANOVA. Following ANOVA, Tukey's *post hoc* test was performed as appropriate using JMP 9 software (SAS Institute, Cary, NC). Data is presented as mean ± 95% confidence interval. Bars shown in graphs or averages displayed in a table without sharing the same letter denote significantly different groups.

Results

Self-assembling tissues resemble native tissues

Engineered constructs resembled the geometry of the knee meniscus, including its wedged profile, after culture (Fig. 1). Ligament fibrocyte constructs appeared slightly more curved than tenocytes constructs, which, in turn, appeared slightly more curved than meniscus cell constructs. Ligament fibrocyte constructs were slightly larger than constructs from other experimental groups, displaying a significantly greater construct width ($P = 0.011$), inner length ($P = 0.032$), and inner width ($P = 0.006$) than meniscus cell constructs (Table I). All constructs stained positively for the major components of native meniscus fibrocartilage (Fig. 2). Constructs displayed the presence of collagen, GAG, and collagen I and II, with particularly intense staining for GAG. Slightly more collagen staining was seen in constructs seeded with ligament fibrocytes or meniscus cells as compared to those seeded with tenocytes.

Ligament fibrocyte co-cultures exhibit superior tensile properties

Tensile properties of constructs seeded with ligament fibrocytes were significantly greater than those from other experimental groups (Fig. 3). Ultimate tensile strength of ligament co-cultures in the circumferential direction reached a value of 1.80 ± 0.39 MPa compared to 1.40 ± 0.17 MPa for tenocyte co-cultures, representing an increase of 29%. Ligament fibrocyte co-cultures also displayed the highest Young's modulus in the radial direction, reaching 3.24 ± 0.37 MPa compared to values of 2.63 ± 0.44 MPa and 2.44 ± 0.65 MPa for meniscus cell and tenocyte co-cultures, respectively, representing a 23–33% increase. No differences were observed among groups when comparing values for Young's modulus measured in the circumferential direction. The ligament fibrocyte co-culture group displayed the greatest ultimate tensile strength when measured in the radial direction. Ligament fibrocyte co-cultures achieved values of 1.58 ± 0.22 MPa, compared to 1.18 ± 0.17 MPa and 1.35 ± 0.35 MPa for meniscus cell and tenocyte co-cultures, respectively, representing a 17–34% increase. Overall, ligament fibrocyte co-cultures maintained greater tensile properties than meniscus cell or tenocyte co-cultures.

Ligament fibrocyte co-cultures display enhanced compressive properties

Constructs seeded with ligament fibrocytes also displayed greater properties when tested in compression (Fig. 4). Constructs seeded with ligament fibrocytes displayed significantly greater relaxation moduli than constructs seeded with meniscus cells or tenocytes. At 10% strain, relaxation modulus for the ligament fibrocyte constructs reached 87 ± 28 kPa, representing a significant 61% increase over the meniscus cell constructs' value of 54 ± 12 kPa. Similarly, at 20% strain, ligament fibrocyte constructs displayed a relaxation modulus of 249 ± 51 kPa, compared to 181 ± 51 kPa and 183 ± 33 kPa for meniscus cell constructs and tenocyte constructs, respectively. Thus, ligament fibrocyte construct relaxation modulus at 20% strain increased 36–38% compared to other groups.

Similar trends were observed for instantaneous moduli, but these trends were not significant. Ligament fibrocyte co-cultures reached 206 ± 66 kPa at 10% strain, compared to 150 ± 52 kPa and 138 ± 25 kPa for meniscus cell and tenocyte co-cultures, respectively. At 20% strain, ligament cell co-cultures also displayed the greatest instantaneous moduli, reaching 539 ± 125 kPa compared to 446 ± 123 kPa and 411 ± 66 kPa for meniscus cell and tenocyte co-cultures, respectively.

