Induction of Fiber Alignment and Mechanical Anisotropy in Tissue Engineered Menisci with Mechanical Anchoring

Jennifer L. Puetzer^a, Esther Koo^b, Lawrence J. Bonassar^{a,c,1}

^aDepartment of Biomedical Engineering, ^bBiological and Environmental Engineering, and ^cMechanical and Aerospace Engineering at Cornell University, Ithaca, NY, 14853, USA

Original Article

Word Count: 3,913

5 Keywords: Meniscus, Tissue Engineering, Collagen, Boundary Conditions, Fiber Alignment

¹ Corresponding author: 149 Weill Hall, Cornell University, Ithaca, NY 14853, USA. Tel: +1 607 255 9381; Fax: +1 607 255 1222
Email addresses: lb244@cornell.edu (Bonassar, LJ), jlp295@cornell.edu (Puetzer, JL)

Abstract:

This study investigated the effect of mechanical anchoring on the development of fiber organization and anisotropy in anatomically shaped tissue engineered menisci. Bovine meniscal fibrochondrocytes were mixed with collagen and injected into molds designed to produce meniscus implants with 12 mm extensions at each horn. After a day of static culture, 10 and 20 mg/ml collagen menisci were either clamped or unclamped and cultured for up to 8 weeks. Clamped menisci were anchored in culture trays throughout culture to mimic the native meniscus horn attachment sites, restrict contraction circumferentially, and encourage circumferential alignment. Clamped menisci retained their size and shape, and by 8 weeks developed circumferential and radial fiber organization that resembled native meniscus. Clamping also increased collagen accumulation and improved mechanical properties compared to unclamped menisci. Enhanced organization in clamped menisci was further reflected in the development of anisotropic tensile properties, with 2-3 fold higher circumferential moduli compared to radial moduli, a similar ratio to native meniscus. Ten and 20 mg/ml clamped menisci had similar levels of organization, with 20 mg/ml menisci producing larger diameter fibers and significantly better mechanical properties. Collectively, these data demonstrate the benefit of using bio-inspired mechanical boundary conditions to drive the formation of a highly organized collagen fiber network.

1. Introduction

Menisci are primarily anchored by ligament-like extensions at the horns which play a major role in menisci's ability to withstand and distribute loads in the knee. These extensions prevent extrusion, while allowing movement to conform to the condyles during each gate cycle (Kale et al., 2010; Messner and Gao, 1998). Collagen organization is also fundamental to the distribution of complex loads of the knee (Kawamura et al., 2003; Messner and Gao, 1998). Native menisci consist largely of circumferentially aligned collagen bundles anchored by perpendicular radial tie-fibers (Kambic and McDevitt, 2005; Petersen and Tillmann, 1998; Rattner et al., 2011). This organization results in anisotropic properties, with the circumferential tensile modulus 3-10 times larger than the radial modulus (Makris et al., 2011; Tissakht and Ahmed, 1995).

Tears that disrupt this organization result in pain, swelling, mechanical instability and degradation of surrounding cartilage (Hasan et al., 2013). Due to the avascular nature of the meniscus, tears are slow to heal, resulting in over 1 million surgeries a year in the US (Hasan et al., 2013; Khetia and McKeon, 2007). Treatment of large tears are generally not effective, and while cadaveric meniscus transplants show promise, lack of supply, the potential for disease transmission, and geometry constraints limit allograft therapy (Hasan et al., 2013).

Although there has been great effort to generate whole tissue engineered menisci to serve as an alternative to allografts (Balint et al., 2012; Ballyns et al., 2008; Higashioka et al., 2014; Huey and Athanasiou, 2011; Kon et al., 2008; Mandal et al., 2011; Puetzer and Bonassar, 2013; Tienen et al., 2006; Zur et al., 2011), none have achieved clinical use. Such implants often lack the native collagen fiber organization and anisotropic properties essential to distribute the loads of the knee appropriately. Recently, we have developed high density type I collagen menisci that have significant improvements in biochemical and mechanical properties with time in culture, but lack native organization (Puetzer and Bonassar, 2013).

It has been well established that contraction of collagen gels under a variety of mechanical boundary conditions can guide the formation of aligned collagen fibers (Bell et al., 1979; Bowles

et al., 2010; Costa et al., 2003; Grinnell and Lamke, 1984; Thomopoulos et al., 2005). It is believed cellular traction forces align collagen fibers along free edges (Costa et al., 2003; Grinnell and Lamke, 1984). Further, these aligned collagen gels develop anisotropic mechanical properties (Gould et al., 2012; Lee et al., 2008; Thomopoulos et al., 2005). However, all of this work has been done with low density collagen gels (1-6 mg/ml) and very thin simple geometries. To date, mechanical boundary conditions have not been investigated in high density collagen gels (10-20 mg/ml) or used with large complex geometries such as the meniscus.

