Harnessing Cellular-Derived Forces in Self-Assembled Microtissues to Control the Synthesis and Alignment of ECM

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Abstract

The alignment and blend of extracellular matrix (ECM) proteins give a tissue its specific mechanical properties as well as its physiological function. Various tissue engineering methods have taken purified ECM proteins and aligned them into gels, sponges and threads. Although, each of these methods has created aligned ECM, they have had many limitations including loss of hierarchical collagen structure and poor mechanical performance. Here, we have developed a new method to control ECM synthesis using self-assembled cells. Cells were seeded into custom designed, scaffold-free, micro-molds with fixed obstacles that harnessed and directed cell-mediated stresses. Cells within the microtissue reacted to self-generated tension by aligning, elongating, and synthesizing an ECM whose organization was dictated by the strain field that was set by our micro-mold design. We have shown that through cell selection, we can create tissues with aligned collagen II or aligned elastin. We have also demonstrated that these self-assembled microtissues have mechanical properties in the range of natural tissues and that mold design can be used to further tailor these mechanical properties.

Keywords

Self-assembly; spheroid; cell-derived ECM; mechanical properties; strong tissues; aligned ECM

Introduction

The extracellular matrix (ECM) is a complex biocomposite that serves diverse functions including the provision of biological cues and mechanical support for cells. This complex interplay between the structure and composition of the ECM is crucial to the utility of a tissue as a whole. For decades, investigators have been trying to recapitulate the complexity of the ECM with ambitions of creating natural and strong tissue equivalents/biomaterials with precise spatial organization and alignment. Collagen, silk, fibrin, and elastin are some of the naturally derived polymers that have been fabricated, with various levels of success, into aligned biomaterials/tissues (1-5). Much research has focused on collagen because of its abundance and because early successes demonstrated its biocompatibility and ability to restore cells to a more natural phenotype (6). Although purified collagen has been successfully fabricated and aligned into a variety of materials including gels, porous sponges, and threads, and has been populated by a variety of cell types, major barriers arise when using purified collagen (7). Specifically, biomaterials made from purified collagen are far weaker than natural tissues, they lose their complex hierarchical structure and natural cross-links, and they do not have the biocomplexity (composition, blend) of the ECM that cells synthesize (8-10).

On the other end of the spectrum, researchers have investigated decellularizing natural tissues and organs. These materials have preserved much of the precise structural architecture of the ECM and can serve as complex scaffolds for cell attachment or cell growth (11, 12). However, there are serious limitations with decellularized organs including availability, lot-to-lot variability, and transmission of diseases. Tissue grafts obtained from cadavers have inherent variability due to the fact that each graft is from a unique donor (genetics, age, size, shape, medical history, etc.) as well as a unique anatomical site (or maximum of two) of that donor.

And, although these materials can be crudely processed (cutting, shaping) there is no possibility to truly engineer their underlying mechanical or biological composition. Missing is a natural, strong, material that can be tailored in terms of mechanical properties, composition, and architecture.

An increasing amount of evidence has demonstrated that self-assembled microtissues more closely match the complexity of *in vivo* tissues including the architecture, differentiated functions, metabolic activity as well as the synthesis of ECM (13-17). We have previously demonstrated that through micro-mold design, we can form complex shaped microtissues such as toroids, rods, honeycombs, and loop ended dogbones (18-20). We have also demonstrated that self-assembling cells generate measurable contractile forces and that micro-molds can harness these cell-mediated stresses during self-assembly and direct the strain that develops in the resulting tissue (21, 22). Specifically, we have shown that when cells are seeded in a loop-ended dogbone recess, the cells will align and stretch uniaxially. Our hypothesis is that micro-mold design dictates cell alignment which in turn controls the alignment of the ECM that these cells synthesize and this ECM alignment dictates the mechanical properties of the tissue. The objectives of this study was to form self-assembled microtissues in five different mold designs, characterize the cell and ECM alignment in these five designs and test mechanical properties.

Here, we have shown that by simply altering mold design (trough size/shape and peg constraint placement) we can recapitulate a number of tension environments to create aligned tissues. We designed different micro-molds to create tissues with uniaxial tension (loop-ended dogbones tissues), circumferential tension (toroid tissues), biaxial tension (cross tissues), equibiaxial tension (trampoline tissue), and multiaxial tension (triangle tissue) as these alignments represent a number of alignments that are found in natural tissues. When cells were

seeded into these non-adhesive, scaffold-free molds, they formed cell-cell adhesions, exerted contractile forces, aligned, and over time, formed aligned ECM. The alignment of the cells and their ECM varied according to the design of the micro-mold and the kind of tension that it was designed to induce in the tissue. The resulting tissues were strong and exhibited mechanical properties in the range of natural tissues. Furthermore, we demonstrated that through cell selection, we can elicit the synthesis of aligned collagen II and elastin, and through mold design, we can further tailor mechanical properties.

