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Publication Date

2026-06-01

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating Fiber-Enabled Long-Range Stiffening and Stiffness Anisotropy using Optical
Tweezers

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Michelle Lanterman

Dissertation Committee:
Professor Elliot Botvinick, Chair
Professor Wendy Liu
Assistant Professor Herdeline Ann Ardoña

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DEDICATION

To

My family of bing bongs.

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor and committee chair Professor Elliot Botvinick. Your enthusiasm and outlook on scientific exploration is inspiring. I am so grateful to have had your support as a mentor. Thank you for making me a better scientist and engineer.

I would also like to thank all the committee members from both my advancement and defense committees including Professor Wendy Liu, Professor Herdeline Ann Ardoña, Professor John Lowengrub, and Vice Provost, Dean, and Professor Michael Dennin. Thank you for all your insightful feedback.

Next, thank you to all my fellow BEAMS lab members. You all remind me that we can do hard things, and also have fun doing them. To my AMR predecessors, Alicja Jagiello, Qingda Hu, and Mark Keating, thank you for all the hours of training, troubleshooting, and guidance. You paved the way for all the work in this thesis. To my mechanobiology mates and especially Sarah Albassri and Rahul Sreedasyam, I would not have made it through without your support and friendship. You are both incredible scientists and it was invaluable to be able to bounce ideas off of you throughout this process.

I also had the pleasure of working with collaborators in the Putnam Lab at the University of Michigan including Professor Andy Putnam, Irene Zhang, and Atticus McCoy. Thank you for all the interesting scientific discussions and for teaching me everything I know about vascular development.

Finally, I would like to thank my family. To my mom and dad, I would be nowhere if it wasn't for you. Thank you for all your sacrifices and for your constant love and support. To Brian and Michael, thank you for always putting up with your little sister. I'm forever aiming to be as smart and talented as my big bros. To Bryce, thank you for going on this long journey with me. I wouldn't be able to do it without you.

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Surface-Enhanced Raman Spectroscopy (SERS) for Disease Detection
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Optical System for Minimally-Invasive Surgery 2017
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ABSTRACT OF THE DISSERTATION

Investigating Fiber-Enabled Long-Range Stiffening and Stiffness Anisotropy using Optical Tweezers

By

Michelle Lanterman

Doctor of Philosophy in Biomedical Engineering

University of California, Irvine, 2026

Professor Elliot Botvinick, Chair

Biophysical properties of the fibrous extracellular matrix (ECM) are critical regulators of cell behavior. Bulk measurements at single time points have often been used to investigate matrix stiffness as an instructive cue. However, our lab has demonstrated that local pericellular stiffness can be heterogeneous, anisotropic, and vary greatly from the measured bulk properties. Thus, we developed a multi-axis optical tweezers-based active microrheology (AMR) system which is capable of measuring ECM properties on a length scale relevant to cells.

The fibrillar architecture of the native ECMs is well known to give rise to nonlinear mechanical properties that govern force transmission and long-range cell-cell communication. Despite this, direct comparisons of pericellular stiffening in fibrous and amorphous hydrogels remain limited. In this thesis work, I used multi-axis AMR to investigate how ECM architecture regulates the emergence of long-range stiffening and stiffness anisotropy.

In an initial study in collaboration with Professor Andrew Putnam, we used AMR in a fibrin hydrogel vascular self-assembly model to assess how different supportive stromal cells (SCs), in co-culture with endothelial cells (ECs), differentially stiffen the ECM and mediate vascular morphogenesis. We found that lung fibroblasts (LFs) lead to enhanced vascular

development, concurrent with significant anisotropic stiffening of the fibrous ECM in the direction of capillary growth, as well as extensive ECM remodeling compared to bone marrow-derived mesenchymal stem cells (MSCs). Notably, this stiffening extended far beyond the immediate pericellular region, consistent with long-range force transmission mediated by strain-hardening in fibrous matrices.

To further explore the direct impact of a fibrillar ECM, we used AMR to map pericellular stiffness around LFs and MSCs in stiffness-matched fibrin and amorphous gelatin methacryloyl (GelMA) hydrogels. While both cells stiffened fibrin relative to cell-free controls, they reduced stiffness in GelMA. Inhibitor studies implicated cell contractility as a predominant mediator of pericellular stiffness changes. Additionally, fibrin supported significant stiffness anisotropy around cells while GelMA remained relatively isotropic. Together, these data highlight the potential link between fibrous ECM structure and the development of stiffness anisotropy and long-range stiffening, which may be important mediators of complex biological processes like vascular morphogenesis.

Chapter 1

Introduction and Background

1.1 Extracellular Matrix

The extracellular matrix (ECM) is an organized 3D network of macromolecules that gives structural and functional support to cells and tissues. The ECM provides critical biochemical and biophysical cues that regulate the development and homeostasis of tissues by altering cell migration, proliferation, and differentiation. [33] As cells sense and respond to their extracellular environment, they are also actively modifying the structure and composition of the ECM dynamically. These reciprocal cell-matrix interactions govern tissue structure and function, and dysregulation of this complex feedback system has been shown to influence pathogenic processes such as fibrosis and cancer. [69] Understanding this dynamic mechanical crosstalk between cells and the surrounding ECM remains a challenge in mechanobiology and lies at the core of this thesis work.

1.1.1 ECM Structure

The ECM is comprised of water, collagens, proteoglycans, and many other glycoproteins as shown in **Figure 1.1**. [33, 70] The ECM is highly heterogeneous, fibrillar, and macroporous.

In most tissues, the most prevalent component of the ECM is type 1 collagen (T1C), however the structure and composition of the ECM is tissue-specific and defines its function. [146] The fibrillar structure of the ECM is highly heterogeneous, with fibers in the ECM ranging from 10s to 100s of nms in diameter and pore sizes ranging from 10s of nms to several μ ms. [97] Importantly, this fibrillar structure formed by proteins such as collagen, fibronectin, and laminin plays an important role in the transmission of cell-generated forces and is discussed further in **Chapter 1.6.2**.

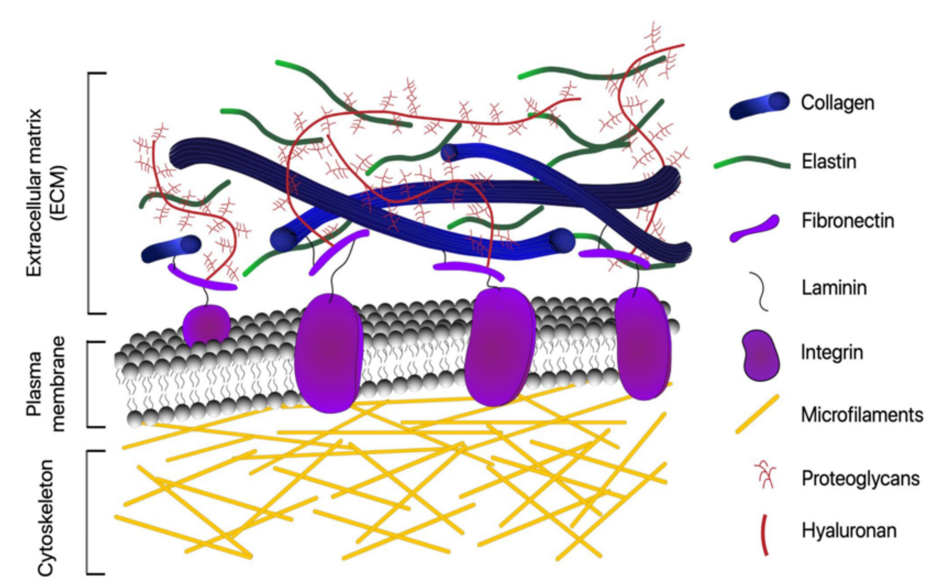


Figure 1.1: Structure of the ECM. [121]

1.2 Cell-Matrix Interactions

The ECM is a highly dynamic environment that is constantly changing due to reciprocal biochemical and mechanical interactions between cells and ECM components. Cells can mechanically interact with the ECM in a variety of different ways. Transmembrane integrin molecules engage and bind to matrix proteins, forming focal adhesions. [23] These adhesion complexes are sites of two-way mechanical information exchange between the cell and the ECM and mediate the process of mechanotransduction. [149] Mechanotransduction is the process by which mechanical stimuli are converted into biochemical cues. [117] The ECM,

cytoskeletal actin network, and the adhesion complexes that connect the two lie at the heart of mechanotransduction pathways that lead to changes in gene expression as shown in **Figure 1.2**.

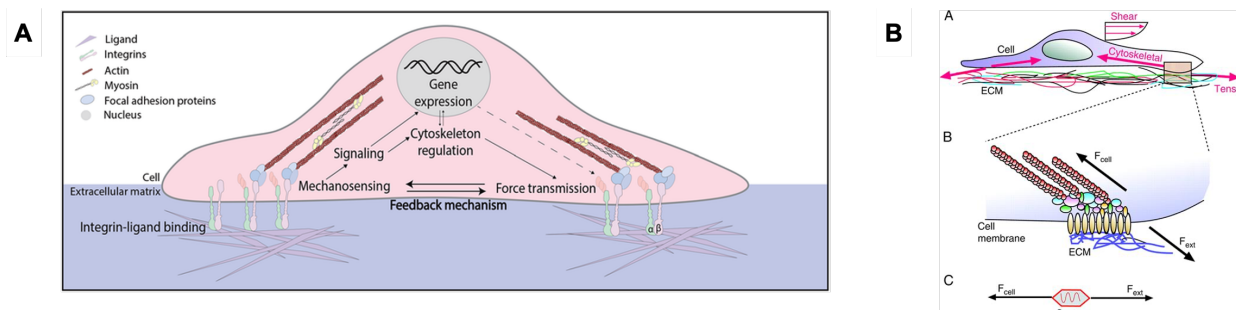


Figure 1.2: (A) Mechanotransduction pathways between the cell and the ECM.[135] (B) Focal adhesions are 2-way force sensors. [19]

At these focal adhesions, cells are not only sensing the mechanics of their surrounding ECM, but they can also apply tractional forces onto the matrix through cytoskeletal contractility. [19, 48] The applied tractional forces have been shown by our lab and others to stiffen the ECM, in part due to strain-hardening of the fibrous ECM. [72, 151, 65] Other ways in which cells can dynamically alter ECM mechanics are through new matrix deposition, remodeling and rearrangement of fibers, and degradation through either secreted or membrane-bound proteinases. [10, 91] A summary of these bi-directional cell-matrix interactions is shown below in **Figure1.3X**.

