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Enhancing the mechanical properties of engineered tissue through matrix remodeling via the signaling phospholipid lysophosphatidic acid

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Abstract

Knee meniscus fibrocartilage is frequently injured, resulting in approximately 1 million procedures annually in the U.S. and Europe. Its near-avascularity contributes heavily to its inability to heal, and places it as a prime candidate for replacement through regenerative medicine. Here, we describe a novel approach to increase extracellular matrix organization, rather than content, in order to augment the mechanical properties of engineered tissue. To synthesize fibrocartilage, we employ a self-assembling process, which is free of exogenous scaffolds and relies on cell-to-cell interactions to form all-biologic constructs. When treated with the signaling phospholipid lysophosphatidic acid (LPA), tissue constructs displayed increased tensile properties and collagen organization, while total collagen content remained unchanged. LPA-treated constructs exhibited greater DNA content, indicative that the molecule exerted a signaling effect. Furthermore, LPA-treated cells displayed significant cytoskeletal reorganization. We conclude that LPA induced cytoskeletal reorganization and cell-matrix traction, which resulted in matrix reorganization and increased tensile properties. This study emphasizes the potential of non-traditional stimuli, such as signaling phospholipids, for use in tissue development studies. The extension of these results to other collagen-rich tissues represents a promising avenue for future exploration.

Keywords

Knee meniscus; Tissue engineering; Biomechanics; Extracellular matrix; Cell traction

1. Introduction

Fibrocartilage throughout the human body frequently experiences degeneration, leading to health problems as diverse as back pain, osteoarthritis, and difficulty speaking and eating [1,2,3]. Of particular interest clinically is the knee meniscus, a wedge-shaped, semi-circular fibrocartilage situated between the distal femur and the tibial plateau. Meniscal lesions alone are responsible for approximately 1 million surgeries annually in the U.S. and Europe, representing the most common set of procedures practiced by orthopedic surgeons [4,5]. The

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knee meniscus performs its biomechanical functions by virtue of the biochemical content and organization of its extracellular matrix (ECM). The inner portion of the meniscus resembles articular cartilage and specializes in bearing compressive loads, while the outer portion is more fibrous and suited to withstanding these loads after they are transformed into tensile stresses. Glycosaminoglycans (GAGs) in the meniscus confer compressive resistance, while collagens, especially type I and type II, are responsible for its capacity to bear tensile loads [6,7]. ECM organization also enhances meniscus tensile properties, as bundles of collagen fibers are aligned circumferentially and radially within the tissue [8]. Although the organization and content of their ECM makes the meniscus and other fibrocartilages well equipped for their demanding biomechanical functions, these tissues' unique avascularity and hypocellularity render them unable to mount healing responses following trauma or disease [9].

Their lack of regenerative capacity positions fibrocartilages as ideal candidates for replacement or restoration through tissue engineering. Within tissue engineering, many scaffold-based approaches have been reported, forming a large body of research concerning the synthesis of fibrocartilage and other tissues [10,11,12]. However, these methods often face issues, such as a lack of cell-to-cell communication, the prevention of mechanotransduction, and the impairment or absence of ECM remodeling [13,14]. For this reason, scaffoldless tissue engineering strategies, such as bio-printing and cell sheet engineering, have recently emerged [15,16].

As an alternative to scaffold-based approaches, our laboratory has developed a completely biological method of tissue synthesis, which avoids several problems associated with scaffolds. This scaffoldless technology, termed the *self-assembling process*, employs a non-adherent mold to promote the direct, biophysical interaction of seeded cells [17,18]. This non-adherent surface prevents cell-to-substrate attachment, instead encouraging cell-to-cell cadherin binding, resulting in minimization of free energy [19]. During culture, cells synthesize an ECM reminiscent of the development and composition of native cartilage. As ECM content is directly related to mechanical properties, tissues engineered using this method have reported compressive properties approaching those of native tissue values [20]. Thus, tissues engineered with the self-assembling process have seen success in the creation of all-biologic constructs, the recapitulation of appropriate ECM biochemical content, and the generation of compressive properties comparable to native tissue values.