Materials and Methods

Micro-Mold Design and Gel Casting

Micro-molds were designed using the computer aided design (CAD) software SolidWorks (SolidWorks Corporation, Concord, MA). Dogbone molds have been previously described (22). Briefly, dogbone recesses consisted of two tori with inner diameters of 800 μm and outer diameters of 2.4 mm, set 5 mm apart and connected by a trough (connecting rod) of width 400 μm or 600 μm . Toroid molds have also been previously described (19). Each toroid feature was a cylindrical well, 1.4 mm in diameter and contained a circular trough and a cylindrical peg that extended upward from the center of the circular trough. The circular trough was 400 μm wide and the cylindrical peg was 600 μm in diameter. The small trampoline mold was a hexagon shaped recess with six toroid like features at the vertices of the hexagon. The total surface area of the bottom of the small trampoline recess was 7.91 mm². The cross molds were designed as a single cylindrical well with four pegs spaced 500 μm from the cylinder's edge aligned in a cross formation at 0°, 90°, 180° and 270°. The cross feature had a bottom surface area of 8.2 mm². The triangle mold was an equilateral triangle (bottom surface area of 4.1 mm²) with rounded vertices that contained pegs 400 μm from the edge of the vertices. The large

trampoline molds were designed as a hexagonal shaped tissue with three outer orbitals of pegs spaced 600 μm apart. The central portion of the large trampoline had a surface area of 22 mm^2 .

CAD files were used to produce thermowax molds with a rapid prototyping machine (3D Systems Corporation, Valencia, CA). Wax molds were replicated using OOMOO® 25 Silicone Rubber (Smooth-On, East Texas, PA). Silicone molds were submerged overnight in 100% ethanol, rinsed in distilled water, and autoclaved. To create agarose hydrogel micro-molds, 2% (weight/volume) sterile molten agarose was added to each silicone mold, allowed to gel, removed from the silicone molds, and then placed in a sterile 6-well tissue culture dish. The gels were rinsed with fresh culture medium and then equilibrated in culture media at 37°C.

Cell Culture and Micro-mold Seeding

Human chondrocytes (passage 3-9) were purchased from PromoCell (Heidelberg, Germany) and were expanded in PromoCell Chondrocyte Growth Medium supplemented with 1% pen/strep at 37°C and 5% CO_2 . Cells were removed from flasks using a standard trypsin process. Briefly, cells were exposed to 0.04% trypsin for 10 minutes, quenched with medium containing serum, spun down at 800 rpm for 6 minutes, resuspended, and counted. Dogbone molds contain 8 dogbone recesses per a mold, and each dogbone was seeded with 0.835 and 0.121 million cells for the 400 μm and 600 μm width dogbone molds, respectively. The small trampoline, triangle, toroid, and large trampoline, cross molds were seeded with 0.3, 0.2, 0.3, and 2 million cells, respectively. After 30 minutes, 3 ml of chondrogenic media (DMEM supplemented with 1% pen/strep, 64 $\mu\text{g/ml}$ L-ascorbic acid 2-phosphate, 200 $\mu\text{g/ml}$ L-proline, 5 mg/ml ITS Premix Universal Culture Supplement, 10 ng/ml human recombinant TGF- β 1, and

1.0 mM dexamethasone) was added. Chondrocyte microtissues were fed every 2-3 days with 3 ml of chondrogenic media and were maintained at 37°C and 5% CO₂. All chondrocyte tissues (passage 3-9) matured in micromolds immunostained positive for collagen II, consistent with prior studies showing that chondrocyte phenotype is restored when cells cultured in 2D and are then cultured in 3D (23).

Normal human fibroblasts (NHF) (passage 3-7) were purchased from PromoCell (Heidelberg, Germany) and were expanded in high glucose DMEM with 10% fetal bovine serum (FBS), 1% pen/strep at 37°C and 10% CO₂. Cells were removed from flasks using a standard trypsin process. Briefly, cells were exposed to 0.05% trypsin for 10 minutes, quenched with serum containing medium, spun down at 800 rpm for 6 minutes, resuspended, and counted. NHFs were seeded in 400 µm dogbone molds at a density of 0.871 million cells per dogbone and into small trampoline molds at a density of 0.3 million cells. After 30 minutes, 3 ml of high glucose DMEM supplemented with 1% pen/strep, 50 µg/ml L-ascorbic acid 2-phosphate, 30 µg/ml L-proline, and 7.5 ng/ml human recombinant TGF-β1 was added to each well and media was changed every 2-3 days. NHF microtissues were maintained at 37°C and 10% CO₂.

Immunohistochemistry, Confocal Microscopy, and Nuclei Alignment Analysis

To analyze nuclei alignment, chondrocyte microtissues were matured for 4 days and then fixed in 4% paraformaldehyde. Microtissues were labeled with 0.3 mM 4', 6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) and 6.6 µM Oregon Green® 488 Phalloidin (Life Technologies, Grand Island, NY) and confocal images were captured with a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA). Using the analyze

particle function in Image J Software (Rasband, W.S., ImageJ, NIH), an ellipse was fit to each nuclei, and the angle of the major axis of each ellipse was measured. Angle bins were set at 30° intervals for the cross, trampoline, and triangle microtissues, and at 15° intervals for the dogbone microtissues. For each field of view (n=8, 9, 7, and 6 for the dogbone, triangle, cross, and trampoline, respectively, with an average of 60 nuclei for each field of view) the number of nuclei in each bin was counted and the percentage of nuclei in each angle bin was averaged for all fields of view to create angle distribution histograms. For each shape, to determine if each angle bin had statistically more nuclei than each of the other bins, a student t-test (two-tail, unequal variance), was performed with a 95% confidence interval.