1.3 Hydrogels as ECM Mimetics

While 2D in vitro culture has long been used and is a useful model to study cells, there is a shift towards the use of 3D culture models to better mimic the microenvironment in vivo. [50, 13] As discussed, the complex 3D ECM is highly critical to cell behavior. Thus, it follows that 2D monolayer cell culture on glass or plastic surfaces can lead to divergent cell behavior. [107] In particular, cell spreading, migration, proliferation, and intercellular interaction have all been shown to change in 2D vs 3D environments. [9, 168, 23] Additionally, 3D environments are

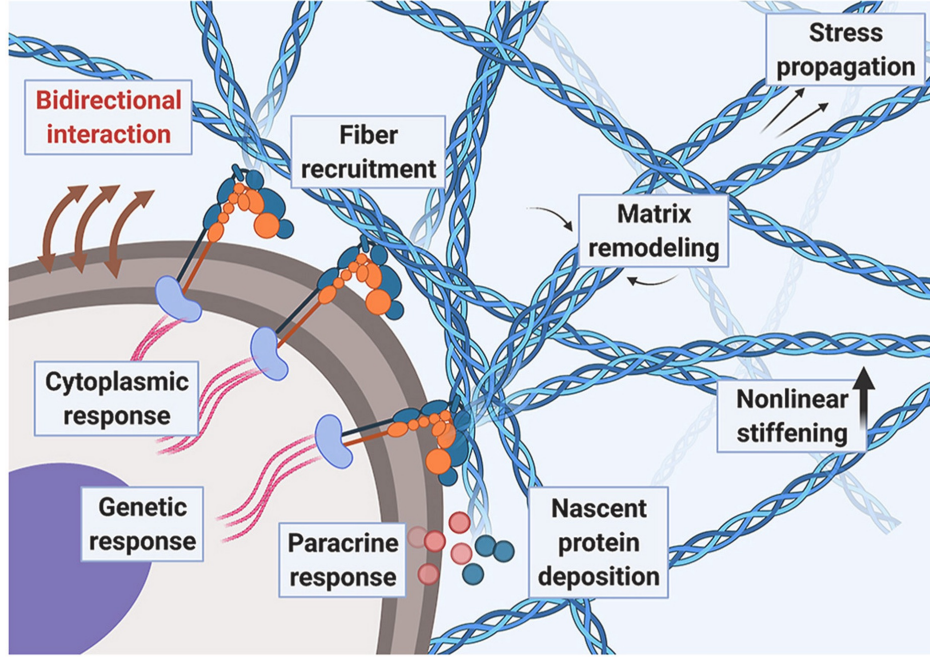


Figure 1.3: Reciprocal cell-matrix interactions.[121]

necessary to replicate the structure of tissues and expression of tissue-specific genes, making a 3D model necessary for tissue engineering (TE) applications. [39] Hydrogel platforms have emerged as highly attractive biomaterials for use in 3D in vitro studies and as TE scaffolds. Hydrogels are crosslinked polymer networks with high water content that recapitulate the mechanics and transport properties of soft tissues. [148] Hydrogel design must consider the physical and chemical properties of the material that can affect cell behavior. These include, but are not limited to, the stiffness, viscoelasticity, porosity, degradability and bioactivity of the biomaterial as represented in **Figure 1.4**. [86, 167]

1.3.1 Natural vs. Synthetic Hydrogels

Hydrogels can broadly be split into either naturally derived or synthetic. (**Figure 1.5** Examples of naturally derived hydrogels include gels made of fibrillar proteins like collagen and fibrin, or polysaccharides like hyaluronic acid (HA). [170] Naturally derived hydrogels are widely used due to their inherent biocompatibility, degradability, and cell interaction. [170, 25] Although chemical modification of natural gels is possible to tune their mechanics

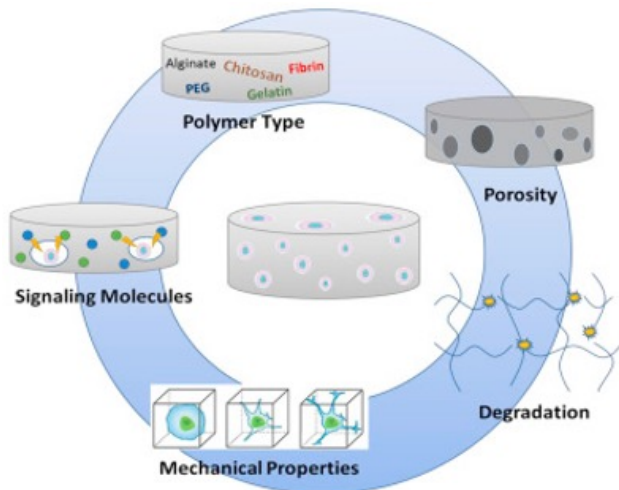


Figure 1.4: Important hydrogel design criteria.[167]

and biological performance, these materials suffer from high variability and could lead to lead to adverse immune responses if implanted due to xenogenic protein components. [129]

Due to these limitations of natural ECMs, synthetic hydrogel platforms have been explored for their high degree of tunability and the ability to decouple the mechanical and chemical effects of the matrix. [82] Examples of synthetic hydrogels include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(acrylamide) (PAAm). [54] Synthetic hydrogels are easily and more reproducibly functionalized, and common modifications to synthetic hydrogels include addition of cell binding motifs such as RGD and proteolytic degradation sites. [53] However, synthetic hydrogels inherently lack key features of the native ECM, which is fibrillar, macroporous, and mechanically heterogeneous. [86]

Synthetic hydrogels without modification are generally non-fibrous, relatively homogeneous, and exhibit linearly elastic mechanical properties. This linearly elastic material behavior limits the propagation of mechanical forces relative to strain-stiffening fibrous materials. [161, 91] Although the amorphous structure and homogeneity of these synthetic hydrogels are essential for their utility in techniques such as 3D traction force microscopy [84], the full effect of their non fibrous structure on cell-matrix interactions, and further cell-cell

communication through matrix interactions which are critical to many cellular processes remains unknown.

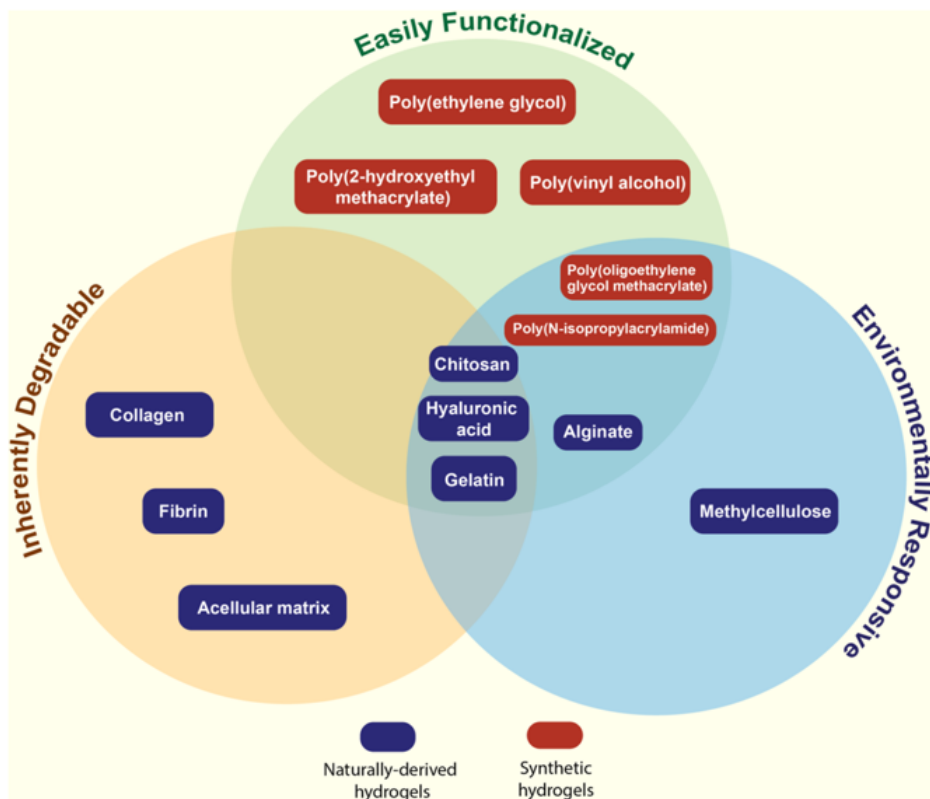


Figure 1.5: A comparison of synthetic and naturally-derived hydrogels.[164]

1.3.2 Fibrin

Fibrin hydrogels are commonly used 3D *in vitro* models of the ECM and were used throughout this work. Fibrin is naturally derived and plays a critical role in tissue homeostasis and wound healing *in vivo*. [1] Fibrin gels are prepared from the primary proteins involved in blood clotting, fibrinogen and thrombin. [64] Polymerization of fibrin is initiated by the thrombin cleavage of fibrinopeptides from fibrinogen, eventually forming fibers that elongate, thicken, and branch to form a 3D fibrous network as shown in **Figure 1.6**. [159]

Fibrin is a popular choice for an artificial ECM substitute due to its inherent cell interactions and has been used as a biological scaffold to support regeneration of various tissues. [1]

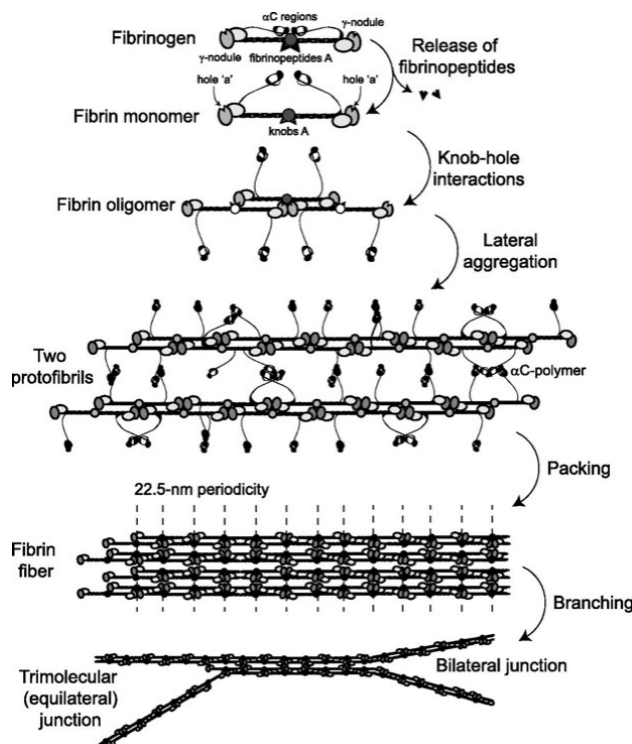


Figure 1.6: Method of fibrin polymerization. [159]

Fibrin contains cell adhesive sites such as the Arg-Gly-Asp (RGD) that can interact with cell surface receptors. Additionally, fibrin is susceptible to proteolytic degradation by the enzyme plasmin and various MMPs. [55]

Fibrin hydrogels are fibrillar and macroporous in their structure. Fiber width and pore size vary with polymerization conditions such as fibrinogen concentration, thrombin concentration, and pH. [128] As a result of their fibrillar structure, fibrin gels exhibit nonlinear, strain-hardening behavior. [64, 30] Given their popularity and potential as a tissue engineering scaffold, as well as our lab's previous experience with them, [61, 73, 57] fibrin hydrogels were used here as a fibrillar hydrogel model in both **Chapters 3 and 4**.