The objective of this study was to investigate the effect of long-term anchored culture on the organization and development of tissue engineered meniscal constructs at multiple high density collagen concentrations. We hypothesize that high density type I collagen menisci, cultured with native horn-anchored boundary conditions, will result in alignment of collagen fibers and development of anisotropic tensile properties

2. Methods

2.1 Construct Fabrication

Sixty-eight meniscal constructs were fabricated as previously described (Puetzer and Bonassar, 2013). Briefly type I collagen was extracted from Sprague-Dawley rat tails (Pel-Freez Biologicals, Rogers, AZ) and reconstituted at 20 or 30 mg/ml using previously established techniques (Bowles et al., 2010; Cross et al., 2010). Meniscal fibrochondrocytes were isolated within 24 hours of slaughter of 1-3 day old bovine with 0.3% collagenase digestion and suspended in media. Sets of menisci were created from 15 different batches of cells and divided equally between clamped and unclamped, with each batch consisting of cells pooled from 2-3 bovine. The stock collagen solution was mixed with appropriate volumes of 1N NaOH, 10x phosphate-buffered saline (PBS), and 1x PBS to begin the gelling process at pH 7.0 and 300 mOsm osmolarity (Bowles et al., 2010; Cross et al., 2010). This collagen solution was

immediately mixed with the cell/medium solution and injected into ovine meniscus micro-computed tomography-based molds (Ballyns et al., 2008) to obtain 10 and 20 mg/ml meniscal constructs at 25×10^6 cells/ml. The molds were allowed to gel for 1 hour at 37°C (Figure 1). After 1 hour, the meniscal constructs were removed from the molds and cultured in media composed of DMEM, 10% FBS, 100 µg/mL penicillin, 100 µg/mL streptomycin, 0.1 mM non-essential amino acids, 50 µg/mL ascorbate, and 0.4 mM L-proline, changed every 2-3 days.

2.2 Application of Boundary Conditions

In order to achieve anchorage of the implants at the horns, meniscal molds (Ballyns et al., 2008) were redesigned with a 12x2.5x2 mm (LxWxH) extension at each horn. Further, previously described ABS loading trays (Ballyns and Bonassar, 2011) were redesigned to have a trough at the base of the meniscus that extensions lay in. Stainless steel clamps infused with bronze (Shapeways, NY) were screwed down over the extensions to secure them throughout culture (Figure 1). This boundary condition of anchoring the meniscus at the horns was chosen to mimic the native tibial attachment sites *in vivo* and to restrict contraction of collagen gels circumferentially. By restricting contraction circumferentially we hope to induce a residual hoop stress to encourage alignment. After a day of static culture, 10 and 20 mg/ml collagen menisci were either clamped or unclamped and cultured for up to 8 weeks.

2.3 Construct Analysis

At 0, 2, 4, and 8 weeks 4-6 samples from each group (clamped or unclamped, 10 mg/ml or 20 mg/ml) were removed from culture. Upon removal from culture all meniscal constructs were analyzed for organization, biochemical composition, and mechanical properties, with attention to radial vs. circumferential directions. For gross analysis, serial photographs taken throughout culture were analyzed using ImageJ to calculate change in area of samples, and the final weight of the constructs were compared to determine change in mass with time.

2.3.2 Confocal Image Analysis

Cross-sections from throughout the scaffolds were fixed in 10% buffered formalin, stored in 70% ethanol, and imaged with confocal microscopy in the circumferential and radial direction to visualize collagen fiber and cell organization as previously described (Bowles et al., 2010; Carey et al., 2012; Puetzer and Bonassar, 2013). Briefly, confocal reflectance imaging was performed in conjunction with fluorescence imaging by splitting a 488 nm laser on a Zeiss 710 confocal microscope using a 40x/1.2 C-Apochromat objective. Confocal reflectance microscopy was performed at 475-510 nm by collecting backscattered light reflected by collagen fibers through a 30 μ m pinhole and using a pixel dwell time of 1.58 μ s. Auto-fluorescent cells were captured at 500-580 nm. In the images, green represents collagen content, while cells are red.

Four to six circumferential confocal images from 4-6 cross-sections throughout the meniscus (total 16-36 images/treatment group) were analyzed with a custom MATLAB code based on a series of 2D fast Fourier transforms (FFT) to determine the degree of alignment and mean fiber diameter (Figure 2). Degree of alignment is reported using an established alignment index (AI) measure (Bowles et al., 2010; Chaudhuri et al., 1987; Ng et al., 2005). An initial FFT was performed to determine the maximum angle of alignment by summing the intensity of the FFT at 5° increments from 0–180°. This angle was further confirmed using the MATLAB radon transform function. The AI was determined by summing the intensity $\pm 20^\circ$ from the maximum alignment angle as previously described (Bowles et al., 2010). AI values range from 1(unaligned) to 4.5(completely aligned).

$$AI = \frac{\int_{\theta_m - 20^\circ}^{\theta_m + 20^\circ} I d\theta}{\left(\frac{40^\circ}{180^\circ}\right) * \int_{0^\circ}^{180^\circ} I d\theta}$$

To determine the diameter of fibers, the original image was rotated according to the radon transform to fix the maximum angle of alignment at 90°, ensuring that fibers are primarily vertical

in the image (Figure 2). The image was cropped and resized to avoid border effects and another FFT was performed, providing information on the frequency of sinusoidal patterns along the x-axis. To determine average fiber size a histogram of intensity along the x-axis was created and averaged. The histogram span corresponding to the average intensity was converted to pixels and further microns using the image size. The code was validated using control sinusoidal phantom images and by relating results to ImageJ calculated fiber diameters.

2.3.3 Histological and Biochemical Analysis

Histological and biochemical analysis were performed as previously reported (Ballyns et al., 2008; Puetzer and Bonassar, 2013). Histologically, radial and circumferential cross-sections of the menisci were fixed, embedded into paraffin, sectioned, and stained with picosirius red. They were imaged under polarized light to observe large-scale collagen organization.