To analyze ECM synthesis, chondrocyte microtissues matured for 4 and 8 weeks were fixed with 4% paraformaldehyde and permeabilized for 30 minutes with 0.2% Triton x-100. Microtissues were then immunostained by incubation in a blocking solution containing PBS with 5% normal goat serum, 1% (weight/volume) bovine serum albumin, and 0.05% Triton x-100 agitated for 3 hours on a shaker table (120 rpm). Samples were then incubated with a rabbit anti-collagen type II antibody (Abcam, Cambridge MA) (20 µg/ml), and a mouse anti-collagen type I antibody (Abcam, Cambridge MA) (10 µg/ml), agitated on a shaker table at room temperature for 2 hours, and then incubated for 2-3 days at 4°C. The primary antibody was removed, samples were washed for 30 minutes (3x every 10 minutes on shaker table) in PBS, and then blocking solution was added. Samples were incubated in blocking solution for 3 hours at room temperature with shaking, and then incubated on a shaker table with a Cy2 goat anti-rabbit IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) (20 µg/ml) and a Cy3 goat anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) (20 µg/ml) for 2 hours, and then incubated for 2-3 days at 4°C. . Samples were taken out of the secondary

solution, and washed for 1 hour (6x every 10 minutes on a shaker table). Chondrocyte samples were then labeled with 0.3 mM DAPI.

NHF microtissues were matured for 4 weeks and followed the same fixation, permeabilization, immunostaining procedures described above, except they were immunostained with a rabbit anti-elastin antibody (20 µg/ml) (Abcam, Cambridge MA) and a mouse anti-collagen type I antibody (Abcam, Cambridge MA) (10 µg/ml). The secondary antibodies used were a Cy2 goat anti-rabbit IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) (20 µg/ml) and a Cy3 goat anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) (20 µg/ml).

Mechanical Testing

To analyze the mechanical properties of self-assembled microtissues, dogbone microtissues with connecting rod widths of 400 and 600 µm (n=11 and 5, respectively) were matured in chondrogenic media for 8 weeks. Prior to mechanical testing, each dogbone sample was imaged in its gel from the top and side using a Carl Zeiss Axio Observer Z1 microscope equipped with an AxioCam MRm camera (Carl Zeiss MicroImaging, Thornwood, NY). Top and side view images were used to get an aspect ratio (width/thickness) of chondrocyte microtissues. Each microtissue was then individually released from its gel and loaded into a laser cut polymethyl methacrylate (PMMA) frame, and the microtissues were dusted with ground allspice to track local variations in strain. Due to the high water content of our samples, we eliminated the use of soluble dyes that might diffuse or insoluble paints with toxic vehicles (24). Instead, samples were speckled on their surfaces with allspice. The frame was then loaded into the

grippers of a custom built mechanical testing device and the edges of the frame were cut to allow for direct tissue loading. The custom built mechanical testing setup, described previously (25), consisted of a centrally positioned linear actuator (Series A, Ultramotion) with a built in encoder, which recorded motor displacement to determine the crosshead displacement. Coupled to the moving end of the linear actuator was a 50 gram tension/compression force transducer (LCFA-50G, Omega Engineering Inc.) which provided force measurements during the tensile test. Force measurements were converted into nominal engineering stresses via the measured initial cross-sectional area for each specimen. The apparatus was controlled by a custom LabVIEW VI program (National Instruments Corporation, Austin, TX, USA). A loading rate of 0.05 mm/s was used for the dogbones seeded in the 600 μm mold (n=5) and at both 0.05 mm/s (n=7) and 0.1 mm/s (n=4) for the dogbones seeded in the 400 μm mold. Tissue strain was visually recorded using a Canon Eos Rebel T3i camera. To analyze local tissue strain over the course of tensile loading, marker displacement was measured at various locations along the dogbone microtissue with respect to a fixed origin (n=6, 400 μm samples only). Local strain was calculated as the change in distance between successive particles divided by the initial inter-particle distance.

Stress analysis was computed using MATLAB (The MathWorks, Inc., Natick, MA, USA). True strength was calculated from the maximum force and final cross-sectional area of the tissue at the location of failure. “Average engineering strength of the rod” was calculated from the maximum force and the initial average cross-sectional area of the rod of the tissue.

“Minimum strength of rod” was calculated from the maximum force and final cross-sectional area of the thinnest portions of the rod. For all samples, a bulk tangent modulus was calculated from the slope of the stress-strain curves (average engineering strength plots) for the last 10% of

tissue strain as this coincided with the linear region of the curve. As a control, Sylgard 184 polydimethylsiloxane (PDMS) was cast at a 10:1 (base:curing agent) ratio and cut into thin strips and subjected to mechanical loading at 0.05 mm/s.