1.3.3 GelMA

Gelatin methacryloyl (GelMA) is a naturally derived hydrogel also used in this thesis work. Gelatin is a natural polymer derived from hydrolysis of collagen proteins that maintains RGD

and MMP degradable motifs and can form a hydrogel through thermo-reversible physical gelation. [3] To overcome thermal stability challenges with gelatin, GelMA is a chemically modified form that allows for covalent crosslinking and has gained popularity. Gelatin is functionalized with methacryloyl side groups using methacrylic anhydride (MA) to form GelMA. [153] This modification allows for irreversible photopolymerization using a free radical photoinitiator like lithium phenyl-2,4,6-trimethyl-benzoyl phosphinate (LAP) or 2-hydroxy-4-(2-hydroxyethyl)-2-methylpropiophenone (Irgacure-2959). (**Figure 1.7**) [43] The degree of substitution (DS) of methacryloyl groups is controlled by modifying the feed ratio of gelatin and MA during the reaction. The mechanical properties of GelMA are tunable and affected by the DS, polymer concentration, photoinitiator concentration, and UV irradiation time and intensity. [94, 144] Importantly, the structure of GelMA lacks the fibrillar structure present in the collagen it is derived from. [139] Instead, GelMA hydrogels are amorphous, but heterogeneity in the local structure can be present due to random areas of low local crosslinking density. [92] GelMA also behaves more linearly elastic, similar to covalently crosslinked synthetic hydrogels and in contrast to fibrous natural hydrogels. [3, 143] In **Chapter 4**, GelMA is used as an amorphous, non-fibrillar hydrogel model in contrast to fibrin.

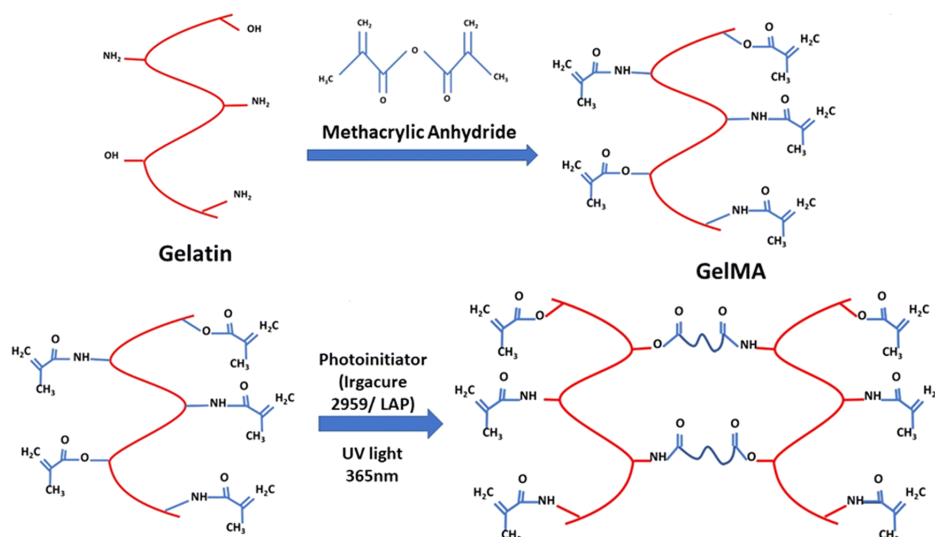


Figure 1.7: GelMA synthesis and polymerization. [43]

1.4 Mechanobiology

As discussed, cells respond to the mechanical cues of their surrounding microenvironment. The field of mechanobiology is the study of this process, focusing on how cells and tissue mechanics, as well as external mechanical forces, influence cell behavior. [65] The role of the ECM and cell-matrix interactions as described in **Chapter 1.2** are a large focus of this field and will be investigated throughout this thesis work.

1.4.1 ECM Stiffness

The effect of ECM stiffness on cell behavior specifically has been of great interest within mechanobiology. It has been shown and well appreciated now that cells respond to the stiffness of the ECM, and this can alter basic cellular processes such as apoptosis, proliferation, spreading, adhesion, and migration. [156, 165, 118, 42, 93] ECM stiffness has also been shown to affect larger-scale processes such as differentiation of stem cells into divergent lineages, pathogenic process like cancer progression and fibrosis, and vascular morphogenesis. [31, 169, 52, 89] Importantly, ECM stiffness is highly dynamic due to the reciprocal cell-matrix interactions discussed in **Chapter 1.2**. Thus, cell-mediated modulation of the matrix stiffness should be examined since it can change neighboring cell behavior. In fact, cell-generated traction forces that stiffen the ECM have been shown to induce alignment and migration of surrounding cells. [161, 126, 116] In **Chapter 3**, I will explore the dynamic mediation of ECM mechanics in the context of vascular morphogenesis due to cell-generated traction forces.

1.4.2 Stiffness Anisotropy

Stiffness anisotropy of the matrix has also emerged as a potentially important mechanobiological signal. Studies in our lab have shown that cells can sense and respond to induced stiffness anisotropy in the surrounding ECM. [147, 62] Additionally, stiffness anisotropy has

recently been shown to be an important potential regulator in tissue morphogenesis, leading to myotube alignment in a skeletal muscle model. [138] We’ve also shown that various cell types can establish ECM stiffness anisotropy differentially depending on ECM type and density within fibrous hydrogels. [61] To build upon these findings, stiffness anisotropy will be explored throughout **Chapters 3 and 4**.

1.4.3 ECM Fibers as Force Highways

The transmission of cell-generated traction forces through the ECM is a mode of cell-cell communication. There is evidence that the fibrillar structure of the ECM is critical to the long-range transmission of these forces. Numerous studies have shown experimentally and also through material modeling that cells embedded in amorphous synthetic polymer hydrogels can only sense each other within distances 10s of μms as opposed to 100s of μms – mms in fibrous natural protein matrices like fibrin and collagen. [126, 15, 137] In a recent study investigating the propagation of Ca^{2+} signaling after mechanostimulation with a microcavitation bubble, our lab also showed a reduced radius of signaling in amorphous PVA compared to fibrous collagen. [141] Together these data demonstrate the role of fibers as what we like to call “force highways”, transmitting mechanical forces over long ranges within the ECM. The role of ECM fibers in the development of pericellular stiffening and stiffness anisotropy will be investigated in **Chapter 4**.

Chapter 2

Microrheology

As discussed and central to the work in this thesis, evidence suggests that the ECM is a critical regulator of cell behavior. Most studies investigating the mechanobiological effects of the ECM mainly focus on the effect of initial bulk stiffness. However, cells are constantly both responding to and dynamically regulating the properties of the ECM and previous investigations from our lab have demonstrated the extensive mechanical heterogeneity that exists across the ECM relative to a single bulk measurement. [72] As seen in **Figure 2.1**, the stiffness around a single cell can span orders of magnitude and even exceed the measured bulk stiffness in comparable systems.

In classical bulk rheology, a material's deformation and flow behavior is measured using a mechanical rheometer such as a rotational parallel plate rheometer. Under a known stress or strain, the material response is recorded and the bulk mechanical properties of a viscoelastic material, such as a hydrogel, can be calculated. [142] In contrast, microrheology is the measurement of local viscoelastic properties on the micron scale and is most often performed by monitoring the motion of embedded particles.

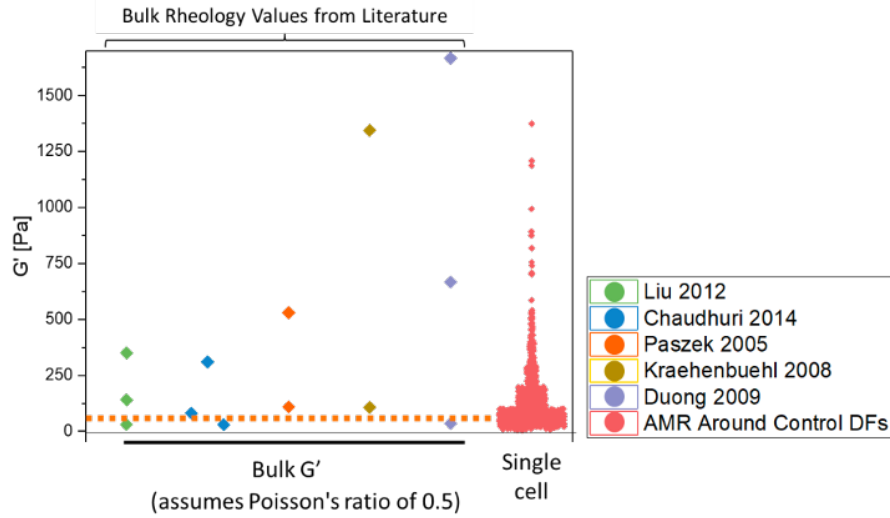


Figure 2.1: Bulk shear storage modulus measured in cell-laden hydrogels compared to AMR-measured G' around single dermal fibroblast cells. (Courtesy of Dr. Mark Keating)

2.1 Passive vs. Active Microrheology

Microrheology techniques are defined as either passive (PMR) or active (AMR). [21] In PMR, particle motion is observed as it undergoes thermal Brownian motion and the generalized stokes-einstein relation (GSER) is applied to extract local viscoelastic properties. [140] Although PMR allows for effective spatial mapping of local mechanical properties, it is limited to soft materials on the orders of 100s of Pascals. [38] In AMR, particles are externally driven often by optical, magnetic, or micromechanical forces. [66] The active force element on the particles in AMR allows for accurate measurements of much stiffer materials than in PMR. [75]

AMR is a promising technique, capable of measuring spatiotemporal differences in local ECM mechanical properties on the same length scale as single cells in 3D. In our lab, we utilize optical tweezers to perform AMR as described in **Chapter 2.2**. Using optical forces exerted by a high-power trapping beam, we oscillate $2\mu\text{m}$ silica beads embedded in 3D hydrogels and monitor the bead responses using a laser detection system to non-destructively find local ECM mechanical properties on the same length-scale as a single cell.

2.2 Optical Tweezers System

In our lab, we employ a dual-beam optical tweezers system to perform active microrheology (AMR). Modified from a previously described system [72], the current AMR system schematic is shown in **Figure 2.2**. The system relies on two laser beams, a 1064nm continuous-wave fiber trapping laser (YLR-5-1064-LP, IPG Photonics) used to oscillate probe beads, and a 785nm single mode fiber-pigtailed detection laser (LP785-SF100, ThorLabs) to monitor probe bead movement. These beams are co-aligned to enter the white light path of a motorized IX81 inverted microscope (Olympus) that is equipped with the Zero Drift Compensation package (Olympus) and a filter cube placed below the objective lens. The laser/detector unit is replaced by a stock dichroic beam splitting mirror and a short pass dichroic beam splitting mirror (Chroma) that reflect our two additional lasers through the 1.45NA 60x TIRF oil objective lens (Olympus), focusing the co-aligned beams into the sample plane. This microscope modification allows the additional lasers to pass through the microscope objective lens while also passing visible light for confocal and brightfield microscopy.