For biochemical analysis 4 samples from each meniscus were weighed wet (WW), frozen, lyophilized, and weighed dry (DW). The samples were digested in 1.25 mg/ml papain solution and analyzed for DNA, GAG and collagen content via the Hoechst DNA assay (Kim et al., 1988), a modified 1,9-dimethylmethylene blue assay at pH 1.5 (Enobakhare et al., 1996), and a hydroxyproline assay (Neuman and Logan, 1950), respectively. Media samples taken every 2-3 days were analyzed with the same assays to track the release of biochemicals to the media.

2.3.4 Mechanical Analysis

The equilibrium and tensile moduli were determined as previously described (Ballyns et al., 2010; Gleghorn et al., 2007; Puetzer and Bonassar, 2013). Briefly, 2-4 4x1 mm plugs from each construct were obtained for compression testing, while radial and circumferential dogbone punches were taken to determine directional tensile properties. The equilibrium modulus was determined in confined compression via a stress relaxation test of 10x50 μ m steps fit to a poroelastic model using MATLAB (Gleghorn et al., 2007). The tensile modulus was determined

at a strain rate of 0.75%/s and calculated as the slope of the linear region of the stress-strain curve (Ballyns et al., 2010). Quasi-static loading was assumed and it was ensured failure was not near clamps. Circumferential and radial punches from neonatal bovine menisci were tested using the same procedure for native comparison.

2.4 Statistics

All data are expressed as mean $\pm$ SD and were analyzed by 3- and 2-way-ANOVA using Tukey's t-test for post hoc analysis. Significance was determined with $p < 0.05$.

3. Results

3.1 Construct Gross Appearance

Gross inspection of constructs demonstrated that 10 and 20 mg/ml clamped samples maintained their size and shape throughout culture, while unclamped menisci contracted significantly (Figure 3A). Unclamped menisci contracted to ~20%-40% of their original area and mass by 8 weeks of culture, while clamped menisci maintained 100% area for 8 weeks. This contraction is believed to be primarily due to water loss, as total collagen content within scaffolds remained relatively constant throughout culture (Figure 8). Ten mg/ml clamped menisci did decrease to ~80% of original mass, while 20 mg/ml clamped menisci maintained their initial mass throughout culture (Figure 3B).

3.2 Collagen Organization

Confocal reflectance microscopy, image analysis, and picrosirius red staining demonstrated clamped menisci developed native-like sized and aligned circumferential fibers and location-dependent radial morphologies similar to native tissue by 8 weeks of culture (Figure 4-7). Further this organization was present over a range of length scales.

Confocal reflectance in the circumferential direction demonstrated that at 0 weeks 10 and 20 mg/ml collagen gels start out as unorganized, homogeneous mixtures of collagen and cells (Figure 4). With time in culture, unclamped menisci had some improvement in collagen organization, but nothing resembling circumferential alignment (Figure 5). Clamped menisci (10 and 20 mg/ml) began to develop areas of aligned fibers at 2 weeks (data not shown), by 4 weeks the fibers were strongly aligned circumferentially throughout the entire scaffold (Figure 5), and by 8 weeks these fibers grew to match the size of native fibers (Figure 4).

Image analysis demonstrated clamped menisci increased fiber alignment and size with time over unclamped menisci ($p < 0.001$, Figure 6). By 4 weeks, clamped menisci matched the AI of native menisci. By 8 weeks, clamped meniscus fiber diameter increased ~3.5 fold to match native diameters, while unclamped samples had ~10 μ m diameter fibers throughout culture. Further, after 2 weeks, 20 mg/ml clamped menisci maintained larger diameters than 10 mg/ml clamped menisci ($p < 0.001$).

Confocal reflectance imaging demonstrated that the outer-portion of native menisci contains radial tie-fibers that extend perpendicular from the surface, while the central-portion of the meniscus contains larger collagen fibers with branches of smaller, randomly oriented fibers (Figure 4). Initially, 10 and 20 mg/ml collagen gels are unorganized mixtures of collagen and cells. With time in culture, unclamped menisci improved organization slightly, with similar morphologies in circumferential and radial directions. Clamped menisci (10 and 20 mg/ml) developed radial fiber morphologies perpendicular to the surface, similar to the native radial tie-fibers by 4 weeks. Further, by 8 weeks clamped menisci developed larger native-like collagen fibers in the central-portion of the menisci with smaller fibers branching off (Figure 4).

Picrosirius red staining revealed that the native-like circumferential and radial morphologies in clamped menisci were also present at a larger length-scale (Figure 7). Again, unclamped menisci demonstrated little improvement in organization aside from compaction of collagen. Clamped menisci developed circumferentially aligned fibers by 4 weeks that grow in size by 8

223 weeks. In the radial direction, clamped menisci develop larger fibers with smaller randomly
224 oriented fiber branches in the center of the construct similar to native tissue by 4 weeks. By 8
225 weeks, large radial fibers running perpendicularly across the scaffold were visible in 10 and 20
226 mg/ml clamped menisci.