From strain mapping, force data, and initial tissue dimensions, the average engineering stress was calculated for the different regions of the dogbone tissue. To determine the modulus for each region of the tissue, a linear regression was performed for the linear portion of the stress-strain curve.

To determine if mold width (400 or 600 μm) and loading rate (.05 or .1 mm/s) independently had an effect on tissue strength, “average engineering strength of the rod,” “minimum strength of rod,” and bulk tangent moduli, student t-tests (two-tail, unequal variance), were performed with a 95% confidence interval.

Principle Stress Modeling

Stress analysis for the toroid and dogbone molds has been previously described (26, 27). For each of the new mold designs, the stresses in microtissues were computed in the finite element framework using the package ABAQUS v6.10 (SIMULIA, Providence, RI) and was based on the micro-mold dimensions. The model contained two parts: the posts and the tissue. The posts were treated as rigid body, as they are much stiffer than the tissue, and the tissue was modeled as a neo-Hookean material. The interaction between tissue and the posts were treated as frictionless contacts. During the cellular self-assembly process, the contractile cells cooperated with each other and therefore generated tissue level tension. To mimic this process, homogeneous contraction was applied to the tissue portion of the model until it reached the final

shape observed experimentally. The maximal principle stress was plotted for the contracted state to show the stress in tissue level and the maximal principle stress direction in certain regions was plotted to show the local stress direction.

Results

In directed self-assembly, the obstacles in a non-adhesive micro-mold induce tension in the self-assembling 3D tissue. To determine to what extent mold design controls this tension, we designed five different micro-molds to create tissues with uniaxial tension (loop-ended dogbone tissues), circumferential tension (toroid tissues), biaxial tension (cross tissues), equibiaxial tension (trampoline tissues), and multiaxial tension (triangle tissues) (**Figure 1A-E**). The mold designs were modeled using ABAQUS to compute the maximal principle stress (**Figure 1F-J**) and the local stress directions (**Figure 1K-O**). Simulation results indicate that the direction of the local stress varies for each designs.

To confirm that the different designs can direct cell-mediated tension, we seeded normal human chondrocytes into the non-adhesive micro-molds. The cells aggregated, rapidly self-assembled, and generated tension (**Figure 2A-E, Supplemental Video 1**). Within 24 hours, the cells had self-assembled a stable microtissue, with the tension continuing to mount through 96 hours (**Supplemental Videos 2 and 3**).

To investigate if the different mold designs caused cells to align in the predicted directions, chondrocyte microtissues were matured for four days, fixed, and stained for nuclei and F-actin (**Figure 2F-J**). From confocal images, we quantified the angle of the major axis of the nuclei in the five different designs (**Figure 3**). For the dogbone microtissue, it was predicted the cells would align uniaxially, thus parallel with the horizontal. The analysis of nuclei showed that $72 \pm 13\%$ of the cells were aligned between 0° to 14° from the horizontal with an average nuclei angle of $10 \pm 3^\circ$. For the cross microtissue, it was predicted the cells would align around 0° and 180° in the left and right arms of the microtissue and that the cells would align at 90° in the top and bottom arms. Nuclei analysis indicated that cells aligned at these angles with peaks in

the nuclei distribution at 0-30° and 150-180° for the horizontal arms, and between 90-120° for the vertical arms ($p < .05$). The trampoline microtissue was predicted to have equibiaxial tension, with cells aligning equally along all directions. Nuclei analysis showed a random arrangement of cell alignment in the x - y plane (0-180°), with no statistically significant difference in the number of cells within each angle bin. For the triangle microtissue with peg constraints at the vertices of an equilateral triangle, alignment was predicted along the arms of the tissue at 60°. Analysis showed that the nuclei in the 30-60° angle bin and the 60-90° angle bin were statistically more than the other angle bins ($p < .05$), but not significantly different from each other, with the nuclei aligned at $72 \pm 12^\circ$.

To determine if mold design's control over cell alignment also extended to the alignment of the extracellular matrix (ECM) proteins synthesized by the cells, we stained for collagen II. Chondrocytes were seeded into the toroid, dogbone, cross, trampoline, and triangle molds and after 4 weeks, the resulting microtissues were fixed and immunostained for collagen II. In all five of the mold designs, the chondrocytes produced collagen II (**Figure 2K-O**) that was aligned in the direction corresponding to the nuclei.

In self-assembled tissues, the type of ECM synthesized is determined by the cells which are present. To establish if mold design can control the alignment of other cell types that synthesize different ECM proteins, we tested normal human fibroblasts (NHF). NHFs were seeded into dogbone and trampoline molds and matured for 4 weeks. Similar to chondrocytes, NHFs self-assembled into complex shaped tissues. However, as a more contractile cell type, the gross morphology of the NHF tissue differed slightly from that of the chondrocytes. Specifically, NHF dogbone microtissues had more cell elongation and as a result created a thinner connecting rod in the dogbone tissue, whereas the trampoline tissues had a higher density of cells in the

central portion of the tissue (**Figure 4A-B**). These tissues were fixed and immunostained for elastin (**Figure 4-D**). Confocal imaging revealed that in a self-assembled environment, these NHFs produced elastin and that both the nuclei and elastin were aligned in the direction set through mold design. Specifically, these tissues produced uniaxially aligned elastin fibers in the dogbone mold, and unaligned elastin in the trampoline mold.