Prior to entering the microscope, the lasers pass through a variety of different optics. First, the trap laser power is reduced by a polarization-based attenuator composed of a half wave plate and polarizer which ensures the laser power is 240mW going into the scope. Then, the trapping beam is steered using a set of galvanometer mirrors (GVS012, ThorLabs) placed conjugate to the back focal plane and a small portion of the beam is split off by the beam sampler (ThorLabs), allowing the beam position to be recorded by a quadrant photodiode (trapQPD; 2903, Newport). The rest of the trapping beam passes through a telescoping lens pair (L1 and L2) and passes through a dichroic (D3) to combine with the detection beam path into the scope. Prior to entering the scope, the detection beam also passes through its own telescoping lens pair (L3 and L4). These telescoping lens pairs expand the beams to ensure they overfill the back aperture of the objective lens. As the trapping beam

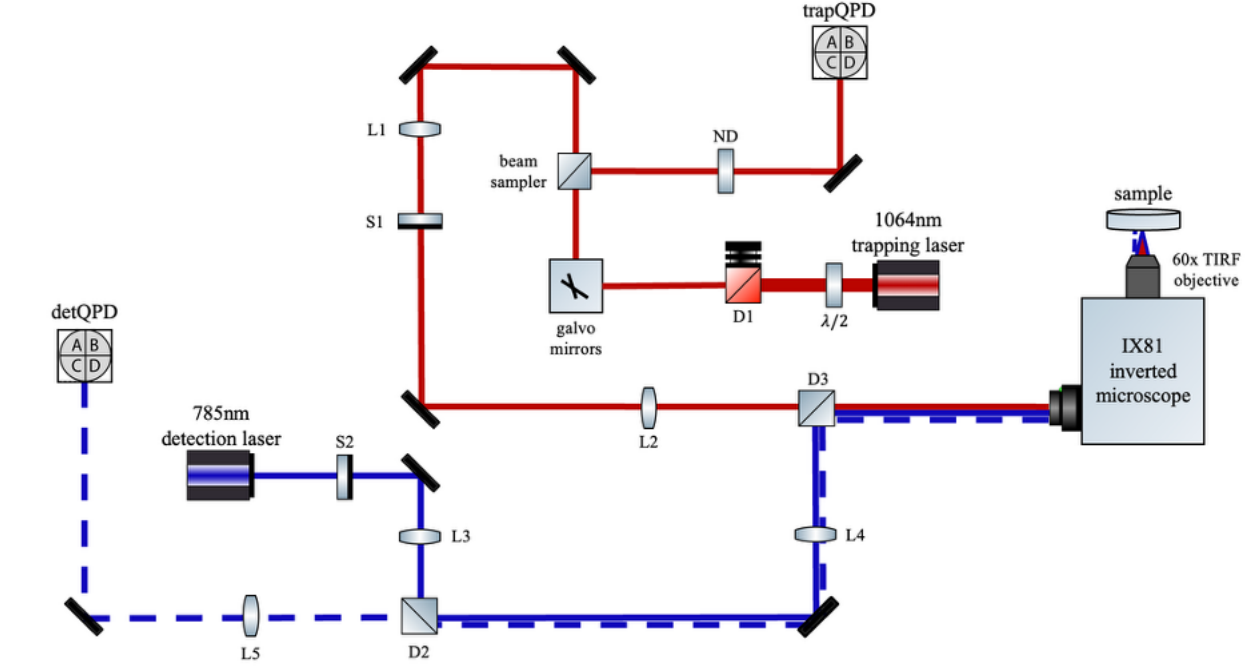


Figure 2.2: Schematic of the AMR system.

oscillates a trapped bead, the back reflection of the detection beam is slightly deflected. The back reflection of the detection beam reflects off a dichroic (D3) and passes through another dichroic (D2) where it is focused onto a second quadrant photodiode (detQPD; 2901, Newport) by a lens (L5). The detQPD signal oscillates in response to the deflections of the back-reflection of the detection beam as the probe bead sweeps through the focal point of the beam.

Samples are placed into a custom stage-top incubator system that is attached to a piezoelectric XY nanopositioning stage (P-722.2CL, Physik Instrumente) within a large XY stepper motor stage (MS-2000, Applied Scientific Instruments). This dual-stage setup allows for precise sample movements across a large area ($100\mu\text{m} \times 100\mu\text{m}$). To visualize the samples during testing, the image path of microscope is split using a magnifying achromatic doublet pair (MAP1030100-A, ThorLabs) and a beamsplitter onto two cameras. The unaltered image is reflected onto an EO-4010 Monochrome USB 3.0 Camera (Edmund Optics) to visualize a larger field of view (FOV) while navigating around the sample, while the 3.3x magni-

fied image is passed onto a high-speed digital CMOS camera (ORCA-Flash4.0 C13440, Hamamatsu) to improve the resolution of each individual bead which is essential for bead centering to the laser trap. The microscope system is also equipped with the FluoView 1200 laser confocal scan head system which allows for confocal imaging of samples between AMR measurements. In this work, the confocal system was used to perform reflection confocal microscopy (RCM) to visualize the ECM structure within hydrogels samples.

The AMR system is run using custom LabVIEW (NI) software, adapted from a previous version as described in *Keating et. al.* [72] The AMR LabView program allows for stage movement between beads, navigation around each sample and automated, accurate positioning of the optical trap to the center of each bead. After centering the bead to the optical trap, the trapping beam is oscillated sinusoidally at a given frequency, amplitude, and axis for 5s. The system allows for any given number of oscillation waveforms, but for this thesis work all beads were oscillated at 50Hz using a sinusoidal waveform with an amplitude of 150nm in both 0° and 90° relative to the image FOV (what we refer to as the X' and Y' axes). The detQPD and trapQPD signals are recorded during bead oscillations, allowing for calculation of the local material response function as described in **Section 2.4**. The custom program also handles the calibration sequence of the system as outlined in **Section 2.3**.

2.3 System Calibration

Prior to taking stiffness measurements in samples, the AMR system is calibrated in water following the workflow outlined in **Figure 2.3**. A solution of microbeads is diluted into water within a 35mm petri dish (MatTek). A single bead is trapped in both the detection and trap laser beams, and the bead is brought to the desired height above the glass where AMR measurements will be taken. The laser beams are co-aligned and the signals on both the detQPD and trapQPD are centered by adjusting mirrors in the optical path.

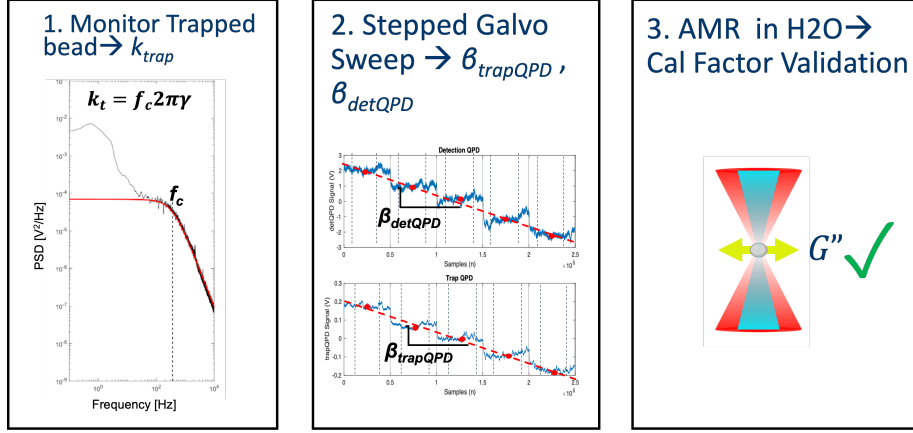


Figure 2.3: AMR calibration workflow.

After alignment, a minimum of 5 beads go through the three-step calibration sequence to find three calibration factors in both the X' and Y' axes of the measurement system. First, the trap stiffness k_t is attained using the power spectrum method. [8, 108, 119] The Brownian motion of the bead trapped in both laser beams is recorded for 30s and the power spectrum is fit with a Lorentzian equation. The corner frequency f_c of the Lorentzian fit is directly proportional to the trap stiffness (2.1)

$$k_t = f_c 2\pi\gamma \quad (2.1)$$

$$\gamma = 6\pi\eta r \quad (2.2)$$

Where the viscous drag coefficient is calculated by Eq. 2.2 with η , the temperature-dependent viscosity of water, and r , the radius of the bead.

Next, to find the voltage-to-displacement calibration factors, $\beta_{trapQPD}$ and β_{detQPD} , for both QPDs, a stepped sweep is performed. The galvos are swept across 600nm with 150nm steps and a dwell time of 5s per step. The slope of a linear fit of each QPD response is calculated as β . The average calibration factors across all successful calibration trials are saved as k_t , $\beta_{trapQPD}$ and β_{detQPD} for each axis tested. Lastly, these values are validated by performing AMR in water and comparing the measured viscosity with the theoretical viscosity.

2.3.1 Characterization of G2M Factor

In order to find the voltage-to-displacement calibration factors, $\beta_{trapQPD}$ and β_{detQPD} , the known trap beam positional response to a given galvo command voltage, what we refer to as the G2M factor, must be characterized. In previous iterations of the system, rather than detecting the positional change in the trap beam directly through imaging, the detQPD response was recorded with known piezo stage movements along one axis to find β_{detQPD} . Then, as the galvos were swept during daily calibration, $\beta_{trapQPD}$ could be found. However, this methodology did not take into account axes-dependent differences and is highly dependent on the alignment of the lasers to each QPD which are adjusted daily.

As an alternative, I directly characterized the G2M factor by observing the trap position at different galvo command voltages through image processing. Briefly, after alignment of the system, beads were trapped in water with only the trapping beam. Galvo command voltages were swept using 1mV steps from +/- 5mV, beyond the +/-3mV range used during AMR measurements. Images of the trap bead were recorded for 30s at each position to account for random Brownian motion within the trap, and the average bead position was computed. Linear regression was performed to find a relationship between the galvo command voltage and bead position which indicates the position of the trapping laser where the slope of the line fit gives the galvo voltage to trap position conversion factor, G2M. Example of these linear regressions are in **Figure 2.4A-B**. To account for any deviation across days due to laser alignment, this process was repeated on 5 separate days. The average G2M factor in each axis was computed and used to calculate $\beta_{trapQPD}$ and β_{detQPD} moving forward, where G2Mx and G2My were found to be -63.31 and -69.16 $\mu\text{m}/\text{mV}$ respectively. To verify the G2M values characterized, the measured G' in the X' and Y' axes within cell-free GelMA hydrogels prepared as detailed in **Chapter 4.3.3** were compared. Since these hydrogels are expected to be isotropic, we used them to check for anisotropic bias in the AMR system. Using the G2M factor to calculate the daily calibration factors, AMR-measured G' was found

to be isotropic in cell-free GelMA hydrogels, verifying the use and validity of this calibration scheme for AMR measurements. (Figure 2.4C; Mann-Whitney U-test: $p > 0.05$)

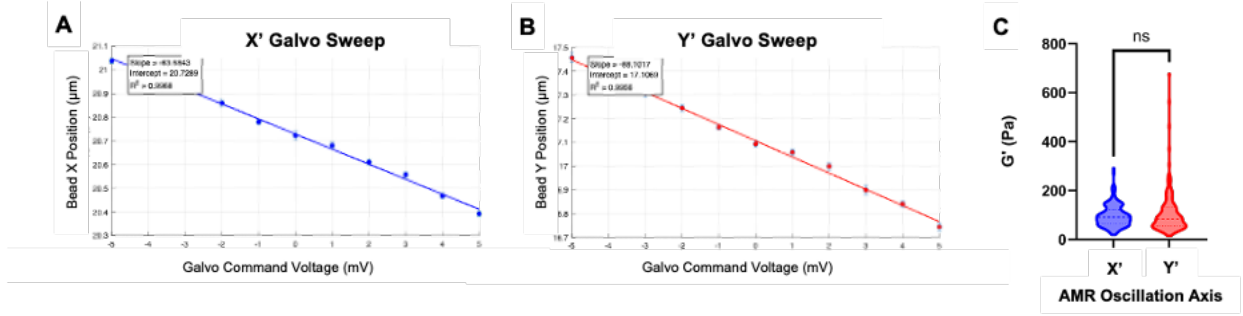


Figure 2.4: Characterization of G2M Factor. Linear regression of measured probe bead position vs. galvo command voltage in the (A) X' and (B) Y' direction. (C) AMR measured G' in the X' and Y' axes within cell-free GelMA using G2M values for calibration.