228 3.3 Biochemical Analysis

229 Clamping had little effect on biochemical synthesis with 10 and 20 mg/ml clamped menisci
230 maintaining consistent GAG and collagen properties throughout culture (Figure 8). Clamped
231 menisci had a significant decrease in DNA content compared to unclamped menisci. At 2
232 days, all menisci had a DNA wet weight concentration of ~0.8 mg DNA/meniscus. By 2
233 weeks of culture, this dropped to 0.4 mg DNA/meniscus for unclamped samples and 0.1
234 mg DNA/meniscus for clamped samples. This level of DNA was maintained throughout the
235 remainder of culture (Supplemental Figure 1). The final DNA concentration of 0.1 μ g/mg WW in
236 clamped menisci is a tenth of the reported native DNA concentration of 0.10% WW (Herwig et
237 al., 1984).

238 Clamped menisci maintained consistent GAG content throughout culture reaching only 2-3%
239 GAG content of native tissue, while unclamped menisci had significant improvements in GAG
240 beginning at 2 weeks (Figure 8). However, normalizing GAG content to DNA content showed
241 that both clamped and unclamped menisci had a similar ~1.5 fold increase by 8 weeks
242 ($p < 0.001$, data not shown). Additionally, both clamped and unclamped menisci released the
243 same amount of GAG to the media throughout culture (Figure 8). Interestingly, between weeks
244 2-4 all menisci had a similar significant increase in GAG release ($p < 0.001$). After 4 weeks, all
245 menisci decreased GAG release to levels below that recorded in the first two weeks (Figure 8).

246 As expected, both clamped and unclamped 20 mg/ml menisci had significantly higher
247 collagen concentration than 10 mg/ml menisci. Unclamped menisci maintained collagen

content throughout culture, with a slight decrease by 8 weeks. Both concentrations of clamped menisci however demonstrated a slight decrease in collagen concentration at 2 weeks and then a progressive increase in collagen with time. By 8 weeks, 10 and 20 mg/ml clamped menisci had significantly more collagen content than unclamped ($p<0.05$, Figure 8) and had 10-20% collagen of native tissue. There were no differences in collagen release to the media between clamped and unclamped menisci (Figure 8).

3.4 Mechanical Analysis

Clamped menisci improved both equilibrium and tensile moduli with time, and developed anisotropic mechanical properties by 4 weeks (Figure 9). All menisci significantly improved the equilibrium modulus throughout culture ($p<0.001$), with clamped menisci having significantly higher equilibrium moduli than unclamped menisci by 8 weeks ($p<0.001$). Further, the 20 mg/ml clamped menisci equilibrium modulus increased 17-fold over 8 weeks to be significantly higher than 10 mg/ml clamped menisci, which improved only 14-fold ($p<0.001$). Both concentrations of clamped menisci reached native equilibrium modulus values by 8 weeks (Sweigart et al., 2004).

Tensile analysis demonstrated both concentrations of clamped menisci significantly improved the circumferential tensile modulus over the unclamped controls by 4 weeks ($p<0.05$), and further improved this difference through 8 weeks (Figure 9). By 8 weeks, 10 and 20 mg/ml clamped menisci improved the circumferential tensile modulus 5- and 12-fold, respectively. Further, all clamped menisci improved radial tensile modulus ~7-fold by 8 weeks ($p<0.001$).

Unclamped menisci maintained isotropic properties through 4 weeks of culture; however radial 8 week analysis was unable to be performed due to the significant contraction of unclamped scaffolds. Clamped menisci became anisotropic after 4 weeks, with ~2-3 fold differences between circumferential and radial directions ($p<0.001$, Figure 9). This ratio of anisotropy matches neonatal meniscus levels of anisotropy at 3-fold (circumferential modulus 13 ± 2.5 MPa, radial modulus 4.1 ± 0.43 MPa)

4. Discussion

This study demonstrated that anchoring anatomically-shaped tissue engineered menisci at the horns induces the development of circumferentially and radially organized collagen fibers that resemble native tissue at multiple length scales. Clamped menisci maintained size and shape while developing circumferential fibers throughout the entire meniscus and radial morphologies that varied from the outer to the inner portion of the meniscus, similar to native tissue (Aspden et al., 1985; Kambic and McDevitt, 2005; Petersen and Tillmann, 1998; Rattner et al., 2011; Vanderploeg et al., 2012). This is the first study to our knowledge, to demonstrate development of native-like radial fibers in tissue engineered meniscus. Further, this organization was reflected in the development of anisotropic tensile properties similar to the level of anisotropy of neonatal and adult menisci (Makris et al., 2011; Tissakht and Ahmed, 1995).

It is well known that mechanical constraints and construct boundaries guide the alignment of fibers in cell-seeded low density (1-6mg/ml) collagen gels by harnessing cellular traction (Bell et al., 1979; Bowles et al., 2010; Costa et al., 2003; Grinnell and Lamke, 1984; Thomopoulos et al., 2005). In this study, anchoring meniscus implants at the horns restricted contraction, creating circumferential hoop stress to encourage the alignment of cells and fibers. To our knowledge, this study is the first to demonstrate mechanical boundary conditions to guide the organization of fibers by meniscal fibrochondrocytes. Additionally, clamped menisci maintained their size throughout culture, contrary to the contraction typically observed in low density boundary culture (Bell et al., 1979; Bowles et al., 2010; Costa et al., 2003; Grinnell and Lamke, 1984; Thomopoulos et al., 2005). Contraction is known to decrease with increasing concentrations of collagen (Bell et al., 1979; Cross et al., 2010; Puetzer and Bonassar, 2013), and thus the higher concentration of collagen may be responsible for the lack of contraction in clamped menisci.