Even with these recent successes, enhancing tensile properties remains a key challenge in engineering the knee meniscus and other collagen-rich tissues [21]. Biophysical ECM remodeling via cell traction forces, which are involved in biological processes such as wound closure, angiogenesis, and tendon formation [22,23], remains one novel route towards increasing mechanical properties. Similar strategies employing biophysical ECM modulation via collagen crosslink promotion, the removal of excessive GAGs, and non-enzymatic glycation, have all proven successful in enhancing tensile properties of engineered tissues [21,24,25]. In parallel, the identification of novel signals or stimuli, beyond traditional agents such as protein growth factors, is necessary for the field of regenerative medicine to advance. Thus, ECM modification through exogenous traction-promoting stimuli represents a promising area for investigation. However, it is important to recognize that excessive ECM contraction can lead to undesirable tissue construct sizes or geometries [26].

Lysophosphatidic acid (LPA) is a phospholipid mediator that has been shown to induce a variety of signaling effects *in vivo*. LPA has been previously studied for its ability to promote cell survival and proliferation, and is also a significant constituent of mammalian sera, being present at concentrations of 1–10 μM [27,28]. In addition to its proliferation-

inducing capacity, LPA has also been reported to initiate cell cytoskeleton contraction, causing decreased cell sizes and retraction of dendrite-like cell extensions or processes [29]. Moreover, LPA-treated fibroblasts seeded within collagen gels dramatically contract the volume of their collagen gels [30]. Encouraged by these findings, we set out to examine LPA to induce cell traction, increase ECM density, and enhance the tensile properties of self-assembling meniscus fibrocartilage.

The objective of this study was to increase the tensile properties of self-assembling knee meniscus constructs by employing LPA as an exogenous cell-traction inducing agent to remodel the ECM. Constructs were cultured for 5 weeks and treated with LPA for 1 week or not. It was hypothesized that 1) treatment of constructs with LPA would increase tensile properties, 2) no aberrant or excessive contraction to undesirable sizes or shapes would occur, 3) an increase in construct tensile properties would be accompanied by cytoskeletal contraction of cells, and 4) ECM remodeling and an increase in collagen density or alignment would be achieved.

2. Materials and methods

2.1 Articular chondrocyte and meniscus cell isolation

Primary bovine articular chondrocytes and meniscus cells were isolated from eight individual 4-week-old calves (Research 87). Articular cartilage from the distal femur was minced and digested in 0.2% collagenase type II (Worthington) for 18 hours at 37° C. Meniscal tissue was isolated from the knee joint and digested according to a procedure published prior [31]. Briefly, the outer meniscal rim was removed, and the remaining meniscal tissue was minced and digestion in 0.25% pronase (Sigma-Aldrich) for 1 hour followed by 0.2% collagenase type II for 18 hours. After isolation, cells were seeded immediately.

2.2 Self-assembly and culture of constructs

Non-adherent agarose wells in the shape of the native leporine meniscus were prepared using a design adapted from previous work [32]. Wells were saturated prior to and during culture in serum-free chondrogenic medium. To create each fibrocartilage construct, a 1:1 co-culture of articular chondrocytes and meniscal cells was seeded at a density of 20 million cells per 180 μL of chondrogenic media [17,18]. Cells were allowed to sit undisturbed for 4 hours before additional media was added, after which it was changed daily. After 7 days, constructs held enough mechanical integrity to be removed from their agarose wells and kept in free-floating culture. For the treated group, 10 μM LPA (Enzo Life Sciences) was added during $t = 21–28$ days (week 4) of culture. All constructs were cultured for 5 weeks at 37° C and 5% CO_2.