2.4 Calculation of G^*

After the system is aligned and calibration is completed, the bead is brought to the laser trap center and AMR is conducted at a given frequency and amplitude along specified axes using the custom LabView software. Using the detQPD and trapQPD signals that were recorded during trapping beam oscillation, the local complex shear modulus, G^* , can be calculated as described [108, 80, 72, 61] and summarized in **Figure 2.5**. The calibrated trapQPD and detQPD signals correspond to the trap position $x_t(t)$ and bead position $x_b(t)$ over time respectively, and the optical force acting on the bead in 1-D for a single axis is given by

$$f(t) = k_t(x_t(t) - x_b(t)) \quad (2.3)$$

Where k_t is the trap stiffness that was found during calibration through the power spectrum method. The Fourier transform of each signal can be taken to represent each function in the

frequency domain as shown and the force function becomes

$$f(t) = k_T (x_T(t) - x_B(t)) = k_T (A_t \sin(\omega t) - A_b \sin(\omega t - \theta_b(\omega))) \quad (2.4)$$

$$f(t) = A_f \sin[(\omega t) - \theta_f(\omega)] \quad (2.5)$$

Where A_f is the amplitude of the force function and $\theta_f(\omega)$ is the phase lag between the force function and the trap position function. The apparent complex response function $A_f(\omega)$ is then given as

$$A_f(\omega) = \frac{x_b(\omega)}{x_f(\omega)} \quad (2.6)$$

where $x_b(\omega)$ and $x_f(\omega)$ are the Fourier transform of $x_b(t)$ and $x_f(t)$ respectively. The contributions of the trap in the complex response must be corrected for, and the resulting corrected response function becomes $\alpha(\omega)$.

$$\alpha(\omega) = \frac{A(\omega)}{1 - k_t A(\omega)} \quad (2.7)$$

Note that we consider the contribution of the trapping force of the detection beam to be negligible as it is an order of magnitude lower in terms of trap stiffness compared to the trapping beam. Thus, we correct $\alpha(\omega)$ by k_t , the trap stiffness of just the trapping beam as opposed to $k_t + k_d$. After trap correction, the Generalized Stokes-Einstein Relation (GSER) is used to calculate the complex shear modulus $G^*(\omega)$ assuming a continuum. [140]

$$G^*(\omega) = \frac{1}{6\pi r \alpha(\omega)} \quad (2.8)$$

G^* can then be broken down into the real and imaginary components where $G'(\omega)$ is the elastic or storage modulus $G''(\omega)$ is the loss modulus. For simplicity moving forward, I will

be referring to $G'(\omega)$ as the colloquial term, stiffness.

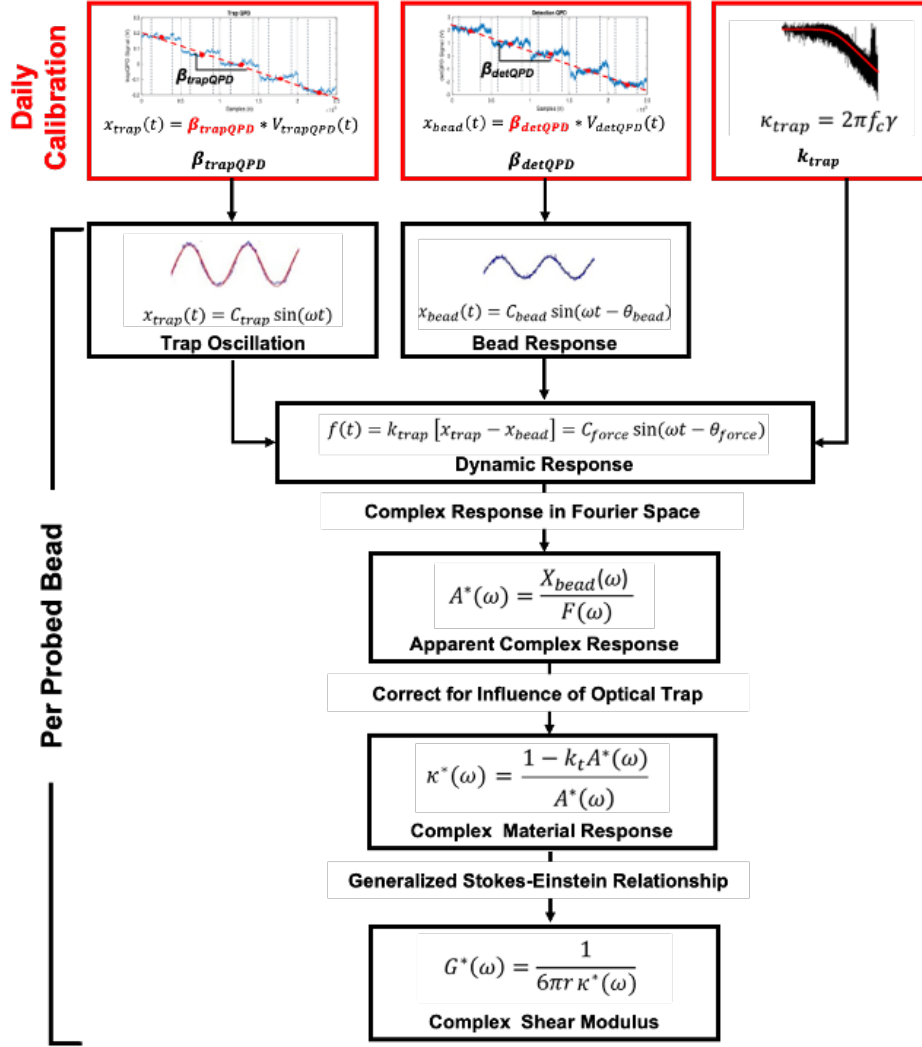


Figure 2.5: Diagram outlining the calculation of complex shear modulus G^* from AMR signals.

2.5 Alternative Micromechanical Characterization Techniques

While AMR was used in this thesis work, alternative methods for micromechanical characterization of biomaterials are available. Atomic Force Microscopy (AFM) is one commonly used method for mapping the topography and local mechanical properties of materials at

the nano-micro level. The AFM consists of a tip mounted to the end of a cantilever that probes the surface of a sample and is used as a nanoindenter to measure local elastic properties. [6] The tip is driven into the surface of the sample while a laser is typically used to measure the cantilever deflection giving force-displacement measurements across the sample. [96, 127] While this technique is well-established and provides high sensitivity, it is limited to measuring surface properties rather than probing the 3D bulk environment that cells are experiencing.

Alternatively, methods such as multi-particle tracking (MPT) or 3D traction force microscopy (TFM) can interrogate 3D volumes. Both MPT and 3D TFM involve monitoring the motion of embedded particles within the sample. In MPT, a type of passive microrheology, the thermal motion of embedded particles is tracked over time to estimate the local viscoelastic properties within the bulk material. [105, 136] However, as discussed in **Chapter 2.1**, this passive technique is limited to very soft materials. [38] 3D TFM provides spatial maps of cell-generated forces by tracking matrix deformations, as beads around these embedded cells are displaced. [32, 120] However, this technique is highly dependent on, and limited by, the material model used for force reconstruction and is computationally intense. [107] Additionally, this technique measures the forces exerted by cells onto the matrix, rather than intrinsic material properties. In contrast, optical tweezers-based AMR is well suited for characterizing the heterogeneous viscoelastic properties in 3D hydrogels with and without embedded cells due to the precise control of applied optical forces onto probe particles.

2.6 Limitations of AMR

Although AMR serves as a powerful tool to map micromechanical properties in 3D hydrogels, limitations of the technique must be considered. Optical tweezers-based AMR is ultimately limited by the optical force exerted on the probe particles by the optical trap. Optical tweezers use highly focused light to exert a localized trapping force onto probe particles that

is largely dependent on the index of refraction mismatch between the probe particle and the surrounding medium, probe bead size, the laser power, and how tightly the beam is focused. [5, 109, 14] These optical forces are limited to the pN range.

Another important limitation is the signal to noise ratio (SNR) of the particle tracking system. Particles can be tracking using various methods such as video-based tracking or laser-based detection systems as used here, which are generally more sensitive than video-based systems.[114] The minimum detectable displacement drives the measurement limit of an AMR system and is dependent on the SNR. [51]

Finally, the continuum assumption made in the calculation of the complex shear modulus G^* using the GSER limits the interpretation and validity of AMR measurements. This assumption starts to break as the particle size approaches the characteristic length, or mesh size, of the hydrogel. [100, 140] Thus, care must be taken when interpreting AMR-measured G^* in fibrous hydrogels where the pore size is larger than the probe particles. The continuum assumption can also break down if there are physical or chemical interactions between the probes and the material, or if the probes are not dilute enough and affect the structure of the material. [133, 140] Understanding these limitations of an optical tweezers-based AMR system is critical to the design and interpretation of microrheology experiments. Exploration of the limit of detection in our specific AMR system and the practical mitigation of its effects is discussed in **Chapter 3.3.5**.

Chapter 3

Active Microrheology Reveals Distinct ECM Mechanical Signatures Induced by Stromal Cells of Different Tissue Origins

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*ML and IWZ are co-first authors on this manuscript +ELB and AJP are co-corresponding authors on this manuscript

The work contained in this chapter has been submitted and is under review with ACS Biomaterials Science & Engineering.

3.1 Abstract

Stromal cells (SCs) provide important instructive cues to endothelial cells (ECs) during both normal and neoplastic vascularization. While the tissue-specific origins of ECs are important for function, the impact of SC identity on microvascular function remains unclear. We previously showed robust microvasculature forms by co-delivery of ECs and supportive SCs, that SC identity regulates the rate of neovascularization and vessel functionality, and such SCs of different identities exert varied traction forces on their surroundings. Here, we used active microrheology (AMR) and traditional macrorheology to evaluate dynamics of both local and global ECM mechanics in a 3D EC-SC co-culture model of vascular morphogenesis. Human umbilical vein ECs were co-embedded with either highly contractile lung fibroblasts (LFs) or significantly less contractile bone marrow-derived mesenchymal stromal cells (MSCs) within fibrin gels across various cell-seeding densities. By day 14, interconnected vascular networks developed, with rates of capillary morphogenesis higher in EC-LF than in EC-MSC co-cultures. Vascularization in EC-LF co-cultures was accompanied by ECM stiffening across length scales, in part due to cell contractility. AMR revealed highly heterogeneous local stiffness, with values ranging over two orders of magnitude in the same construct. AMR also identified the emergence of local stiffness anisotropy in the direction of capillary growth for EC-LF but not EC-MSC co-cultures by day 14, which was accompanied by significant matrix remodeling and local degradation. Together, these data suggest different SC populations, through active cell contractility-dependent stiffening and matrix degradation, induce local mechanical cues that differentially influence vascular development. These results highlight the importance of mechanobiological effects of SCs on the ECM in vascularized engineered tissues.