There appeared to be no adverse effect of collagen concentration on degree of organization. Ten and 20 mg/ml clamped menisci had similar levels of organization, with 20 mg/ml menisci producing larger diameter fibers and significantly better mechanical properties. Recently, doughnut-shaped boundary constraints were used with a scaffold free construct to develop meniscus-like scaffolds; however without collagen fibrils present, collagen fiber development was limited (Higashioka et al., 2014). In this study, self-assembled collagen fibrils are present within hours of gelling the menisci, providing an infrastructure for cells to align and build on.

Image analysis demonstrated the circumferential fibers developed in clamped menisci had similar alignment and size as native tissue. Collagen gels self-assemble into fibrils with diameters of 20-70 nm within a few hours of gelation (Kadler et al., 1996). This process is thought to be intrinsic to collagen gels; however the growth of these fibrils to fibers is primarily controlled by cells (Kadler et al., 1996). It has been reported cell-seeded collagen gels form collagen bundles ~2-10 μ m in diameter and that collagen concentration has no effect on the fiber diameters (Cross et al., 2010). Similarly, in this study, clamped and unclamped menisci developed 2-10 μ m fibers by 2 days with no difference between concentrations. Unclamped menisci maintained fiber diameters of 10 μ m throughout culture, while clamped menisci significantly improved fiber diameters to ~30-40 μ m by 8 weeks. Interestingly, 20 mg/ml clamped menisci maintained larger diameter fibers throughout culture compared to 10 mg/ml clamped menisci.

Clamped meniscus fiber size measurements were similar to those of neonatal bovine tissue, as well as those in dog and human menisci (30-50 μ m)(Kambic and McDevitt, 2005; Petersen and Tillmann, 1998). Further, it has been reported native menisci (independent of species, age, or sex) organize fibrils into ~5-10 μ m core bundles which group together to form the larger collagen fibers commonly seen in meniscal sections (Rattner et al., 2011). In this study, the smallest collagen fiber diameter in clamped menisci (found using the entire span of the rotated FFT histogram) increased from 2 μ m to 10 μ m by 2 weeks of culture, and remained at 10 μ m

through 8 weeks (data not shown). This data provides further support to the idea that meniscal fibrochondrocytes may have an intrinsic ability to form specific meniscal tissue structures (Ibarra et al., 1997; Puetzer and Bonassar, 2013).

Image analysis also demonstrated clamped menisci fiber diameters grew the most between 4-8 weeks. It has been reported in both tendon/ligament (Lee et al., 2005; Wang et al., 2003) and meniscus (Baker and Mauck, 2007), that substrate alignment enhances the organization of newly synthesized collagen. This current study is consistent with these data. By 4 weeks clamped menisci had circumferentially aligned fibers throughout the entire scaffold, but little improvement in fiber size. The largest growth in fiber size was after 4 weeks, once the fibrochondrocytes had aligned the fibers. Further, a similar trend was seen in collagen concentration of clamped menisci. From 2 weeks on, when fibers first begin to align in clamped menisci, collagen content progressively increased to be significantly more than the unclamped counterparts at 8 weeks. Collectively, these data suggest a feed-forward process by which cellular remodeling of existing collagen signals further formation of larger, more organized collagen fibers.

Clamping had much less of an effect on biochemical composition compared to organization and mechanical properties. Collagen and GAG content in clamped samples changed by <20% over 8 weeks, while fiber size increased 3-4 fold, compressive modulus increased 14-17 fold, and tensile modulus increased 5-12 fold. These data show that the alignment, growth and organization of collagen fibers in clamped menisci were primarily responsible for the increase in mechanical properties. This provides further support to the idea that organization of ECM rather than amount of ECM dictates the functional maturation of meniscal scaffolds (Baker and Mauck, 2007). Further, this work is consistent with recent work that demonstrates cartilage mechanics can be understood in the context of rigidity percolation theory (Silverberg et al., 2014). This theory predicts that non-percolating networks composed of thin, poorly connected fibers form

very compliant networks. Increasing the size and connectivity of the fibers can move these networks over the percolation threshold, causing large (10-100 fold) increases in stiffness.

By 4 weeks, clamped menisci developed anisotropic tensile properties. This mirrored the development of distinct circumferential and radial organizations. Further, at 8 weeks clamped menisci developed equilibrium moduli equal to native values (110-490 kPa) (Sweigart et al., 2004). However, although clamped menisci developed native sized and aligned fibers by 8 weeks, the tensile moduli remained orders of magnitudes below immature native values (circumferential 13-26 MPa (Eleswarapu et al., 2011), radial 4 MPa). This suggests further development of collagen fibers is needed which could be facilitated by using mechanical or chemical stimulation to improve collagen fiber density and crosslinking.

In addition to enabling the generation of organized tissues for therapeutic interventions, anchored meniscus cultures may also serve as a useful meniscus development model. During development, menisci start out as dense unorganized, condensed mesenchyme. At 10 weeks the attachments to the tibial plateau are formed (Merida-Velasco et al., 1997). By 14 weeks, cells begin to align and form collagen bundles, which with time grow in size as perpendicular radial fibers appear. Throughout this process DNA concentration decreases monotonically (Clark and Ogden, 1983; Fukazawa et al., 2009). Similarly, in this study, clamped menisci start out as unorganized, homogeneous mixtures of cells and collagen. With time in culture DNA concentrations decrease as collagen fibers align and then grow in both the circumferential and radial direction. These early stages of development are intrinsically regulated, but later stages as collagen fibers mature require mechanical stimulation (Mikic et al., 2000). Thus additional mechanical stimulation may be needed to further develop these clamped collagen menisci.