2.3 Tissue gross morphology, histology, and immunohistochemistry

At $t = 35$ days, constructs were removed from culture, photographed, and measured via ImageJ (NIH). Wet weights were taken before dividing each construct into pieces for histology, mechanical testing, and biochemical analysis. For histology and immunohistochemistry, construct samples were cryoembedded in Histoprep at −20°C (Fisher Scientific) and sectioned at 12 μm. Safranin-O/fast green and picrosirius red sections were fixed in formalin before staining, and immunohistochemistry was performed with the Vectastain ABC and DAB Substrate Kits (Vector Labs) with rabbit anti-bovine collagen I (US Biologics) and rabbit anti-bovine collagen II antibodies (Cedar Lane Labs).

2.4 Tensile and compressive testing

Uniaxial, strain-to-failure testing was utilized to assess construct tensile properties. Samples were cut to a standard width of 0.80 mm, depth of 1.00 mm, and gauge length of 1.28 mm, photographed and measured using ImageJ, then cut into dog-bone shapes and adhered to paper strips with cyanoacrylate glue. Samples were then clamped within a uniaxial tensile testing machine (TestResources 840LM) and subjected to a 1% s^{-1} strain rate until failure. Young's modulus (E_Y) was calculated from the linear portion of the stress-strain curve, and ultimate tensile strength (UTS) was calculated from the maximum stress.

Unconfined stress-relaxation testing was used to assess construct compressive properties. Punches, 3 mm dia., were cut from constructs and photographed. Sample thickness was determined via a custom program on the testing machine (Instron model 5565). Tissue samples underwent step-wise stress relaxation to 10% and 20% strain while submerged in a bath. Sample viscoelastic properties, including instantaneous modulus (E_I), relaxation modulus (E_R), and coefficient of viscosity (μ), were calculated by fitting data curves to a standard Kelvin solid model [33].

2.5 Analysis of tissue biochemical content

Biochemistry samples were weighed while wet, then frozen and lyophilized to obtain dry weights. Collagen content was measured with the use of a Sircol standard (Biocolor) and a Chloramine-T colorimetric hydroxyproline assay. DNA content was calculated with the use of PicoGreen dsDNA reagent (Invitrogen), assuming a conversion factor of 7.7 pg DNA/cell. GAG content was quantified by a protocol based on the Blyscan assay kit (Invitrogen). All quantification measurements for biochemical content were performed with a GENios spectrophotometer/spectrofluorometer (TECAN).

2.6 Cellular actin staining and polarized light microscopy

Co-cultures of primary articular chondrocytes and meniscus cells were seeded upon tissue culture-treated slides for fluorescent staining. Co-cultures were kept in chondrogenic media for 24 hours, with or without 10 μM LPA. Cells were then washed with PBS, fixed in 3.3% paraformaldehyde (Sigma-Aldrich), stained with phalloidin (Biotium), and mounted in ProLong Gold w/DAPI (Molecular Probes). Using multiple images, cell size and circularity were quantified in ImageJ. For polarized light visualization of collagen, histology samples were sectioned to 30 μm before picrosirius red staining and imaging underneath a U-POT polarizer (Olympus).

2.7 Statistical analysis

For each biochemical and biomechanical test, n = 7 samples were used. Results were analyzed with single-factor analysis of variance followed by a Tukey's HSD post hoc test when merited ($p < 0.05$). All data are presented as means ± standard deviations, with asterisks indicating significance, and n.s. indicating no significance.

3. Results

3.1 Gross morphology, histology, and immunohistochemistry

Tissue constructs resembled native meniscus fibrocartilage in terms of gross appearance and histological staining. After 5 weeks of culture, all engineered tissue constructs maintained the characteristic curvature and wedge-shaped profile of the native knee meniscus (Figure 1A). Wet weights and hydration percentages were not significantly different between groups (data not shown). No constructs displayed aberrant contraction to sizes incompatible with mechanical or biochemical assaying. In terms of construct dimensions, the treated group

displayed a downward trend in comparison to the control group in terms of average construct surface areas and thicknesses, but these changes were not statistically significant.