3.2 Introduction

The ability to successfully vascularize engineered tissues to support parenchymal cell function remains a significant challenge in regenerative medicine.[102] There is also an expanding unmet need for new approaches and therapies for the treatment of peripheral ischemia and other ischemic pathologies.[7, 125] One common cell-based strategy to address these challenges involves delivery of endothelial cells (ECs) in a scaffold or hydrogel to form new capillary structures capable of connecting to host microvasculature. However, delivery of ECs alone can result in the formation of leaky, unstable vessels that regress over time,[45] likely due to EC apoptosis.[130] Perivascular mural cells are essential for the formation of mature, stable, functional vessel networks,[78, 157] and strategies involving co-delivery of ECs with stromal cells (SCs) of different tissue origins have been widely explored.[157, 132, 35, 101, 45] Unfortunately, there is no consensus with respect to the choice of cells to co-deliver with ECs, in part because the definition of a “pericyte” remains contested.[17, 22, 47, 123] The organotypic identity of these SCs has also been shown to alter the transcriptome of ECs in co-culture models[24] and to influence the functional perfusion of microvasculature both in vitro[46, 101] and in vivo.[45]

In addition to paracrine cues from SCs,[110, 112] the mechanical properties of the extracellular matrix (ECM) play a regulatory role in vascular assembly and EC function. ECM elasticity, or stiffness, has long been postulated to be an important instructive cue governing vascular morphogenesis.[60] Many studies have used polyacrylamide substrates to evaluate the influence of ECM elasticity on EC phenotype in 2D, reporting changes in the permeability[58] and metabolism[157] of EC monolayers and in the assembly of microvascular networks. [16] In 3D, increased ECM density in natural protein-based hydrogels,[41, 124] and either increased solids content[154] or cross-linking[134] in synthetic gels, all of which increase the bulk stiffness, serve to attenuate angiogenic sprouting and vasculogenic self-assembly in 3D. By contrast, increased ECM stiffness independent of changes in density

has been shown to promote angiogenic sprouting,[83] and to induce a tumor-like vasculature with impaired barrier function.[11] ECs sense the mechanical properties of the ECM by exerting myosin-mediated contractile forces,[152] thereby controlling directional sprouting[79] and self-assembly into vascular networks.[27] These EC contractile forces are essential for capillary morphogenesis,[76] and the rates at which ECs deform ECM fibers correlates with the rates at which they form vessel-like structures in 3D.[77] EC-generated pulling forces on the ECM influence the multicellular integrity of nascent vascular sprouts[166] and vascular permeability.[99]

Despite widespread recognition of the importance of ECM stiffness, most prior studies correlate the initial bulk stiffness of an acellular material with subsequent EC phenotypic changes, distilling the complexity of the ECM’s mechanical properties down to a single numerical value. However, vascular morphogenesis involves reciprocal mechanical interactions between cells and the ECM that dynamically increase bulk stiffness over time in culture, coincident with the self-assembly of a microvascular network.[35, 67, 160] In this study, we aimed to assess and demonstrate the complexity of the ECM’s mechanical properties during vascular morphogenesis by evaluating the spatiotemporal changes in both bulk and local ECM stiffness via a combination of parallel plate shear rheology and optical tweezers-based active microrheology (AMR). We used a 3D co-culture model in which ECs and SCs are co-encapsulated in a fibrin hydrogel, undergoing a bottom-up self-assembly process that is widely used to fabricate prevascularized tissues.[34, 36, 101, 132] Based on our prior findings that SC identity influences vascular phenotype in vivo,[45] we evaluated the influence of SC identity on vascular assembly and both local and bulk mechanical remodeling.

Our findings show that lung fibroblasts (LFs) induce EC assembly to a greater extent than do bone marrow-derived mesenchymal stromal cells (MSCs). The combination of bulk rheology and AMR show that vascular morphogenesis is accompanied by dynamic mechanical remodeling of the fibrin across length scales over time, with significant differences observed

based on the total cell density and identity of the SCs. AMR detected local stiffness values that spanned nearly 3 orders of magnitude within a single construct, underscoring the wide range of mechanical heterogeneity experienced by cells during vascular morphogenesis in 3D. In the EC-LF co-cultures in particular, AMR revealed a transient softening followed by a collective stiffening of the ECM coincident with microvascular assembly, and enabled identification of a local cell-mediated stiffness anisotropy that may locally guide ECs during vascular morphogenesis. Collectively, this study shows that SC identity influences the magnitude of vascular morphogenesis and the mechanical properties of the ECM across length scales and over time.

3.3 Materials and Methods

3.3.1 Cell Culture

Human umbilical vein endothelial cells (ECs) were isolated from fresh umbilical cords (obtained via an IRB-exempt protocol from the University of Michigan Mott’s Children’s Hospital) as previously described.[40] ECs were cultured in fully supplemented Vasculife VEGF Endothelial Medium (Lifeline Cell Technology LLC, Frederick, MD), and used until passage 7. Normal human lung fibroblasts (LFs; Lonza) were cultured in high glucose Dulbecco’s modified eagle medium (DMEM; Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Gibco) and used until passage 15. Vasculife and DMEM were additionally supplemented with 1% Antibiotic-Antimycotic (Gibco). Bone marrow derived mesenchymal stromal cells (MSCs; RoosterBio, Frederick, MD) were cultured in Rooster Nourish (RoosterBio, Frederick, MD) and used until passage 6. All cells were cultured at 37 °C and 5% CO₂ with medium exchanges every other day.

3.3.2 Fibrin-based vascular morphogenesis assay

A three-dimensional cell culture model of vascular morphogenesis was assembled as previously described.[101, 35, 20] Stromal cells (SCs) and ECs were pelleted and resuspended at a 1:1 ratio across a range of total cell densities in fibrin-based gel precursor solutions (e.g., 500 K/mL (5×10^5 cells/mL) gels received 250 K/mL (2.5×10^5 cells/mL) ECs and 250 K/mL (2.5×10^5 cells/mL) SCs). Fibrinogen from bovine plasma (Sigma) was prepared in PBS and sterile filtered with a 0.22 μ m PES membrane filter (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, IRL). To form gels, sterile bovine thrombin (Sigma) was added to the center of the culture plate to achieve a final concentration of 1 U/mL in the fibrin gel. The cell-laden fibrinogen solution was then added to the thrombin in the plate, pipetting up and down once to mix. The final fibrin gels comprised of 2.5 mg/mL clottable fibrinogen protein and 10% FBS.

For bulk rheology and vascular network quantification, gels (500 μ L total volume) were cast in 24-well plates. For microrheology and reflection confocal imaging, gels (500-1000 μ L total volume) were fabricated in 35 mm glass-bottom dishes (MatTek, Ashland, MA). 100 K/mL (1×10^5 cells/mL) samples were cast directly into 35 mm glass-bottom dishes (1000 μ L total volume). For 500 K/mL (5×10^5 cells/mL) samples, polydimethylsiloxane (PDMS, Dow Corning, Midland, MI) well inserts were fabricated to reduce the size of the gels. Inserts were made from sheets of PDMS, cut with a 5/8" hole punch (McMaster-Carr, Elmhurst, Il) to form the ID of the insert and trimmed down to fit inside the 35mm dishes. 500 K/mL (5×10^5 cells/mL) gels were cast into PDMS inserts within 35mm dishes (500 μ L total volume).

Hydrogels were left undisturbed for 5 minutes at room temperature in the biosafety cabinet before moving to an incubator for 25 minutes. Gels were then cultured in fully supplemented Vasculife, with media exchanged every other day (1 mL media per 500 μ L gel or 2 mL media per 1 mL gel) until each planned timepoint was reached. For experiments involv-

ing the myosin II inhibitor blebbistatin, constructs were re-fed with medium containing 50 μM blebbistatin on either day 13 (overnight treatment) or day 14 (1 hr treatment). After treatment, constructs were subjected to bulk rheology and fixing and staining for vascular quantification.

3.3.3 Bulk rheology of fibrin gels

The mechanical properties of the cell-laden fibrin constructs were assessed by quantifying the bulk shear storage moduli (G') via parallel plate shear rheology as previously described.[68] An AR-G2 rheometer (TA Instruments, New Castle, DE) was used with an 8-mm diameter measurement head with P800 sandpaper and a Peltier stage. Rheology was performed in-plate and at a 300 μm depth from the surface of the fibrin gel. This depth was attained by lowering the measurement head incrementally until contact with the gel surface, after which the measurement head was lowered 300 μm further to compress the gel. Shear storage moduli were measured at 37 $^{\circ}\text{C}$, 6% strain amplitude, and 1 rad/s frequency and averaged over a 1-min time sweep. Depending on the experiment, measurements were taken on day 1, 3, 5, 7 or 14.

3.3.4 Fluorescent imaging and quantification of vasculogenesis

On days 3, 5, 7, and 14, hydrogels were fixed with zinc formalin (Z-fix; Anatech, Battle Creek, MI) for 15 min, then washed three times with PBS or TBS for 5 min. Gels were then stained overnight with rhodamine-conjugated lectin from *Ulex europaeus* agglutinin I (UEA, 1:200; Vector Labs, Newark, CA), 4', 6-diamidino-2-phenylindol (DAPI, 1 $\mu\text{g}/\text{mL}$; Thermo Fisher, Waltham, MA), and AlexaFluor 488 phalloidin (1:200; Thermo Fisher, Waltham, MA) to visualize ECs, nuclei, and F-actin, respectively. Hydrogels were then washed with PBS or TBS overnight prior to imaging. Images were acquired using an Olympus IX81 confocal microscope equipped with a disk scanning unit (DSU; Olympus America, Center Valley, PA) and Metamorph Premier software (Molecular Devices, Sunnyvale, CA). 300 μm confocal

stacks (7 slices/stack, 50 μm step) were acquired via the DSU and collapsed into maximum intensity projections using Fiji. Quantification of vascular density was performed on 4x magnification stacks using the Angiogenesis Tube Formation application in Metamorph. Total vessel network lengths were quantified by the module within a given volume, with final values then reported as vessel densities (length per volume).