To determine if there was collagen I present in our chondrocyte microtissue, all chondrocyte microtissue were stained for collagen I (**Supplemental Figure 1**). Only dogbone samples stained positive for collagen I and the staining was only at the surface of the tissue and not in deeper optical slices. NHF dogbones were also stained for collagen I (**Supplemental Figure 2**). Collagen I staining was much more diffuse than the elastin staining for our NHF tissues which follows previous studies that showed that when NHFs are cultured long term, under tension, with TGF- β , they have diminished collagen I production and enhanced elastin production (28).

To create larger tissues, we designed a scaled-up trampoline mold. This mold had a hexagonal central trough surrounded by 3 orbitals of posts (around the hexagon's perimeter) which act as a constraint to the central portion of the tissue. This design had a contiguous tissue that measured 1.1 cm in its largest dimension and had a sheet of tissue 22 mm² in area. Chondrocytes rapidly self-assembled into this large trampoline structure. Although there were some points of tissue failure located near the posts, the central portion of the tissue was stable through eight weeks. After eight weeks, the tissue was fixed and immunostained for collagen II. Similar to the smaller trampoline structure, there was no preferential alignment of the nuclei, and collagen II was present and also unaligned (**Figure 5**).

To understand the complexity of the mechanical properties of the self-assembled tissues, chondrocytes were seeded into dogbone micro-molds with connecting rod widths of 400 and 600 μm . These samples were matured for 8 weeks, speckled, and subjected to uniaxial tensile testing at a loading rate of 0.05 mm/s for the dogbones seeded in the 600 μm mold and at both 0.05 mm/s and 0.1 mm/s for the dogbones seeded in the 400 μm mold (**Figure 6A and Supplemental Video 2**). Although the dogbone tissues as a whole underwent $55.3 \pm 6.2\%$ strain (as measured by motor displacement) there were local variations in the mechanical behavior of the tissue. By measuring the displacement of the speckle markers over the duration of mechanical loading, we were able to calculate the local strain within different portions of the tissue. We identified three distinct regions of the tissue: the rod portion, the toroid portion, and the region connecting the rod portion to the toroid portion (“Y-junction”). Each of these regions underwent different levels of strain under tensile loading (**Figure 6B**). For all of the samples, the rod portion had the least amount of strain before failure, experiencing strains of $20.8 \pm 8.0\%$, while the locations surrounding the Y-junction experienced the highest levels of strain ($71.6 \pm 8.0\%$). Overall, while the rod portion of the dogbone was very homogeneous, experiencing $29.1 \pm 10.7\%$ strain through the length of the tissue, the dogbone tissue as a whole was heterogeneous in strain behavior when comparing the stiff rod region to the other two regions (3.6 ± 1.3 fold difference in strain). Interestingly, the three regions of the dogbone tissue have different nuclei and collagen II alignment as the collagen goes from circumferentially aligned in the toroid portion, to a forked alignment in the Y-junction, to uniaxially aligned in the rod portion (**Supplemental Figure 3**).

To elucidate the stress response of the dogbone tissue to uniaxial loading, we generated stress-strain curves from the motor displacement, initial tissue dimensions, and load cell readings. The stress-strain curve of our microtissues followed the same pattern of what is seen

with soft biological tissues (**Figure 6C**) with an initial toe-region, a linear region, and then a failure region. From the average initial cross-sectional area of the rod portion of the tissues, we calculated the average engineering stresses experienced by the rod portion of the tissue (**Figure 6C, Table 1**). Coinciding with the pattern of strain, the samples consistently failed at the Y-junction. These areas had the lowest degree of collagen alignment (**Supplemental Figure 3**) and so intuitively would coincide with the weakest portion of the tissue. From the final cross-sectional area of the tissue at the region of failure, the true strength of the tissue was calculated for each test condition. Interestingly, the samples did not fail at the thinnest portion of the tissue (rod region), but at the thick, forked region of the tissue (Y-junction). Since the samples did not fail in the rod portion of the tissue, using the maximum force data, we calculated what would be the “minimum strength” of the thinnest part of the tissue (the rod) (**Table 1**). For the 400 μm samples, there was no difference in minimum strength of the rod portion of the tissue with the different strain rates, but there was a difference between the 400 and 600 μm samples. The 400 μm samples exhibited up to 2X more strength than the 600 μm samples. These 400 μm dogbones were only $156 \pm 58 \mu\text{m}$ in width and were withstanding $39 \pm 11 \text{ mN}$ of tensile force without failing or even necking. By comparing the true strength of the Y-junction of the tissue to the minimum strength of the rod region of the tissue, there was at least a 3.5 and 4.5 fold difference in the strength of these regions for the 400 μm and 600 μm samples, respectively. To compare our tissues with those in the literature, we calculated the bulk tangent moduli from the slope of the linear portion of the stress-strain curves (average engineering strength plots). There was no difference in the moduli for the two strain rates of the 400 μm samples, but the 400 μm samples were stiffer than the 600 μm samples. The stiffness of these dogbones were in the range of

biological tissues and was stiffer than comparable sized strips of PDMS (measured here to be $1.23 \pm .05$ MPa).