Collectively, this study demonstrates the benefit of using bio-inspired mechanical boundary conditions to drive the formation of a highly organized collagen fiber network. These are some of the most organized meniscal constructs developed to date, however clamped menisci only reach 2-3% GAG and 10-20% collagen of native tissue. Future work should focus on improving

377 biochemical composition and tensile properties for these menisci to serve as whole meniscal
378 replacements.

379

Acknowledgments

The authors would like to thank the National Science Foundation's Graduate Research Fellowship Program, the Cornell BME NSF GK-12 program: DGE 0841291, the Microscopy and Imaging Facility, Life Sciences Core Laboratories Center at Cornell University, Dr. Hod Lipson, John Cheeseborough, Dr. Cynthia Reinhart-King, Mary Clare McCorry, Shawn Carey and the members of the Bonassar Lab for their support in this research.

References

- Aspden, R.M., Yarker, Y.E., Hukins, D.W., 1985. Collagen orientations in the meniscus of the knee joint. *J Anat* 140 (Pt 3), 371-380.
- Baker, B.M., Mauck, R.L., 2007. The effect of nanofiber alignment on the maturation of engineered meniscus constructs. *Biomaterials* 28, 1967-1977.
- Balint, E., Gatt, C.J., Jr., Dunn, M.G., 2012. Design and mechanical evaluation of a novel fiber-reinforced scaffold for meniscus replacement. *J Biomed Mater Res A* 100, 195-202.
- Ballyns, J.J., Bonassar, L.J., 2011. Dynamic compressive loading of image-guided tissue engineered meniscal constructs. *J Biomech* 44, 509-516.
- Ballyns, J.J., Gleghorn, J.P., Niebrzydowski, V., Rawlinson, J.J., Potter, H.G., Maher, S.A., Wright, T.M., Bonassar, L.J., 2008. Image-guided tissue engineering of anatomically shaped implants via MRI and micro-CT using injection molding. *Tissue Eng Part A* 14, 1195-1202.
- Ballyns, J.J., Wright, T.M., Bonassar, L.J., 2010. Effect of media mixing on ECM assembly and mechanical properties of anatomically-shaped tissue engineered meniscus. *Biomaterials* 31, 6756-6763.
- Bell, E., Ivarsson, B., Merrill, C., 1979. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci U S A* 76, 1274-1278.
- Bowles, R.D., Williams, R.M., Zipfel, W.R., Bonassar, L.J., 2010. Self-assembly of aligned tissue-engineered annulus fibrosus and intervertebral disc composite via collagen gel contraction. *Tissue Eng Part A* 16, 1339-1348.
- Carey, S.P., Kraning-Rush, C.M., Williams, R.M., Reinhart-King, C.A., 2012. Biophysical control of invasive tumor cell behavior by extracellular matrix microarchitecture. *Biomaterials* 33, 4157-4165.
- Chaudhuri, S., Nguyen, H., Rangayyan, R.M., Walsh, S., Frank, C.B., 1987. A Fourier domain directional filtering method for analysis of collagen alignment in ligaments. *IEEE Trans Biomed Eng* 34, 509-518.
- Clark, C.R., Ogden, J.A., 1983. Development of the menisci of the human knee joint. Morphological changes and their potential role in childhood meniscal injury. *J Bone Joint Surg Am* 65, 538-547.
- Costa, K.D., Lee, E.J., Holmes, J.W., 2003. Creating alignment and anisotropy in engineered heart tissue: role of boundary conditions in a model three-dimensional culture system. *Tissue Eng* 9, 567-577.

422 Cross, V.L., Zheng, Y., Won Choi, N., Verbridge, S.S., Sutermastr, B.A., Bonassar, L.J.,
 423 Fischbach, C., Stroock, A.D., 2010. Dense type I collagen matrices that support cellular
 424 remodeling and microfabrication for studies of tumor angiogenesis and vasculogenesis in
 425 vitro. *Biomaterials* 31, 8596-8607.

426 Eleswarapu, S.V., Responde, D.J., Athanasiou, K.A., 2011. Tensile properties, collagen content,
 427 and crosslinks in connective tissues of the immature knee joint. *PLoS One* 6, e26178.

428 Enobakhare, B.O., Bader, D.L., Lee, D.A., 1996. Quantification of sulfated glycosaminoglycans
 429 in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylen blue. *Anal Biochem* 243,
 430 189-191.

431 Fukazawa, I., Hatta, T., Uchio, Y., Otani, H., 2009. Development of the meniscus of the knee
 432 joint in human fetuses. *Congenit Anom (Kyoto)* 49, 27-32.

433 Gleghorn, J.P., Jones, A.R., Flannery, C.R., Bonassar, L.J., 2007. Boundary mode frictional
 434 properties of engineered cartilaginous tissues. *Eur Cell Mater* 14, 20-28; discussion 28-29.

435 Gould, R.A., Chin, K., Santisakultarm, T.P., Dropkin, A., Richards, J.M., Schaffer, C.B., Butcher,
 436 J.T., 2012. Cyclic strain anisotropy regulates valvular interstitial cell phenotype and tissue
 437 remodeling in three-dimensional culture. *Acta Biomater* 8, 1710-1719.