3.3.5 Optical tweezers-based active microrheology

Optical tweezers active microrheology (AMR) was carried out using a dual-laser, multi-axis optical tweezers system as previously described [63] (see system schematic in Fig. S1). A 1064nm continuous-wave fiber laser (YLR-5-1064-LP, IPG Photonics) micro-beam was steered by a pair of galvanometer mirrors (GVS012, Thorlabs) that were located conjugate to the back focal plane of the microscope objective lens (60x-oil PlanApo TIRFM 1.45NA, Olympus) and were controlled by custom LabView (National Instruments) software. A stationary, co-aligned 785nm fiber-pigtailed laser beam (LP785-SF100, ThorLabs) was used to detect probe bead displacement in response to the driving force from the trapping beam. Optical trap position was monitored by a beam sampling optic and quadrant photodiode (QPD) placed distal to the galvanometer pair. Bead position was monitored by collecting the small fraction of light that is back reflected by the trapped bead, and deflection of this reflected light is detected by a second QPD. (2901 and 2903 respectively, Newport, Irvine, CA). Prior to AMR measurements, the AMR system was calibrated in water, as previously described [72] to find the proportionality constants relating QPD signals to bead displacements and the optical trap stiffness. Then we validated the system can properly measure viscosity of water given the calculated calibration factors. During AMR experiments, the QPD proportionality constants, trap stiffness, and QPD signals were used to calculate the complex material response, α^* . AMR-measured stiffness data herein is presented as the real component, $\hat{G}'$, of the complex shear modulus, which is computed from α^* as previously described.[108] Note, we use the variable $\hat{G}'$ for AMR measurements and G' for parallel

plate rheometer measurements. For AMR experiments, a dispersion of 2 μm diameter carboxylated silica microbeads (Bangs Laboratories, Fishers, IN) at a concentration of 0.08% (w/v) was included in the fibrinogen precursor solution prior to hydrogel polymerization as described above within 35 mm glass-bottom dishes (MatTek, Ashland, MA). A stock solution of microbeads in ultra-pure water was sterilized in a UV oven for 30min prior to dilution in precursor solution. Each sample was mounted in a dish holder placed inside a stage-top nanopositioning piezoelectric stage (P-545.xR8S PInano XYPiezoSystem, PI) on an IX81 inverted microscope (Olympus). $\hat{G}'$ was probed by oscillating individual microbeads in the transverse plane at with the optical trap, driven at 50Hz and an amplitude of 150nm . AMR measurements were performed on days 1, 3, 5, 7, 10, and 14 (N=3 hydrogels, per day, per cell-laden condition). Within each sample, 3 fields-of-view (FOVs) were chosen that contained growing capillaries and 15 probe beads were randomly selected and measured in each FOV. For cell-free gels, 3 hydrogels were incubated for 1 day and 45 beads were measured in each sample. Each measured probe bead was approximately 35 μm above the coverglass and oscillated along the horizontal and vertical axes with respect to the camera image FOV.

Prior to reporting a value for $\hat{G}'$ we conduct one additional triage step to ensure signals are above the noise floor, otherwise the calculated $\hat{G}'$ will have no physical meaning. Beads not having movement within the detection limit of the detection beam QPD were excluded from analysis. Specifically, beads were rejected if the amplitude of the FFT of the detection beam QPD signal (at the drive frequency) was under 3 standard deviations above the noise floor defined below. Further, beads having a calculated $\hat{G}' < 0$ were rejected. The noise floor was defined as the FFT amplitude at frequencies bracketing the drive frequency of 50 Hz, specifically within windows spanning 40-45 Hz and 55-60 Hz (Fig. S2). Fundamentally AMR is limited by the maximum optical force acting on a bead (on the order of several hundreds of pN) that must overcome material resistance in order to displace the bead within the detection limit. Further, the detection limit can be affected by the sensitivity and signal to noise ratio (SNR) of the bead position detection system as well as the alignment of the trapping and

detection lasers.[51] Thus, we control and adjust for laser alignment daily during calibration steps in water.

3.3.6 Reflection confocal microscopy

Reflection confocal microscopy (RCM) images were acquired in fixed and stained samples in order to visualize the fiber structure across different co-cultures. RCM was conducted using the 488 nm laser of a Fluoview3000 laser scanning microscope (Evident) with a UPlanSApo 40x/1.25 NA silicone immersion objective lens (Evident). For the same FOV, confocal fluorescence images were acquired using the 405 nm, 488 nm, and 561 laser lines, where the dichroic and filter settings were predefined by the microscope software for the given fluorescent dyes. Images were collected with a scanning speed of 2 μ s/pixel and a scan resolution of 4096 pixels x 4096 pixels across the 318.2 μ m x 318.2 μ m FOV. The Contrast Limited Adaptive Equalization (CLAHE) method was performed on the reflection images before stacking and stitching the images together with the corresponding fluorescent images using Fiji.

3.3.7 Statistics

Various statistical analyses were performed via Prism (GraphPAD, La Jolla, CA) depending on the nature of the data. Bulk mechanical properties and vascular density data were analyzed using one-way ANOVA or two-way ANOVA as appropriate regarding the number of independent factors compared. Tukey post-hoc testing was used for multiple comparisons. Bulk mechanical and vascular density data are represented as mean $\pm$ standard deviation of at least three independent experimental replicates. AMR data was analyzed using non-parametric tests because most distributions are non-normal. Data are represented as median $\pm$ IQR. Comparisons between two AMR distributions were performed using the Mann-Whitney U test. Comparisons between three or more AMR distributions were performed using Kruskal-Wallis test followed by Dunn's post-hoc test for multiple comparisons. Chi-square tests were used to compare AMR measurement success across groups.

3.4 Results

3.4.1 EC co-culture with different stromal cell populations yields vascular network formation in 3D fibrin constructs.

ECs were co-embedded with different stromal cell types (LF or MSC) in a 3D fibrin matrix to investigate distinctions in vascular morphogenesis (**Figs. 3.1A,3.2A**). Initial cell seeding density was also varied (100 K/mL, 250 K/mL, or 500 K/mL) to study how this affected vascular network assembly for different co-culture conditions. On day 1 of culture, ECs in all conditions remained rounded in morphology, irrespective of SC identity (LF vs. MSC) and initial cell densities (**Figs. 3.1B,3.2B**). By day 3, tubular vessel-like projections formed in all cell density and co-culture conditions, with subsequent elongation and branching occurring over the 14-day culture period (**Figs. 3.1B,3.2B**). More extensive branching was observed earlier for 250 K/mL and 500 K/mL constructs in comparison to 100 K/mL constructs for both EC-LF and EC-MSC co-cultures (**Figs. 3.1B,3.2B**).

EC-LF constructs also exhibited distinctions in vascular morphogenesis in comparison to EC-MSC constructs. Representative images demonstrate progressive vascular network formation for EC-LF conditions, especially in the 250 K/mL and 500 K/mL conditions, where dense branching morphologies were observed by days 7 and 14 (**Fig. 3.1BB**). In contrast, EC-MSC constructs exhibited sparser tubule formation and formed networks of lower density (**Fig. 3.2B**). Both stromal cell populations used in the co-culture exhibited close association with the endothelial cells and populated space in between the endothelial cell networks (**Fig. S.3.3**). Additionally, both co-culture conditions yielded vascular networks with clear, elongated tubular projections in comparison to EC monoculture constructs, which failed to develop persistent elongated projections over the course of culture time (**Fig. S.3.4A**). This was evidenced by comparing the mean tube length of vascular projections quantified in co-culture and monoculture conditions, where EC-LF and EC-MSC co-culture conditions

exhibited significantly longer average tube length by day 5, 7 and 14 of culture (**Fig. S.3.5**).

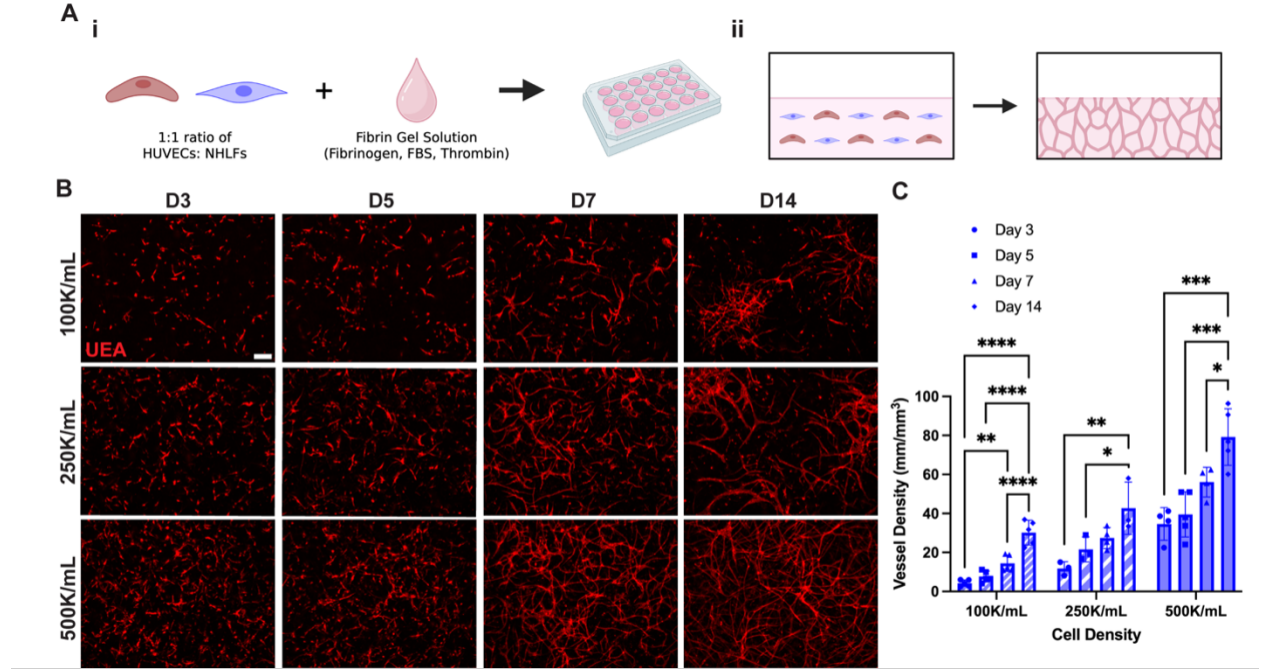


Figure 3.1: A) i) Schematic of combining fibrin with endothelial and lung fibroblast cells and ii) culturing to yield fibrin-based vascular morphogenesis assay. B) Representative max intensity projection (z stack = 300 μm) images of vasculogenesis in fibrin constructs with EC-LF co-culture at 100 K/mL, 250 K/mL, and 500 K/mL total cell density, after 3, 5, 7 and 14 days of culture, are shown stained with UEA (red; scale bar = 200 μm). C) Quantification of vessel density over culture time (days 3, 5, 7, and 14). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$.