Self-assembling co-cultures display differential extracellular matrix content

Variations in biochemical content among different co-culture groups were observed (Fig. 5). Ligament fibrocyte co-cultures displayed the greatest pyridinoline cross-link content per construct wet weight, reaching 152 ± 17.2 nmol/g as compared to 108 ± 28.3 nmol/g and 126 ± 34.2 nmol/g for tenocyte and

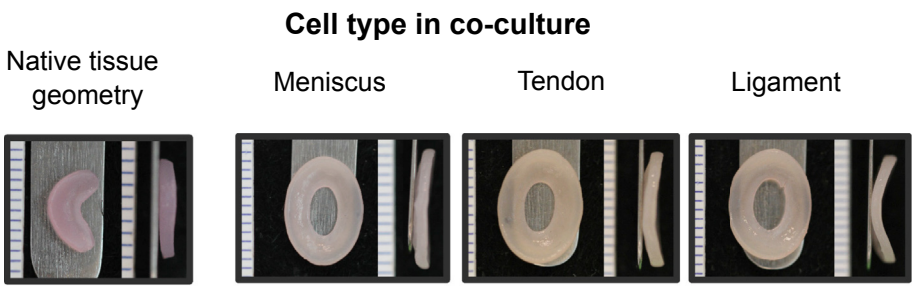

Fig. 1. Gross morphology of self-assembling meniscus tissues engineered via different co-cultures. Each ring-shaped construct can be trimmed to form the two compartments of the native meniscus, as shown on the left hand side. Hash marks indicate millimeters.

Table I
Dimensions and hydration of self-assembling co-cultures at the end of construct culture. Letters denote statistical significance, where groups not sharing the same letter are statistically different

Cell type in co-culture	Construct length (mm)	Construct width (mm)	Inner length (mm)	Inner width (mm)	Hydration (%)
Meniscus	12.0 ± 0.3	8.8 ± 0.2^{B}	5.0 ± 0.2^{B}	3.1 ± 0.2^{B}	84.2 ± 0.9
Tendon	12.2 ± 0.2	9.1 ± 0.2^{AB}	5.3 ± 0.2^{AB}	3.2 ± 0.2^{AB}	84.1 ± 0.9
Ligament	12.1 ± 0.3	9.3 ± 0.2^{A}	5.5 ± 0.2^{A}	3.6 ± 0.2^{A}	84.6 ± 0.9

meniscus cell co-cultures, respectively. This amounted to a 21–41% increase compared to other groups. Similar results were seen when measuring cross-link content per construct dry weight. In terms of collagen, meniscus cell co-cultures displayed 5.00 ± 0.26% per wet weight, as compared to 4.34 ± 0.59% and 4.59 ± 0.25% for tenocyte and ligament fibrocyte co-cultures, respectively. Meniscus cell co-cultures displayed significantly greater collagen per wet weight than tenocyte co-cultures, amounting to an increase of 15%. Similar trends were observed for collagen per construct dry weight. GAG per dry weight was slightly greater in ligament fibrocyte co-cultures as compared to other groups, with ligament fibrocytes displaying a significant 15% increase over meniscus cell co-cultures. No significant differences were observed among groups in terms of DNA content. Total collagen per construct was significantly greater in all co-culture groups in comparison to articular chondrocyte mono-cultures.

Pyridinoline cross-links are correlated with construct tensile properties

Tensile properties were significantly correlated with pyridinoline content (Fig. 6). In the circumferential direction, ultimate tensile strength was correlated with increasing cross-links per wet weight and per dry weight. Similarly, in the radial direction, Young's modulus was correlated with increasing cross-links per wet weight and per dry weight. Radial Young's modulus was slightly more correlated with cross-links per wet weight than cross-links per dry weight.