438 Grinnell, F., Lamke, C.R., 1984. Reorganization of hydrated collagen lattices by human skin
 439 fibroblasts. *J Cell Sci* 66, 51-63.

440 Hasan, J., Fisher, J., Ingham, E., 2013. Current strategies in meniscal regeneration. *J Biomed*
 441 *Mater Res B Appl Biomater*.

442 Herwig, J., Egner, E., Buddecke, E., 1984. Chemical changes of human knee joint menisci in
 443 various stages of degeneration. *Ann Rheum Dis* 43, 635-640.

444 Higashioka, M.M., Chen, J.A., Hu, J.C., Athanasiou, K.A., 2014. Building an anisotropic
 445 meniscus with zonal variations. *Tissue Eng Part A* 20, 294-302.

446 Huey, D.J., Athanasiou, K.A., 2011. Maturation growth of self-assembled, functional menisci
 447 as a result of TGF-beta1 and enzymatic chondroitinase-ABC stimulation. *Biomaterials* 32,
 448 2052-2058.

449 Ibarra, C., Jannetta, C., Vacanti, C.A., Cao, Y., Kim, T.H., Upton, J., Vacanti, J.P., 1997. Tissue
 450 engineered meniscus: a potential new alternative to allogeneic meniscus transplantation.
 451 *Transplant Proc* 29, 986-988.

452 Kadler, K.E., Holmes, D.F., Trotter, J.A., Chapman, J.A., 1996. Collagen fibril formation.
 453 *Biochem J* 316 (Pt 1), 1-11.

454 Kale, A., Kopuz, C., Dikici, F., Demir, M.T., Corumlu, U., Ince, Y., 2010. Anatomic and
455 arthroscopic study of the medial meniscal horns' insertions. *Knee Surg Sports Traumatol*
456 *Arthrosc* 18, 754-759.

457 Kambic, H.E., McDevitt, C.A., 2005. Spatial organization of types I and II collagen in the canine
458 meniscus. *J Orthop Res* 23, 142-149.

459 Kawamura, S., Lotito, K., Rodeo, S.A., 2003. Biomechanics and healing response of the
460 meniscus. *Oper Techn Sport Med* 11, 68-76.

461 Khetia, E.A., McKeon, B.P., 2007. Meniscal allografts: biomechanics and techniques. *Sports*
462 *Med Arthrosc* 15, 114-120.

463 Kim, Y.J., Sah, R.L., Doong, J.Y., Grodzinsky, A.J., 1988. Fluorometric assay of DNA in
464 cartilage explants using Hoechst 33258. *Anal Biochem* 174, 168-176.

465 Kon, E., Chiari, C., Marcacci, M., Delcogliano, M., Salter, D.M., Martin, I., Ambrosio, L., Fini, M.,
466 Tschon, M., Tognana, E., Plasenzotti, R., Nehrer, S., 2008. Tissue engineering for total
467 meniscal substitution: animal study in sheep model. *Tissue Eng Part A* 14, 1067-1080.

468 Lee, C.H., Shin, H.J., Cho, I.H., Kang, Y.M., Kim, I.A., Park, K.D., Shin, J.W., 2005. Nanofiber
469 alignment and direction of mechanical strain affect the ECM production of human ACL
470 fibroblast. *Biomaterials* 26, 1261-1270.

471 Lee, E.J., Holmes, J.W., Costa, K.D., 2008. Remodeling of engineered tissue anisotropy in
472 response to altered loading conditions. *Ann Biomed Eng* 36, 1322-1334.

473 Makris, E.A., Hadidi, P., Athanasiou, K.A., 2011. The knee meniscus: structure-function,
474 pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials* 32,
475 7411-7431.

476 Mandal, B.B., Park, S.H., Gil, E.S., Kaplan, D.L., 2011. Multilayered silk scaffolds for meniscus
477 tissue engineering. *Biomaterials* 32, 639-651.

478 Merida-Velasco, J.A., Sanchez-Montesinos, I., Espin-Ferra, J., Rodriguez-Vazquez, J.F.,
479 Merida-Velasco, J.R., Jimenez-Collado, J., 1997. Development of the human knee joint.
480 *Anat Rec* 248, 269-278.

481 Messner, K., Gao, J., 1998. The menisci of the knee joint. Anatomical and functional
482 characteristics, and a rationale for clinical treatment. *J Anat* 193 (Pt 2), 161-178.

483 Mikic, B., Johnson, T.L., Chhabra, A.B., Schalet, B.J., Wong, M., Hunziker, E.B., 2000.
484 Differential effects of embryonic immobilization on the development of fibrocartilaginous
485 skeletal elements. *J Rehabil Res Dev* 37, 127-133.

486 Neuman, R.E., Logan, M.A., 1950. The determination of hydroxyproline. J Biol Chem 184, 299-
487 306.

488 Ng, C.P., Hinz, B., Swartz, M.A., 2005. Interstitial fluid flow induces myofibroblast differentiation
489 and collagen alignment in vitro. J Cell Sci 118, 4731-4739.

490 Petersen, W., Tillmann, B., 1998. Collagenous fibril texture of the human knee joint menisci.
491 Anat Embryol 197, 317-324.

492 Puetzer, J.L., Bonassar, L.J., 2013. High density type I collagen gels for tissue engineering of
493 whole menisci. Acta Biomater 9, 7787-7795.