Figure 1: Micro-molds were designed (A-E) to harness cell-derived forces to create uniaxial (dogbone; A, F, K), equibiaxial; (trampoline; B, G, L), biaxial (cross; C, H, M), circumferential (toroid; D, I, N), and multiaxial tension (triangle; E, J, O) within the self-assembled tissue. Maximal principle stress (F-J) and max principle stress direction (K-O) were modeled for each of the mold designs. Scale bar is 1 mm.

Figure 2: Human chondrocytes rapidly self-assemble in micro-molds, exert cell-derived forces, align, and produce aligned collagen II. Chondrocytes were seeded into micro-molds designed to create uniaxial (A,F,K), equibiaxial (B,G,L), biaxial (C,H,M), circumferential (D, I,N), and multiaxial (E,J,O) tension. After four days in culture, microtissues were fixed, their nuclei were labeled with DAPI (blue), their f-actin was labeled with a fluorescent Phalloidin (green), and they were imaged with confocal microscopy (F-J). For each of the mold designs, cells aligned according to the direction of stress. After four weeks of maturation, samples were labeled for collagen II (green) and DAPI (blue) (K-O). Synthesized collagen II followed the cellular alignment. Scale Bar is 300 μ m in panel E, and 100 μ m in panels J and O.

Figure 3: Micro-mold design controls nuclei alignment creating four distinct cell alignments. The angle of the major axis of the nuclei of cells within the dogbone (A), cross (B), trampoline (C), and triangle (D) microtissues were quantified and binned in 15° bins for the dogbone, and 30° bins for the three other shapes. Each shape had more nuclei aligned nuclei in the bins predicted for each shape ($p < .05$). Specifically nuclei aligned between 0° to 14° from the horizontal in the rod portion of the dogbones; 0° and 180° (gray bars) for the horizontal portions of the cross (gray circle in inset) and 90° (white bars) for the vertical portions (white circle in inset) of the cross; and 30-60° and 60-90° leg of the triangle. The fraction of nuclei in each of the bins for the trampoline was not statistically different indicating that there was equibiaxial cell alignment.

Figure 4: Self-assembled NHF microtissues produce elastin in uniaxial dogbone molds and equibiaxial trampoline molds. NHF dogbones (A,C) and trampolines (B,D) were matured for four weeks, fixed, the nuclei were labeled with DAPI (blue), and the tissue was stained for elastin (green). Within the dogbone and trampoline molds, NHFs produces uniaxially and equibiaxially aligned elastin, respectively. Scale bars in B and D are 500 and 50 μm , respectively.

Figure 5: Chondrocytes form a scaled-up self-assembled trampoline structure while preserving equibiaxial cell and collagen II alignment. A scaled up (1.1 cm) mold was designed to create a large tissue with equibiaxial alignment (A). Chondrocytes self-assembled a tissue that remained stable through eight weeks of culture (B). The nuclei were labeled with DAPI (blue) and stained for collagen II (green). Confocal imaging demonstrated that collagen II fibers and cell nuclei (C) were randomly aligned through the tissue. Scale bar is 50 μm .

Figure 6: Self-assembled dogbone microtissues under tensile loading exhibit heterogeneous strain behavior according to the region of the tissue. Dogbone samples were speckled, loaded into a custom tensile tester, and loaded at a rate of 0.1 mm/s (A). Marker displacement was measured over time for each sample and the strain between markers was computed over the duration of mechanical testing. (B) Dogbone microtissues exhibited heterogeneous strain by region. Specifically, the dogbone toroid to Y-junction region (“A-B”) and Y-junction to rod region (“C-D”), exhibited much higher levels of strain than the pure rod portions (“D-E”, “E-F”, “F-G”) of the tissue. Stress was calculated from the initial cross-sectional area of the rod portion of the tissue. Shown is a representative stress-strain curve of dogbone under tensile loading (C). From strain mapping, force data, and initial tissue dimensions, the average engineering stress was calculated for each region of the tissue. Shown is a representative stress-strain plot by region (D).

Table 1: Self-assembled dogbone microtissues possess mechanical properties in the range of natural tissues. Chondrocytes were seeded in 600 μ m and 400 μ m molds, matured for eight weeks, and were strained at .05 mm/s (low $\dot{\epsilon}$) and .1 mm/s (high $\dot{\epsilon}$). True strength was calculated from the maximum force and final cross-sectional area at the location of failure (I)(B). “Average engineering strength of the rod” was calculated from the maximum force and the initial average cross-sectional area of the tissue (II)(A). “Minimum strength of rod” was calculated from the maximum force and final cross-sectional area of the thinnest portions of the rod (III) (B). Bulk moduli were calculated from the slope of the upper linear region of the stress-strain curves (average engineering strength plots). *Indicates a significant difference ($p < 0.05$) with the 600 μ m tissues.