In histology, all tissue constructs displayed positive staining for collagen, as indicated by picrosirius red, and GAGs, as indicated by Safranin-O, comparable to native meniscus fibrocartilage (Figure 1B). Overall, treated sections stained slightly darker than control sections. In terms of immunohistochemistry, all constructs stained positively for the presence of collagen type I and collagen type II, as found in native tissue. No differences between control and treated constructs were seen with collagen type I or collagen type II staining.

3.2 Tissue biomechanics and biochemistry

Biomechanical and biochemical data revealed large differences between control and treated constructs. Tensile stiffness, represented by Young's modulus, for control and treated constructs was 247 ± 89 kPa and 503 ± 159 kPa, respectively (Figure 2A). Ultimate tensile strength for control and treated constructs was 122 ± 34 kPa and 204 ± 77 kPa, respectively (Figure 2B). Although the tensile stiffness and strength of engineered menisci increased with LPA treatment, no increase in total collagen content was observed. Total collagen content, normalized by construct dry weights, was 8.10 ± 0.90% and 8.24 ± 0.98% for control and treated tissues, respectively (Figure 2C).

Compressive properties between control and treated constructs were not statistically different. Control and treated groups displayed instantaneous moduli of 274 ± 24 kPa and 255 ± 89 kPa, respectively, and relaxation moduli of 100 ± 14 kPa and 81 ± 28 kPa, respectively. Correspondingly, percent GAG content, as normalized to construct dry weight, was not statistically different, with control and treated tissues exhibiting values of 45.0 ± 8.3% and 53.3 ± 16.6%, respectively. However, DNA content increased with LPA treatment, as evidenced by the Picogreen DNA assay, with an estimated 6.54 ± 2.07 and 10.1 ± 4.51 million cells per control and treated constructs, respectively (Figure 2D). Additionally, alongside this, LPA-treated tissue displayed greater staining under polarized light, indicating an increase in collagen alignment and/or density (Figure 4A).

3.3 Cellular actin staining and polarized light microscopy

Actin staining and polarized light microscopy displayed stark differences in both the cellular cytoskeleton and tissue ECM of control and treated constructs. Control articular chondrocytes and meniscus cells stained for filamentous actin displayed significant cell spreading and large cell sizes, with numerous cells also displaying cell extensions or processes (Figure 3A). In comparison, LPA-treated articular chondrocytes and meniscus cells contracted in size, retracting their filamentous actin cytoskeleton and any cell extensions or processes (Figure 3B). When quantified, control and treated cells had average sizes of 1362 ± 349 and 923 ± 277 pixels, respectively, confirming the observed changes above (Figure 3C). Additionally, control and treated cells exhibited average circularities of 0.78 ± 0.14 and 0.95 ± 0.35, respectively, reflecting a decrease in cell extensions or processes after LPA treatment (Figure 3D).

4. Discussion

This study examined the use of a novel biophysical agent, lysophosphatidic acid (LPA), to exogenously promote cell traction within the ECM and therefore augment tensile properties of engineered tissue. Experimental results supported the hypotheses underlying this study: 1) LPA enhanced the tensile properties of self-assembling meniscus fibrocartilage, more than doubling tensile stiffness and enhancing tensile strength by nearly three-quarters of control. 2) LPA-treated tissue constructs maintained their shapes and dimensions. 3) LPA-treated

cells contracted significantly, reducing their size and retracting cell extensions or processes. 4) LPA-treated tissue displayed greater collagen fiber density and/or alignment than control tissue when visualized with polarized light microscopy. To the best of our knowledge, this is the first study 1) to use biophysical cell traction, resulting from cytoskeleton contraction, to increase the mechanical properties of engineered tissue, 2) to use LPA in cartilage tissue engineering, and 3) to use a phospholipid signaling molecule as a stimulus for self-assembling tissue. This study demonstrates the promotion of cell traction forces and concomitant cytoskeleton contraction as a beneficial strategy in tissue engineering.