Discussion

The results of the present study demonstrate the feasibility and superiority of using tenocytes and ligament fibrocytes in meniscus tissue engineering, making this the first investigation that demonstrates the use of non-meniscus cells for engineering knee meniscus fibrocartilage with substantial mechanical integrity, as quantified via compressive and tensile testing. The findings of this study support our hypotheses: (1) Tenocytes and ligament fibrocytes are indeed capable of undergoing the self-assembling process in co-culture with articular chondrocytes. (2) Constructs engineered from tenocytes as well as ligament fibrocytes compared well with constructs engineered from meniscus cells; it is noteworthy that ligament fibrocytes, in particular, in co-culture with articular chondrocytes, resulted in the formation of the best meniscus-like constructs. (3) Cross-link content in ligament fibrocyte, tenocyte, and meniscus cell co-cultures was well correlated with construct tensile properties.

The successful use of tenocytes and ligament fibrocytes in tissue engineering of the meniscus has strong significance for future clinical and basic research applications. One of the main disadvantages of the use of meniscus cells for meniscus tissue engineering is the limited availability of meniscus tissue[1]. In contrast, ligaments are more available and more accessible as a potential cell source. For instance, patellar ligament and hamstring tendons are the most commonly used grafts in ACL reconstruction, and hold the advantages of relatively easy isolation and limited donor site morbidity[21]. Our findings suggest that tenocytes and ligament fibrocytes can be used in addition to meniscus cells for engineering the meniscus, increasing the flexibility of allogeneic and autologous approaches. Moreover, the use of tendon or ligament offers the advantage of isolating cells from a site other than an injured meniscus, which may improve patient outcomes. In ACL injuries, associated menisci tears are seen in approximately half of the cases[22,23]. As a consequence, in these cases, the remnants of the torn ACL can be harvested for cells which can then be combined in co-culture with articular chondrocytes toward engineering a new autologous knee meniscus. Additionally, an engineered meniscus can be used in transplantation approaches to treat experimental lesions. One method would be partial replacement of the meniscus by suturing an engineered meniscus to the rim of the native tissue. An alternative to this would be replacement of the entire meniscus, in a technique analogous to meniscus transplantation, by fixation of the engineered tissue to surrounding meniscal attachments or underlying bone. Additional research is needed to provide data on efficacy of attachment and quality of integration for these techniques. The successful use of tenocytes and ligament fibrocytes for meniscus tissue engineering points toward new clinical and preclinical approaches which increase the translatability of meniscus tissue engineering.

The results of this investigation reinforce previous observations emphasizing the potential of tissues containing two or more