494 Rattner, J.B., Matyas, J.R., Barclay, L., Holowaychuk, S., Sciore, P., Lo, I.K., Shrive, N.G.,
495 Frank, C.B., Achari, Y., Hart, D.A., 2011. New understanding of the complex structure of
496 knee menisci: implications for injury risk and repair potential for athletes. Scand J Med Sci
497 Sports 21, 543-553.

498 Silverberg, J.L., Barrett, A.R., Das, M., Peterson, P.B., Bonassar, L.B., Cohen, I., 2014.
499 Structure-Function Relations in Neonatal Bovine Articular Cartilage Under Shear.
500 Biophysical Journal 107.

501 Sweigart, M.A., Zhu, C.F., Burt, D.M., DeHoll, P.D., Agrawal, C.M., Clanton, T.O., Athanasiou,
502 K.A., 2004. Intraspecies and interspecies comparison of the compressive properties of the
503 medial meniscus. Ann Biomed Eng 32, 1569-1579.

504 Thomopoulos, S., Fomovsky, G.M., Holmes, J.W., 2005. The development of structural and
505 mechanical anisotropy in fibroblast populated collagen gels. J Biomech Eng 127, 742-750.

506 Tienen, T.G., Heijkants, R.G., de Groot, J.H., Schouten, A.J., Pennings, A.J., Veth, R.P., Buma,
507 P., 2006. Meniscal replacement in dogs. Tissue regeneration in two different materials with
508 similar properties. J Biomed Mater Res B Appl Biomater 76, 389-396.

509 Tissakht, M., Ahmed, A.M., 1995. Tensile stress-strain characteristics of the human meniscal
510 material. J Biomech 28, 411-422.

511 Vanderploeg, E.J., Wilson, C.G., Imler, S.M., Ling, C.H., Levenston, M.E., 2012. Regional
512 variations in the distribution and colocalization of extracellular matrix proteins in the juvenile
513 bovine meniscus. J Anat 221, 174-186.

514 Wang, J.H., Jia, F., Gilbert, T.W., Woo, S.L., 2003. Cell orientation determines the alignment of
515 cell-produced collagenous matrix. J Biomech 36, 97-102.

516 Zur, G., Linder-Ganz, E., Elsner, J.J., Shani, J., Brenner, O., Agar, G., Hershman, E.B.,
517 Arnoczky, S.P., Guilak, F., Shterling, A., 2011. Chondroprotective effects of a
518 polycarbonate-urethane meniscal implant: histopathological results in a sheep model. Knee
519 Surg Sports Traumatol Arthrosc 19, 255-263.

Figure Captions:

Figure 1: Injection molding process and clamping of meniscal constructs. Ovine based meniscal molds had 12 mm long extension added to the horns, high density collagen meniscal constructs were created using injection molding techniques and constructs were clamped at the extensions throughout culture.

Figure 2: Fast Fourier transform based image analysis. An initial FFT is used to determine major alignment angle and alignment index. Image is rotated to major alignment angle and 2nd FFT is converted to a histogram which is averaged to find mean diameter. Mean diameter determined by code matches ImageJ calculated diameters represented by white bars on original image.

Figure 3: A) Photographs of constructs, B) percent area and percent mass of constructs throughout culture. Clamped menisci maintain size and shape throughout culture. Bar = 5mm. Significance between # clamped and unclamped, % 10 and 20 mg/ml clamped menisci ($p < 0.05$)

Figure 4: Circumferential and radial confocal reflectance images of engineered and native menisci. Clamped menisci develop circumferentially aligned fibers by 4 weeks and radial morphologies very similar to native tissue. Scale bar = 50 μm , Collagen = green, cells = red. Arrows point to areas of developing circumferential fibers (CF), radial fibers (RF), and the surface location (S).

Figure 5: Circumferential confocal reflectance images from cross-sections throughout the menisci demonstrate circumferential alignment all the way around the clamped menisci at 4 weeks for both 10 and 20 mg/ml menisci. This was maintained through 8 weeks. No alignment was present in unclamped samples. White scale bar = 50 μm , Collagen = green, cells = red

Figure 6: Circumferential image analysis for alignment index (1 unorganized, 4.5 organized) and mean fiber diameter in engineered vs native menisci. Clamped menisci match native alignment by 4 weeks and diameter by 8 weeks. Significance between # clamped and unclamped, + engineered to native, % 10 vs 20 mg/ml clamped menisci ($p < 0.05$)

Figure 7: Circumferential and radial picrosirius red samples imaged with polarized light. Clamped menisci develop native-like circumferentially aligned fibers and radial morphologies by 4 weeks, which further develop by 8 weeks. Arrows point to areas of developing circumferential fibers (CF), radial fibers (RF). Scale bar = 100 μm

Figure 8: GAG and collagen content per meniscus (top row) and in media per day (bottom row). Significance between # clamped and unclamped, % 10 vs 20 mg/ml clamped menisci, * bracket group, and \$ compared to all other data points ($p < 0.05$).

Figure 9: Equilibrium modulus, tensile modulus, clamped menisci tensile modulus in circumferential and radial direction, and depiction of mechanical samples. Clamped menisci develop superior properties to unclamped menisci by 8 weeks and develop anisotropic tensile properties by 4 weeks. Significance between % 10 vs 20 mg/ml clamped menisci and * bracket group ($p < 0.05$)

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9