It was found that the addition of LPA resulted in beneficial increases to the tensile properties of engineered fibrocartilage, more than doubling stiffness and increasing strength by more than half. Interestingly, no change in collagen content accompanied this effect, implicating that an increase in collagen organization was responsible for the enhanced tensile properties. ECM organization is essential to mechanical strength and stiffness in collagen-rich tissues, and the knee meniscus possesses significant collagen fiber alignment [8]. Indeed, an increase in collagen organization, either through alignment, density, or both, was apparent when picrosirius red sections were imaged under polarized light microscopy. Additionally, although ECM content was unchanged, ECM staining of constructs treated with LPA appeared slightly more intense than controls, corroborating the explanation that an increase in ECM density occurred. Taken together, these results indicate that an increase in tensile properties was due to an increase in organization of the ECM, specifically collagen.

The increase in ECM organization may have been precipitated by an increase in cytoskeletal reorganization. Indeed, when treated with LPA, articular chondrocytes and meniscus cells displayed significant cytoskeletal contraction, leading to retraction of cell extensions or processes, rounder cell morphology, and decreased cell size. LPA has been reported to induce actin polymerization, vinculin accumulation, and focal adhesion formation [29,30]. It is likely that while contracting within their ECM, cells treated with LPA exerted traction forces on their surrounding matrix, remodeling it and increasing collagen fiber density and/or alignment (Figure 4B). Recent work has demonstrated the importance of cell traction forces during development of collagen-rich tissues, corroborating this effect [23]. Therefore, cytoskeleton contraction to promote ECM organization represents a useful tool for tissue engineers, and future studies may quantify the specific traction forces exerted by cells within self-assembling and other engineered tissues.

Although differences in mechanical properties, ECM organization, and cytoskeleton organization were seen in meniscus constructs after treatment with LPA, the size and wedge-shaped profile of these tissues was maintained. ECM contraction, defined as an overall increase in matrix density with a corresponding decrease in matrix volume [29], thus did not occur after LPA treatment. The lack of significant ECM contraction may be due to the high percentage by weight of GAGs in these engineered tissues, which would impart swelling pressure and resist collagen network contraction induced by LPA treatment [10]. Although it is believed that LPA resulted in traction forces being exerted on the ECM, but without contraction, a moderate amount of contraction may be useful in further increasing the alignment and density of the collagen network. Interestingly, separate work with this culture system has demonstrated beneficial increases in tensile properties when excess GAGs are removed [25]. Thus, future work may examine the use of LPA in conjunction with GAG-depleting enzymes to further promote ECM organization and the associated augmentation of mechanical properties.

The biophysical effects seen in LPA-treated tissues are likely precipitated by the large variety of cell signaling effects LPA may exert, which may be beneficial or deleterious. For example, LPA has been well characterized as an anti-apoptotic and pro-survival factor [27],

and an increase in DNA content was indeed observed with its use in this study, indicating the molecule exerted a signaling effect upon engineered tissues. Additionally, LPA is linked to the prevention of osteoarthritis, via its cell surface receptors. It was recently reported that suppression of the LPA1 receptor results in the sensitization of chondrocytes to apoptosis, eventually leading to osteoarthritis [34]. However, other studies also show that LPA can maintain or differentiate cells to a hypertrophic chondrocyte phenotype or an osteoblast phenotype [35]. Regardless of these effects, all tissue constructs in this study, including those treated with LPA, exhibited characteristic fibrocartilage staining for collagen types I and II and GAGs, without degradation of these molecules due to LPA treatment. Future work should investigate the multiple downstream signaling pathways of LPA as well as other traction-inducing stimuli.