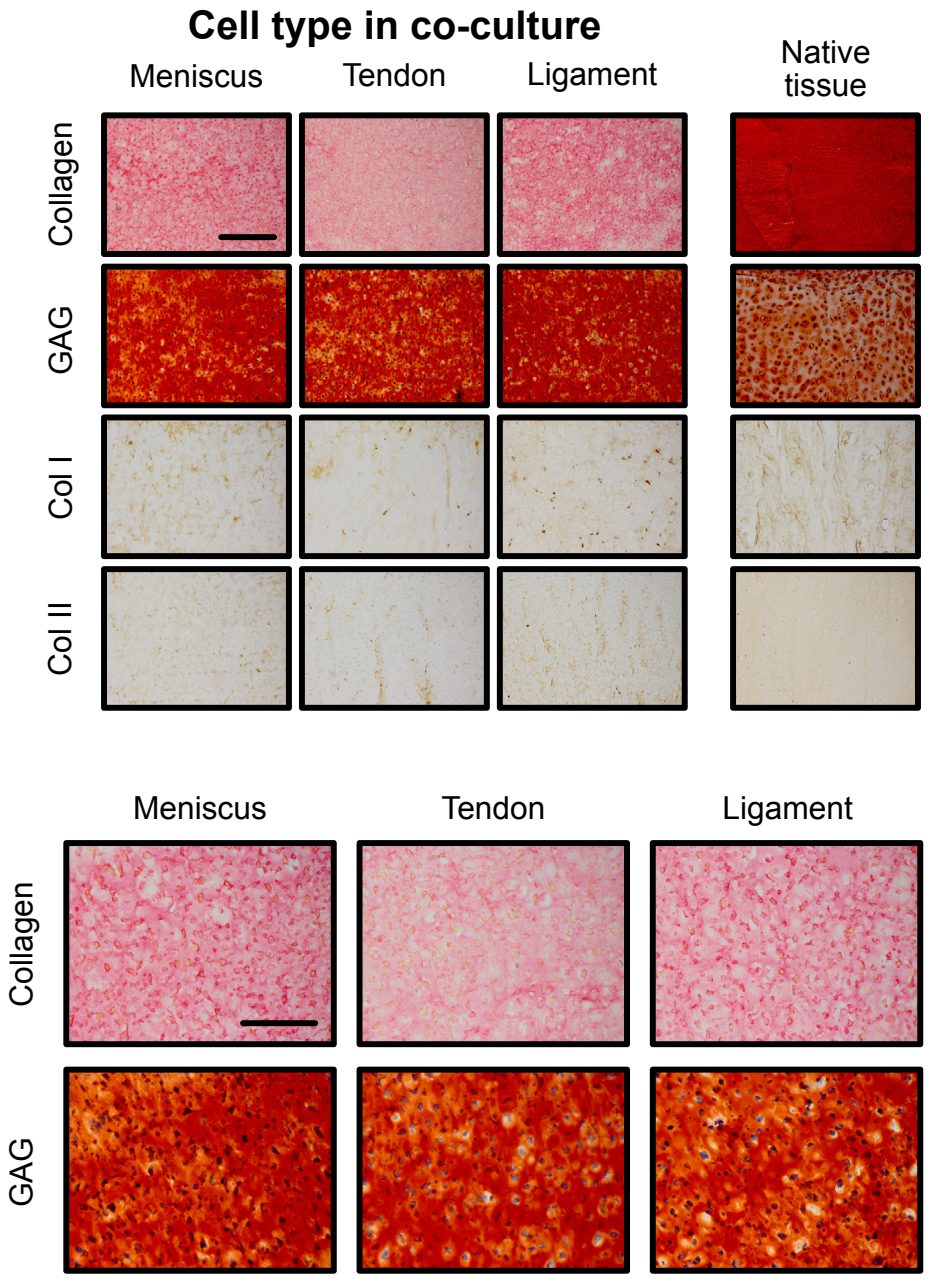

Fig. 2. Histology and immunohistochemistry of self-assembling co-cultures. For comparison, native tissue staining displayed at right hand side. Large magnification images provided for cell morphology. Scale bars represent 200 μm (upper panel) and 100 μm (lower panel).

different cell types. Co-cultures have been successfully used in meniscus tissue engineering over the last decade[13,15,24]. The fact that the meniscus has distinct zones with varying biochemical and biomechanical characteristics encourages the use of co-cultures in engineering this tissue. Previously, co-cultures combining meniscus cells with chondrocytes resulted in engineered menisci that exhibited improved biomechanical properties[13,15]. The use of co-cultures was also successful in creating anisotropy in engineered menisci using a sequential cell seeding methodology[18]. This study indicates the generation of anisotropy among all experimental groups, as values for tensile strength and stiffness are greater when measured in the circumferential direction as compared to the radial direction. This work also demonstrates that novel cell sources can have significant effects on functional properties in co-culture. Stem cells are another cell source that could be combined with this work for future co-culture studies in meniscus tissue engineering[25]. In a study that combined human meniscus cells with human bone marrow stromal cells (BMSCs) in three-dimensional co-culture, a hypoxic environment of 3% oxygen tension improved GAG synthesis by 1.3 fold compared to meniscus cell pellets alone[26]. Combination of different cell sources in a co-culture model represents a successful approach that should be further explored in engineering the meniscus.