	600 μ m, low $\dot{\epsilon}$ (MPa)	400 μ m, all $\dot{\epsilon}$ (MPa)	400 μ m, low $\dot{\epsilon}$ (MPa)	400 μ m, high $\dot{\epsilon}$ (MPa)
True Strength of Dogbone (I)	1.13 $\pm$ 0.44	2.85 $\pm$ 1.94	2.57 $\pm$ 1.47	3.35 $\pm$ 2.77
Engineering Strength of Rod (II)	2.65 $\pm$ 1.07	4.62 $\pm$ 1.95*	4.34 $\pm$ 2.11	5.11 $\pm$ 1.81*
Minimum Strength of Rod (III)	5.08 $\pm$ 2.39	9.17 $\pm$ 3.41*	10.25 $\pm$ 3.85*	8.45 $\pm$ 1.45*
Modulus	8.80 $\pm$ 3.22	15.85 $\pm$ 6.25*	14.58 $\pm$ 6.06	18.8 $\pm$ 6.8*

Discussion

The extracellular matrix (ECM) and the alignment of cells play important roles in the structure and function of a tissue. *In vivo*, there is a range of ECM alignments from uniaxial aligned collagen, as is found in tendons and ligaments, to unaligned collagen, as is found in the dermis, to collagen arranged at prescribed angles, as is found in small intestine submucosa and the myocardium. These different alignments, in addition to the specific composition and blend of ECM proteins, function to give tissues specific mechanical properties (strength, extensibility, stiffness, etc.) as well as physiological functions.

Numerous methods have been developed to control the alignment of various biopolymers and biocompatible polymers with or without the aid of living cells. Polymers that have been aligned include poly (lactic-*co*-glycolic) acid (PLGA), fibrin, and silk and many studies have focused on collagen type I because of its abundance in the body and availability as a purified protein. Methods to align solubilized collagen monomers without the aid of cells include electro-spinning (29), external magnets (1, 30), fluid flow (31), or extrusion (32). Alignment of purified collagen using these mechanical means do not yield material with sufficient mechanical strength so often these materials are chemically cross-linked (e.g., genapin, carbodiimide) which can result in problems with biocompatibility (33-35).

It has been known for many years that living cells can aid in the alignment of collagen monomers if the cells are cast into the gel. Fibroblast seeded into collagen lattices will contract and compact the collagen gel. The cells remodel the exogenous collagen of the gel as they synthesize their own endogenous collagen along with the other ECM proteins they synthesize. Obstacles that interrupt the collagen gel as well as how the collagen gel is tethered (boundary conditions), can modulate how the cells align the collagen (2, 36-38). There are, however,

several significant differences between cells reorganizing a collagen gel and native tissues. First, the protein content of a collagen gel is relatively low (0.1-0.5% by weight) compared to native tissues (i.e. skin, >5%)(39). As the cells reorganize the collagen gel, they are not reconstituting collagen's true native hierarchical structure. The cells are reorganizing the collagen fibrils by simple end-to-end and lateral assembly. Lastly, the cells do synthesize endogenous ECM, but even after four weeks, at least 20% of the original collagen is still present (39). As a result of the residual collagen, its non-hierarchical assembly, and the low protein content, these remodeled collagen gels are at least an order of magnitude weaker than *in vivo* tissues even when subjected to additional external mechanical conditioning techniques, such as cyclic stretch (40).

In this study, we have utilized the cells' innate capacity to form ECM, their contractile nature, and mold design to create strong tissues with both cell and collagen alignment. We have engineered five different mold designs to harness cell-derived contractile forces, thereby creating five distinct tension environments. We seeded mono-dispersed cells into these molds made of non-adhesive hydrogels to create tissues with uniaxial, circumferential, biaxial, equibiaxial, or multiaxial tension. After settling into the non-adhesive micro-molds, the cells were unable to attach to the hydrogel and so formed cell-cell adhesions and exerted contractile forces that drove the self-assembly of a 3D multi-cellular microtissue. The micro-mold design sets the initial position of the cells and provides constraints which direct cell alignment and tissue tension which ultimately directs ECM alignment. Unlike traditional tissue engineering, our tissues were created without exogenous ECM proteins or scaffold and without the use of external mechanical stimulation. Even though our microtissues are initially composed of only cells, after maturation, these tissues have a dense accumulation of aligned collagen II fibers, and have a strength and moduli in the range of soft tissues.