This work demonstrates that treatment with LPA can enhance the biomechanical properties of engineered tissue. An increase in tensile properties was accompanied by cytoskeleton contraction in treated cells as well as an increase in collagen alignment and/or density in treated tissues. Thus, we posit that an increase in ECM organization was caused by stimulation of engineered tissues with LPA, leading to increased tensile properties. We have identified an effective concentration for the use of LPA in tissue engineering, and also established that this particular stimulation regimen has no adverse effects on ECM biochemistry or mechanical properties. Finally, this work highlights the potential of novel biochemical stimuli, such as traction-inducing molecules and phospholipid signaling molecules, for broader investigation in tissue engineering and tissue development studies.

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Highlights

- Collagen-rich tissues require a high degree of matrix organization to function
- Lysophosphatidic acid doubles the tensile properties of engineered fibrocartilage
- Treatment with lysophosphatidic acid increases collagen network organization
- Phospholipid signaling molecules represent a promising area for future work

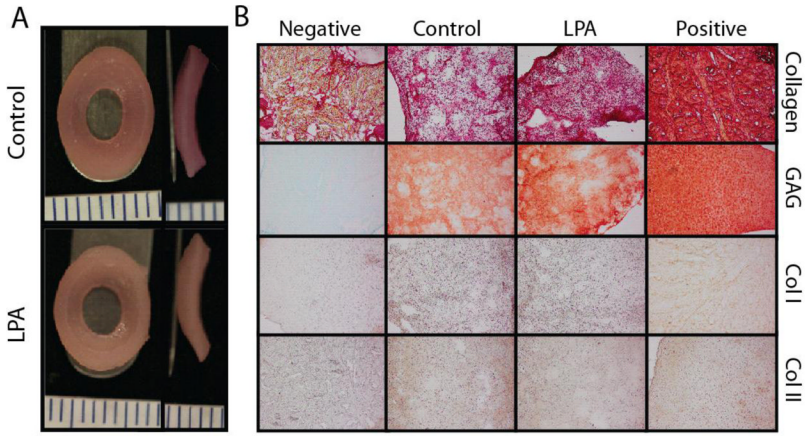

Fig. 1.

Self-assembling tissue morphology and staining after culture. (A) Gross morphology of control and treated constructs. No aberrant contraction occurred, and engineered tissue maintained the curvature of the native knee meniscus during and after culture. (B) ECM histology and immunohistochemistry of engineered constructs. Skeletal muscle, control constructs, LPA-treated constructs, and native meniscus fibrocartilage tissue were stained. All engineered constructs displayed an ECM characteristic of native fibrocartilage, with abundant collagen, GAG, collagen type I, and collagen type II staining. Additionally, collagen and GAG staining of LPA-treated constructs appears slightly denser than that of control constructs.

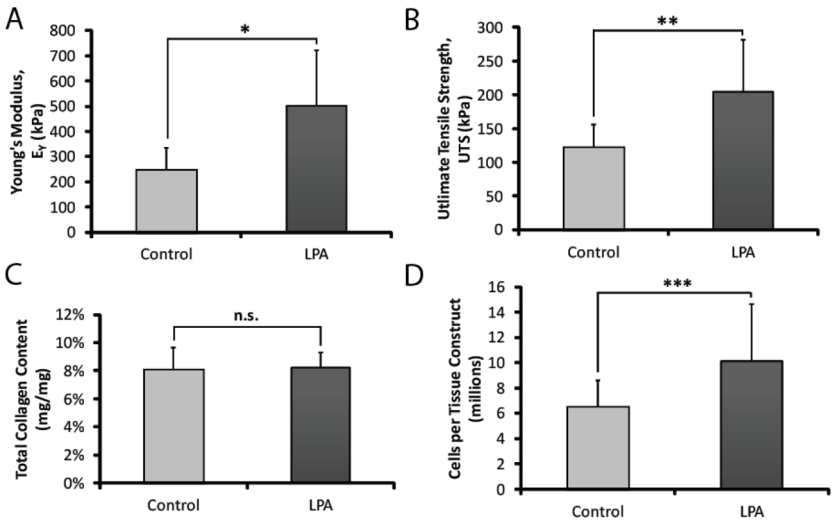

Fig. 2.