The similarities and differences of tenocytes, ligament fibrocytes, and meniscus cells are pertinent for future efforts in engineering the knee meniscus. At least one sub-population of cells in the native knee meniscus is described as being fibroblast-like[27], and thus similar to tenocytes and ligament fibrocytes. At the cellular level, tenocytes and ligament fibrocytes are considered members of the same family of connective fibroblastic cells. However, even with

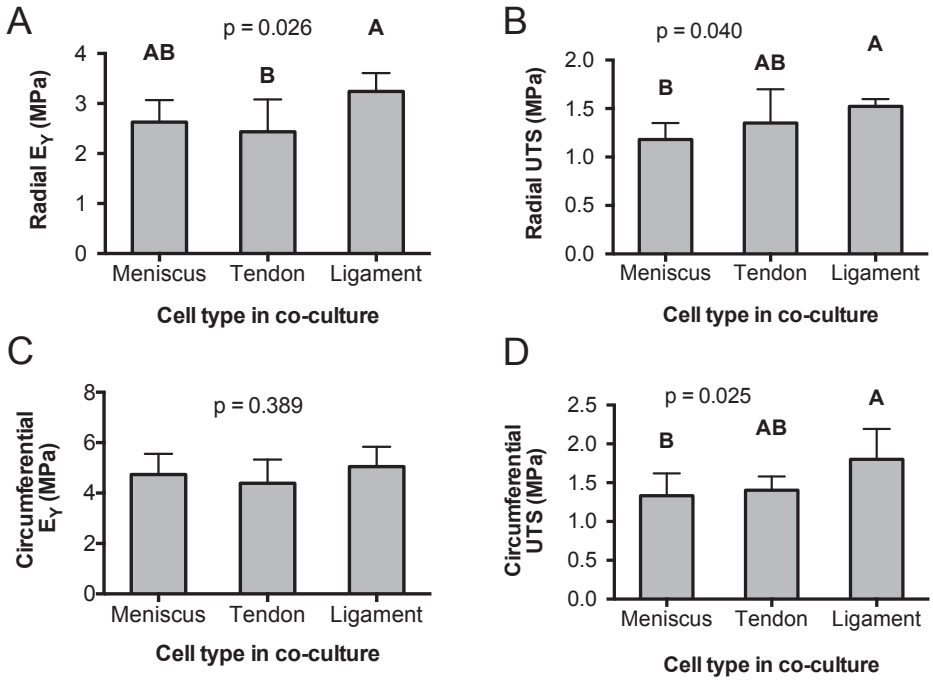

Fig. 3. Tensile properties of self-assembling co-cultures. Graphs display (A) Young's modulus or E_Y and (B) ultimate tensile strength or UTS in the radial direction, as well as (C) E_Y and (D) UTS in the circumferential direction.