We hypothesize that our tissues are stronger than collagen gels with cells, and better match the mechanical properties of native tissues because the 3D self-assembled environment more closely matches the *in vivo* environment. *In vivo*, collagen is produced by cells from the “bottom-up” starting on the molecular level forming fibrils and then fibers (9). The interactions that occur between the different collagen substructures, the organization, and the cross-linking within each fibril are thought to be critical to the overall physiological and mechanical properties of the tissue (39). As well, this process of the cells synthesizing EMC renders a specific fibril/fiber packing and weave that entraps cells and gives tissues their unique properties. The fact that the cells within our self-assembled structures are using this “bottom-up” approach to form a complex interconnected collagen network means that the collagen has the hierarchal structure and natural cross-links found *in vivo*. This is corroborated in that shape of the stress-strain curves of our dogbone microtissues closely resemble that which is seen for soft tissues like tendons and ligaments (39). As well, *in vivo* as cells synthesize collagen, they interweave other types of nonfibrillar collagen and other ECM molecules at specific intervals which modulates ECM function. For example, proteoglycans have been shown to control the size of collagen fibers formed *in vivo*, which also has a role in the strength, stress dissipation, and elasticity of a tissue (41). Most tissue engineered constructs are fabricated from purified collagen, while some researchers have incorporated glycosaminoglycans (42), and others have co-spun collagen and elastin (3). However, these supplemental proteins and molecules cannot be incorporated with the same level of organization and specificity as is done naturally by the cells synthesizing the ECM and as a result, cannot reconstitute the native ECM. Here, our self-assembled chondrocyte microtissues were extremely hydrophilic, suggesting the presence of cell synthesized proteoglycans, which may have also played a role in the superior tissue mechanics. Other groups

have also shown that self-assembling chondrocytes produce other ECM proteins in addition to collagen II (17).

Further recapitulating the *in vivo* environment, our tissues have maximized cell-cell interactions. Cell-cell adhesion and communication are known to be pivotal in a number of cell functions. For example, mechanical stimuli is transmitted through adherens junctions, and this force transmission has been shown to initiate a host of cell signaling pathways including the formation of stress fibers and the upregulation of ECM synthesis (43). We hypothesize that since the forces acting on the cells in our tissues are endogenous cell-derived forces (as compared to applying external stretch/compression), these forces are at a more physiologically relevant level, and so would result in an environment that restores cell phenotype and promotes ECM production. These differences in mechanical stimuli, cell-cell communication, and collagen synthesis could account for the significant increase in strength that is seen in our self-assembled structures as compared to those structures made from collagen gels with cells.

Not only does this method create tissues with relevant tensile strengths and moduli, but it gives us the ability to truly engineer and control the material's biological composition as well as mechanical properties. Using this technology, we have the ability to control and tailor which ECM proteins are formed *in vitro* to fulfill specific applications. Although this study focused on chondrocytes and fibroblasts, our goal was not to engineer cartilage, but rather to show that by choosing a specific cell type, we can dictate the proteins that are formed, in this case collagen II and elastin. By this, we are demonstrating that cell type selection is what determines the type of ECM that is created in self-assembled tissues. To further tailor biological composition, performance, and mechanical properties, we can foresee co-seeding different cell types to create tissues with blends of ECM. By mixing fibroblasts with chondrocytes at different ratios we could

tune mechanical properties (i.e. change elastic properties, increase tensile strength) as collagen (produced by chondrocytes) gives strength to a tissue, while the elastin (produced by the fibroblasts) gives a tissue more elasticity (28). We have also previously demonstrated that we can control how the cells in heterotypic self-assembled tissues organize, manipulating the cells innate propensity to self-sort, and so we could also create layered or blended tissues (44, 45).

As it is well known that fiber alignment dictates mechanical performance, this technology is well suited for creating tissues with specific geometries and thus tailored mechanical properties. In this study, by simply altering the width of the connecting rod of the dogbone micro-mold by 33% we created approximately a 200% increase in both tissue strength and modulus, demonstrating the degree to which mold geometry can tailor mechanical properties. As well, within our dogbone microtissues, we have shown the importance of cell and ECM alignment in the modulation of the mechanical properties of self-assembled tissues, as the less aligned portions of the tissue exhibited only about 20% of the strength and stiffness of the highly aligned rod portion of the tissue. The difference in mechanical properties within a single microtissue is of biological relevance as, for example, single cartilage samples have exhibited a twofold difference in shear modulus over a length of only about 100 μm (46). Similarly, the authors of this study concluded that this sharp difference in mechanics is due to collagen alignment. Here, we used FEA only to model the five different tension environments of our molds. However, FEA could be further utilized to predict tension environments and then inform the design of a number of new molds, with different geometries, for specific tissue engineering applications.

Conclusion

A persistent challenge to the utility of tissue engineering is the lack of strong, natural tissues, with relevant blends and alignments of both ECM and cells. Polymers can be highly aligned and are strong, but lack the important biological signals and properties of native ECM. Biopolymers, such as fibrin and collagen type I, offer a more natural solution, but without chemical cross-linking that can compromise biocompatibility, these biomaterials lack relevant mechanical properties. Directing the assembly of monodispersed cells into complex shaped tissues presents a solution to the shortcomings of synthetic polymers and purified biopolymers, while offering strong, biologically tailorable, mechanically tunable tissues that are simply made. With more self-assembled microtissues already being tested in the clinic (47), and new methods being developed to scale up the size of self-assembled tissues (48), we present here even more control and diversity to this already promising method of tissue engineering.

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