Biomechanical and biochemical properties of constructs. (A) After culture, tensile stiffness of LPA-treated constructs was 203% of control construct values. (B) In parallel, tensile strength of LPA-treated constructs was 167% of control construct values. (C) Although tensile properties increased, collagen content did not change significantly between control and treated tissues. (D) Cellularity of LPA-treated constructs, as calculated from DNA content, increased to 155% of control construct values.

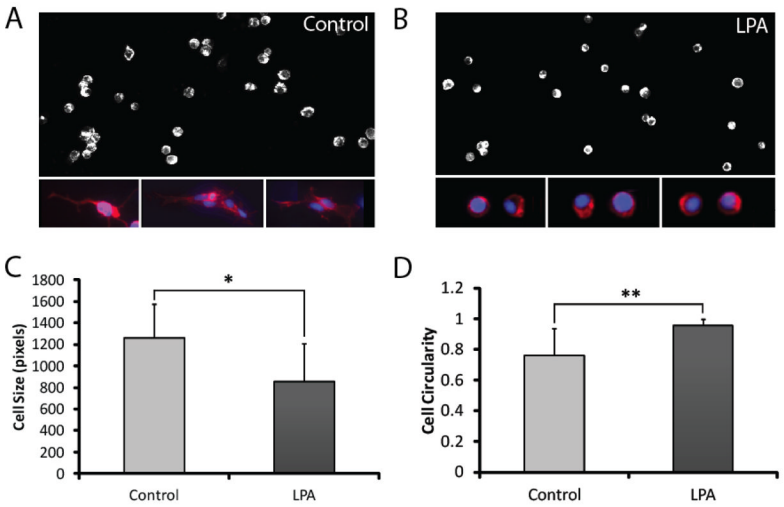

Fig. 3.

Cellular actin staining and morphology quantification. (A) Control cells displayed more spread morphologies, including many with dendrite-like extensions or processes (insets). (B) LPA-treated cells displayed round morphologies, absent of extensions or processes (insets). (C) When quantified for cell size, LPA-treated cells were significantly smaller than control cells, exhibiting a decrease to 66% of control cell size. (D) Additionally, in LPA-treated cells, average cell circularity, a proxy for the absence of cell extensions or processes, was 123% of control values.

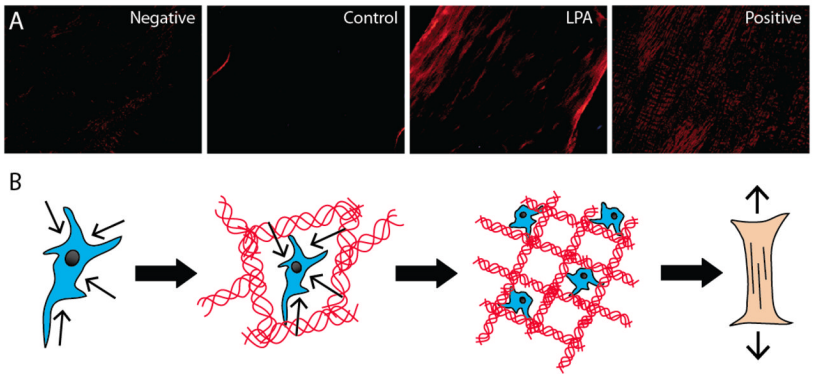

Fig. 4.

Organization of ECM collagen. (A) Polarized light microscopy of native muscle, control construct, LPA-treated construct, and native meniscus fibrocartilage, showing increasing collagen alignment and/or density. Control constructs displayed less collagen alignment and/or density as compared to LPA-treated constructs. (B) Schematic of the proposed mechanism by which LPA increases tensile properties of engineered tissues. Cytoskeletal contraction results in cell traction on the ECM, leading to remodeling of the collagen network and an increase in ECM density and/or alignment, enhancing tensile properties of the associated tissue.