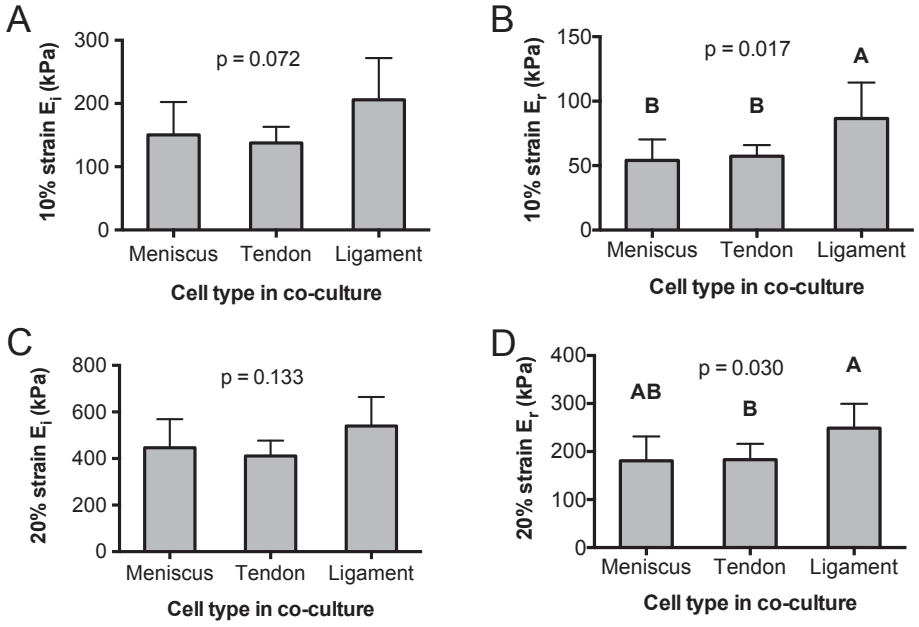

Fig. 4. Compressive properties of self-assembling co-cultures. Graphs display (A) instantaneous modulus or E_i and (B) relaxation modulus or E_r at 10% strain, as well as (C) E_i and (D) E_r at 20% strain.

these similarities, there exist notable differences between tendons and ligaments. A recent investigation of the cytoskeletal composition and compressive properties of cells from various musculoskeletal tissues demonstrated that ligament cells were most similar to meniscus cells[28]. Another study reported a lower proliferation rate for tenocytes in comparison to fibroblasts[29]. Additionally, elastin content, proteoglycan content, and the origin of vascularization[29–31] are all known to differ between tendon and ligament. With specific relevance to the results described above, ligament has been reported as having higher proteoglycan content in comparison to tendon[32–34]. Indeed, when analyzing GAG content as normalized to dry weight, ligament fibrocytes co-cultures grown in this study had significantly higher values than tenocyte co-cultures. Further research should identify the differences between these two cell types to improve understanding of their role in extracellular matrix synthesis for tissue engineering.

The biochemical content and correlations with mechanical properties found in this study confirm the importance of

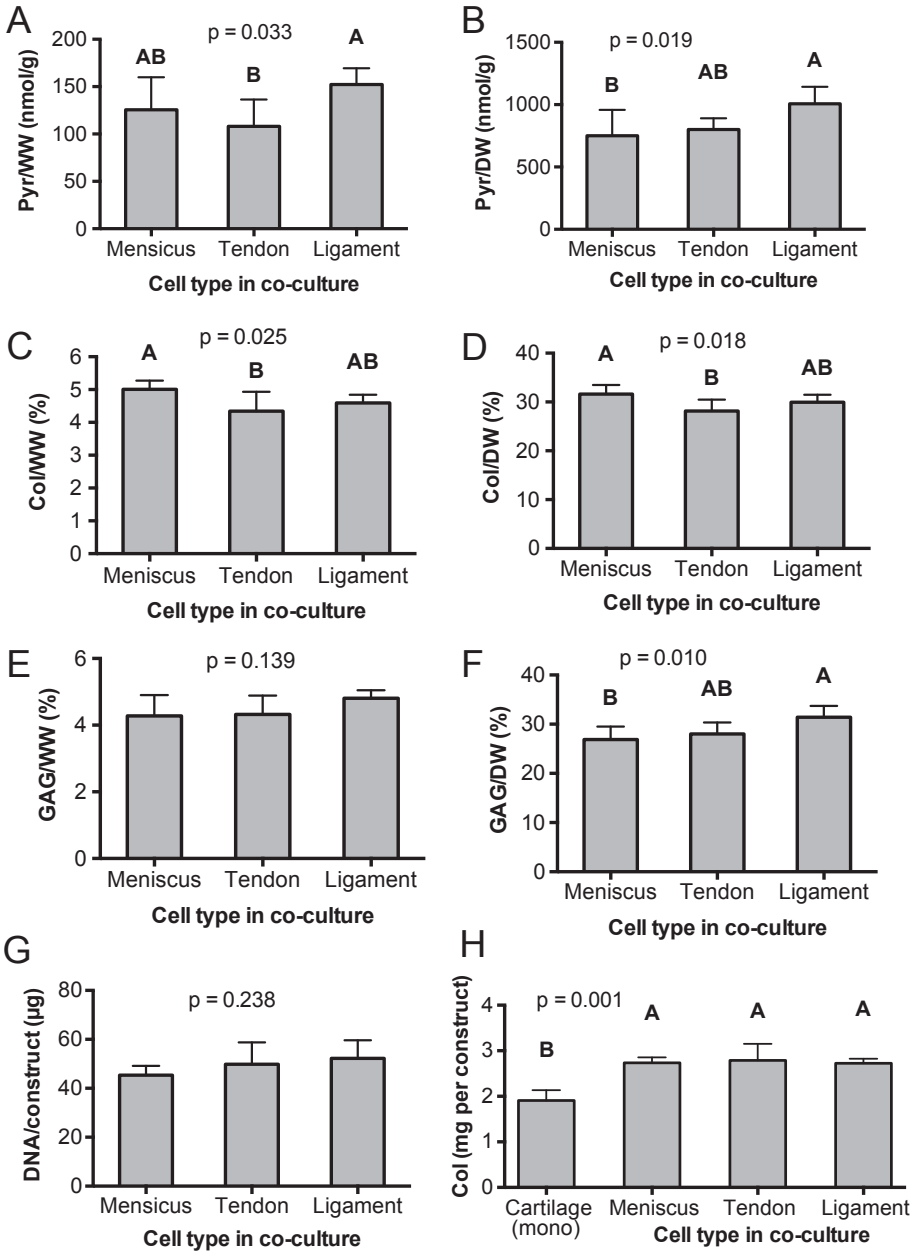

Fig. 5. High performance liquid chromatography and quantitative biochemistry data measured from self-assembling co-cultures. Graphs display (A) pyridinoline cross-link content per construct wet weight and (B) per construct dry weight, (C) collagen content per construct wet weight and (D) per construct dry weight, (E) GAG content per construct wet weight and (F) per construct dry weight, (G) DNA content per construct, and (H) collagen per construct.

pyridinoline cross-linking. In spite of the fact that collagen content was higher for constructs engineered with meniscus cells, tensile properties were greatest in constructs engineered with ligament fibrocytes. Analogously, constructs engineered with ligament fibrocytes displayed the greatest cross-link content of any group. Pyridinoline cross-link content in constructs engineered with ligament fibrocytes approached native values, reaching 75% of numbers reported previously[20]. In addition, tensile properties were found to be most correlated with cross-link content in this study. This is in contrast to prior literature emphasizing the role of collagen content in imparting mechanical properties to cartilage[35,36]. Recent work from our lab has demonstrated the importance of pyridinoline cross-links in improving mechanical properties in engineered and native tissues[20]. In addition, the values for cross-link content exhibited in this study are higher than those described in previous self-assembling cartilages[20]. This is likely because of differences between cell sources and the continuous addition of TGF-β1 in this study. In this study, ligament fibrocyte co-cultures exhibited significantly greater compressive properties than all other groups. Although ligament fibrocyte co-cultures displayed the greatest GAG per dry weight content, this increase was relatively small in magnitude. Instead, it is likely that the increased pyridinoline cross-link content in ligament fibrocyte co-cultures was responsible for the increased compressive properties observed. This is in agreement with previous results from our laboratory illustrating that the presence of collagen may contribute

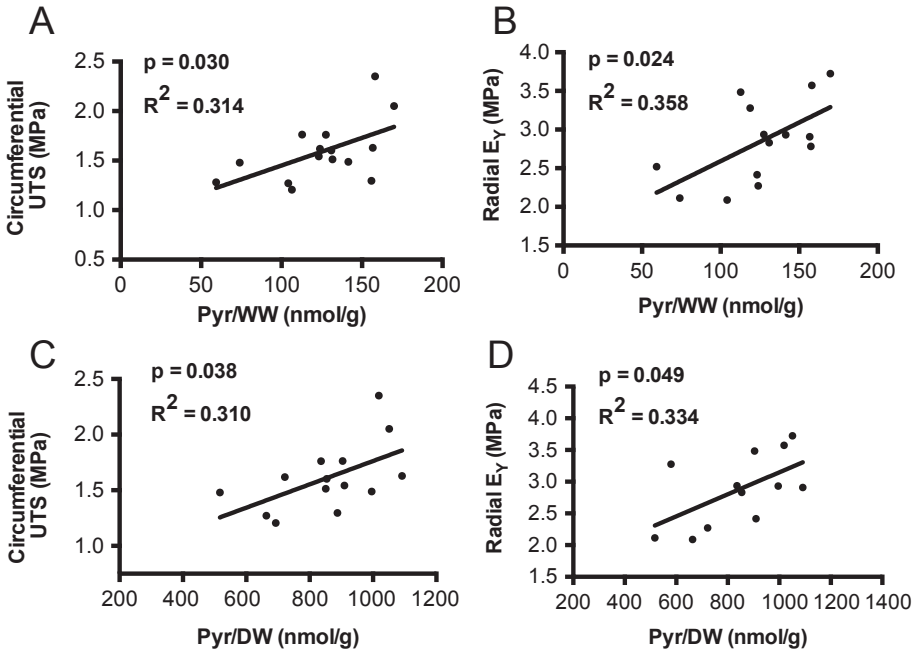

Fig. 6. Correlations between construct mechanical properties and pyridinoline cross-link content. Graphs display (A) circumferential ultimate tensile strength or UTS vs pyridinoline per wet weight, (B) radial Young's modulus or E_Y vs pyridinoline per wet weight, (C) circumferential UTS vs pyridinoline per dry weight, and (D) radial E_Y vs pyridinoline per dry weight.

to tissue compressive properties[37,38]. Overall, this study demonstrates that pyridinoline content drives increases in mechanical properties in co-cultures of various musculoskeletal tissues.

The ability of tenocytes and ligament fibrocytes to undergo the self-assembling process, in conjunction with articular chondrocytes, is a particularly exciting finding. DNA content found in all groups was similar to previous work with meniscus cell co-cultures, and supports that cells survived seeding and were viable during culture[39]. Additionally, previous work has shown that self-assembling tissues seeded with fibroblasts can undergo unwanted deformation, changing the geometry of the engineered tissue significantly[15]. One solution to this has been using seeding constructs in various co-culture ratios[13,24]. Although a pre-stress within constructs was observed in this study (i.e., tissues developed curvature in their gross morphology), constructs did not deform largely during culture. The finding that the co-cultures investigated here can undergo self-assembly without deleterious deformation is promising, and merits future work on the forces generated within tissues engineered with different cell types. Lastly, in this study, it was shown that collagen per construct was significantly lower in articular chondrocyte mono-cultures as compared to all of the co-cultures examined. Since it has been reported that tissue engineered from 100% fibroblast-like mono-cultures do not produce notable amounts of matrix[15], it is likely that a beneficial (additive or synergistic) interaction took place in these co-cultures. Future work should seek to examine this interaction regarding matrix production in co-cultures containing tenocytes and ligament fibrocytes.

This investigation emphasized the potential of different cell sources to engineer knee meniscus fibrocartilage. Overall, constructs engineered using ligament fibrocytes in co-culture were mechanically superior to other groups, including the gold standard approach of using meniscus cells in co-culture. Constructs engineered with tenocytes also resulted in self-assembling tissue, thus increasing the translational potential of using tendon and ligament as additional cell sources in meniscus tissue engineering. Lastly, pyridinoline content was found to be significantly correlated with construct tensile properties among these tissues. Future work should be directed toward using passaged cells, including human cells, to create constructs with similar or greater mechanical properties for implantation in animal models.

Author contributions

PH, NKP, BJH, JCH, and KAA conceived of and designed this study; PH, NKP, BJH, and AA acquired the data, PH, NKP, BJH, AA, JCH, and KAA reviewed and interpreted the data; and PH, NKP, BJH, JCH, and KAA wrote and edited the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflicts to declare.

Role of the funding source

This work was funded by National Institutes of Health grant R01DE019666. This funding source did not play a role in the collection, analysis, or interpretation of the data presented.

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