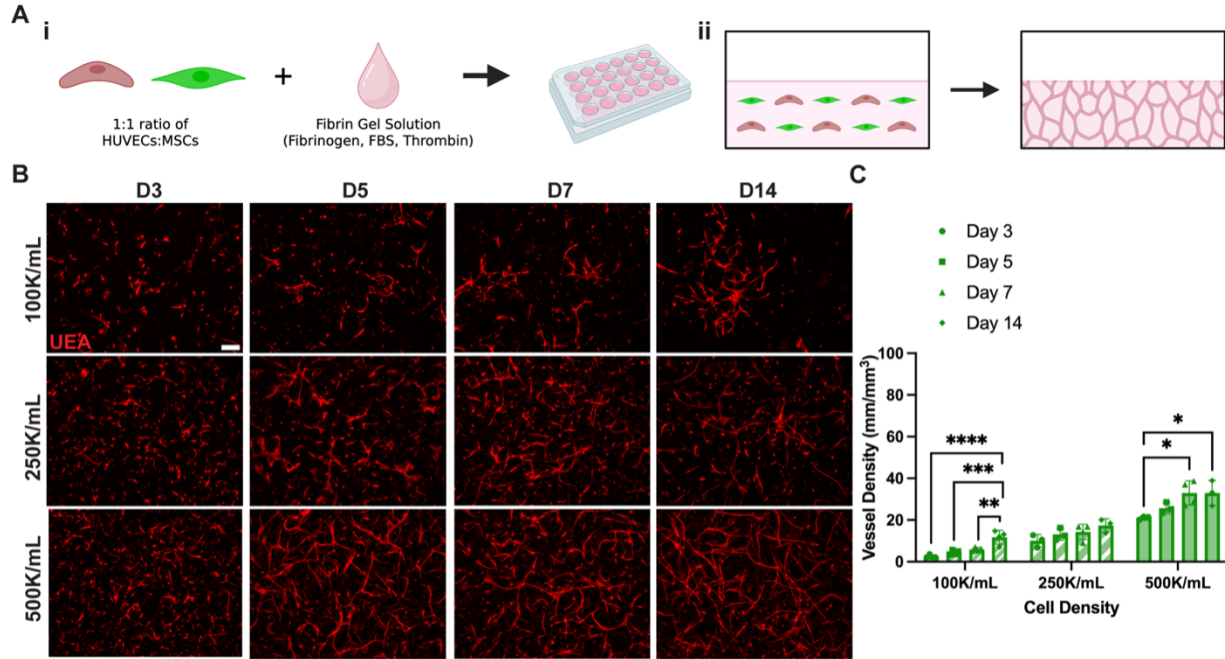


Figure 3.2: A) i) Schematic of combining fibrin with endothelial and mesenchymal stromal cells and ii) culturing to yield fibrin-based vascular morphogenesis assay. B) Representative max intensity projection (z stack = 300 μm) images of vasculogenesis in fibrin constructs with EC-MSC co-culture at 100 K/mL, 250 K/mL, and 500 K/mL total cell density, after 3, 5, 7 and 14 days of culture, are shown stained with UEA (red; scale bar = 200 μm). C) Quantification of vascular density over culture time (days 3, 5, 7, and 14). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$.

3.4.2 Stromal cell identity in EC co-culture affects resultant vascular morphogenesis

Quantification confirmed the increase in vascular density with increased culture time that was observed in representative images (Figs. 3.1B, 3.2B), with significant increases in vessel density for EC-LF (Fig. 3.1C) across all initial cell seeding densities and the 100 K/mL and 500 K/mL conditions of EC-MSC constructs (Fig. 3.2C). In contrast to EC-LF constructs at the same initial cell density, EC-MSC constructs containing 250 K cells/mL exhibited no significant changes in vessel density over time, seemingly plateauing by day 3 of culture (Fig. 3.2C).

To better assess the differences in vascular morphogenesis resulting from different EC-stromal cell co-culture conditions, we reorganized the quantified vessel density data (**Figs. 3.1C, 3.2C**) and performed two-way ANOVA analysis between EC-LF and EC-MSC constructs for a given cell seeding density (**Fig. 3.3**). We specifically evaluated quantitative differences when tubular vessel-like projections first began appearing (day 3) and at the final culture timepoint (day 14). At day 3, only the highest cell seeding density condition (500 K/mL) demonstrated a significant difference in vessel density between stromal cell co-cultures, where EC-LF co-culture exhibited significantly greater ($p < 0.05$) vessel density than the EC-MSC condition (**Figs. 3.3A, C-i**) and EC-monoculture condition (**Fig. S.3.6A**). After 14 days of culture, EC-LF (100 K/mL: $p < 0.001$; 250 K/mL: $p < 0.05$; 500 K/mL: $p < 0.0001$) co-culture conditions demonstrated significantly greater vessel densities in comparison to all EC-MSC conditions (**Fig. 3.3B, C-ii**). EC-MSC vessel densities were less than half the average vessel densities of EC-LF conditions (**Fig. 3.3B**). EC-LF co-culture yielded vessel densities significantly greater than EC monoculture conditions at day 14 as well, with the EC-MSC co-culture also exhibiting significantly greater vessel density than the monoculture in the 500 K/mL condition (**Fig. S.3.6B**).

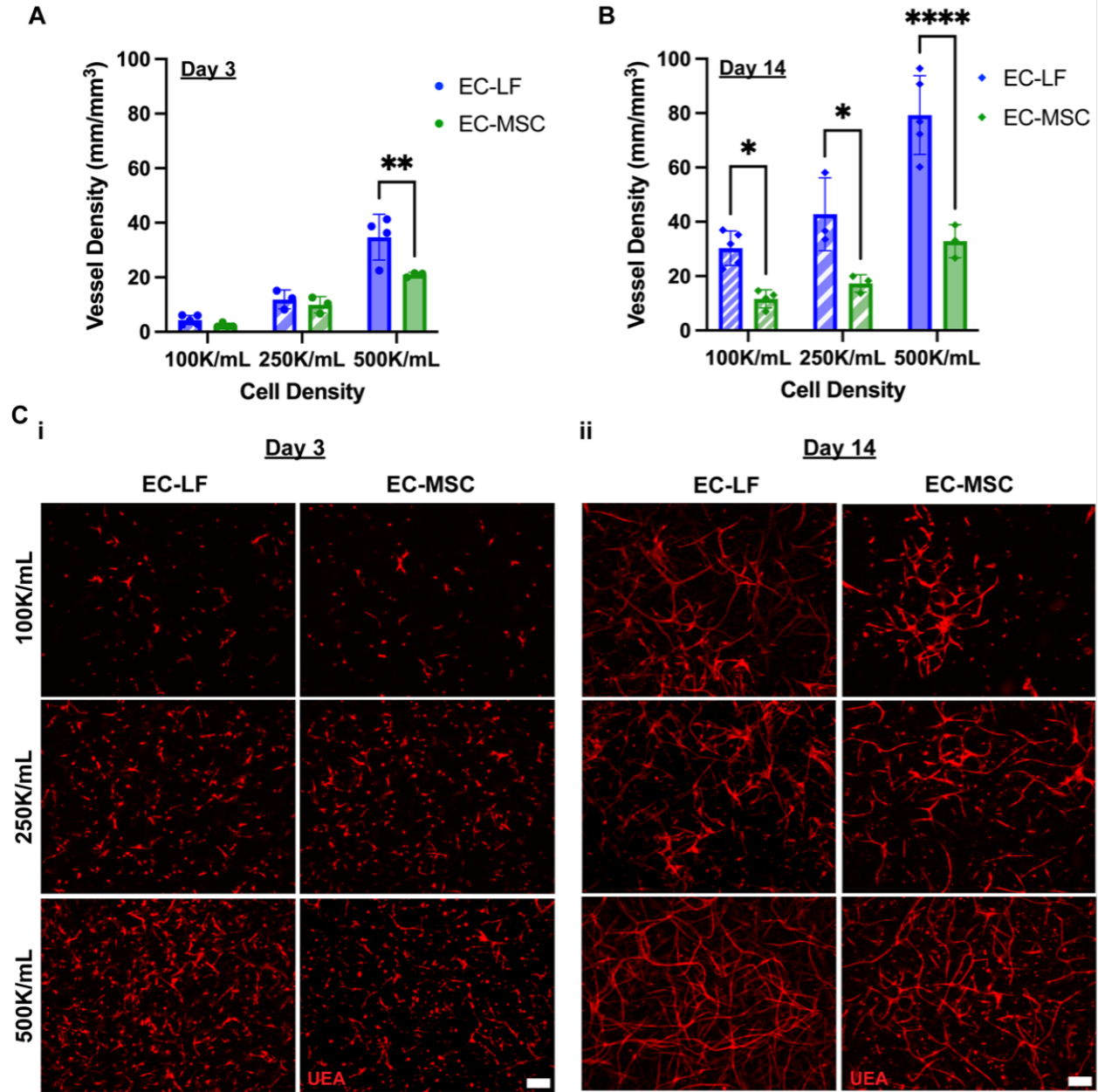


Figure 3.3: Comparison of vessel densities quantified on (A) day 3, and (B) day 14, between co-culture (EC-LF and EC-MSC) conditions at varying initial cell seeding densities (100 K/mL, 250 K/mL, 500 K/mL). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$. C) Representative max intensity projection (z stack = 300 μ m) images of vasculogenesis in fibrin constructs with EC-LF or EC-MSC co-culture at 100 K/mL, 250 K/mL, and 500 K/mL total cell density, after i) 3 or ii) 14 days of culture, shown stained with UEA (red; scale bar = 200 μ m).

3.4.3 AMR demonstrates local ECM stiffness heterogeneities during vascular morphogenesis

Upon confirming the progression of vascular morphogenesis in EC-LF and EC-MSD co-cultures in the fibrin vasculogenic assays, ECM mechanical properties were assessed to uncover changes that occurred alongside vascular self-assembly. Optical tweezers-based active microrheology (AMR) was performed to evaluate local ECM stiffnesses during the process of vascular morphogenesis. AMR measurements along two probe axes were conducted in each co-culture across 14 days. A cartoon schematic of AMR and the probe axes (X' and Y') is shown (**Figs. 3.4A-B**). AMR revealed large heterogeneities in local stiffness surrounding a growing capillary. For example, consider $\hat{G}'$ in the X' direction, $\hat{G}'_x$ for the two beads indicated by arrows in **Fig. 3.4C**. These beads are separated by 25 μm but reflect a 25x difference in $\hat{G}'_x$ as measured via AMR. An aggregate including all co-culture conditions and both probe axes further illustrates the heterogeneity in local ECM stiffness, which spans orders of magnitude (**Fig. 3.4D**). $\hat{G}'$ in cell-laden fibrin gels were considerably larger than that of acellular fibrin gels (**Fig. S.3.7**), whose range of $\hat{G}'_x$ is represented by the gray horizontal rectangle in **Fig. 3.4D**.

Next, the minimum distance between each probe bead and the nearest cell was measured. Beads were then stratified in categories “near” ($\leq 50\mu\text{m}$ away) and “far” ($> 50\mu\text{m}$ away) based on their proximity to the nearest cell. The 50 μm threshold was selected based on the apparent change in slope in the $\hat{G}'$ versus distance relationship. (**Fig. S.3.8**) As expected and consistent with our previous findings of pericellular ECM stiffening,[71] the ECM near cells was stiffer and more heterogeneous ($p < 0.0001$) across all co-culture conditions, suggesting cell-mediated local strain hardening and remodeling of the surrounding ECM (**Fig. 3.4E**). Interestingly, EC-LF constructs initially seeded at the highest cell density (500 K/mL) were stiff, while the ECM far from cells in EC-MSD constructs was indistinguishable from acellular fibrin gels (**Fig. S.3.9**). These data suggest that cell-mediated ECM

stiffening occurred over long ranges in the 500 K/mL EC-LF constructs which had the most vascular development.

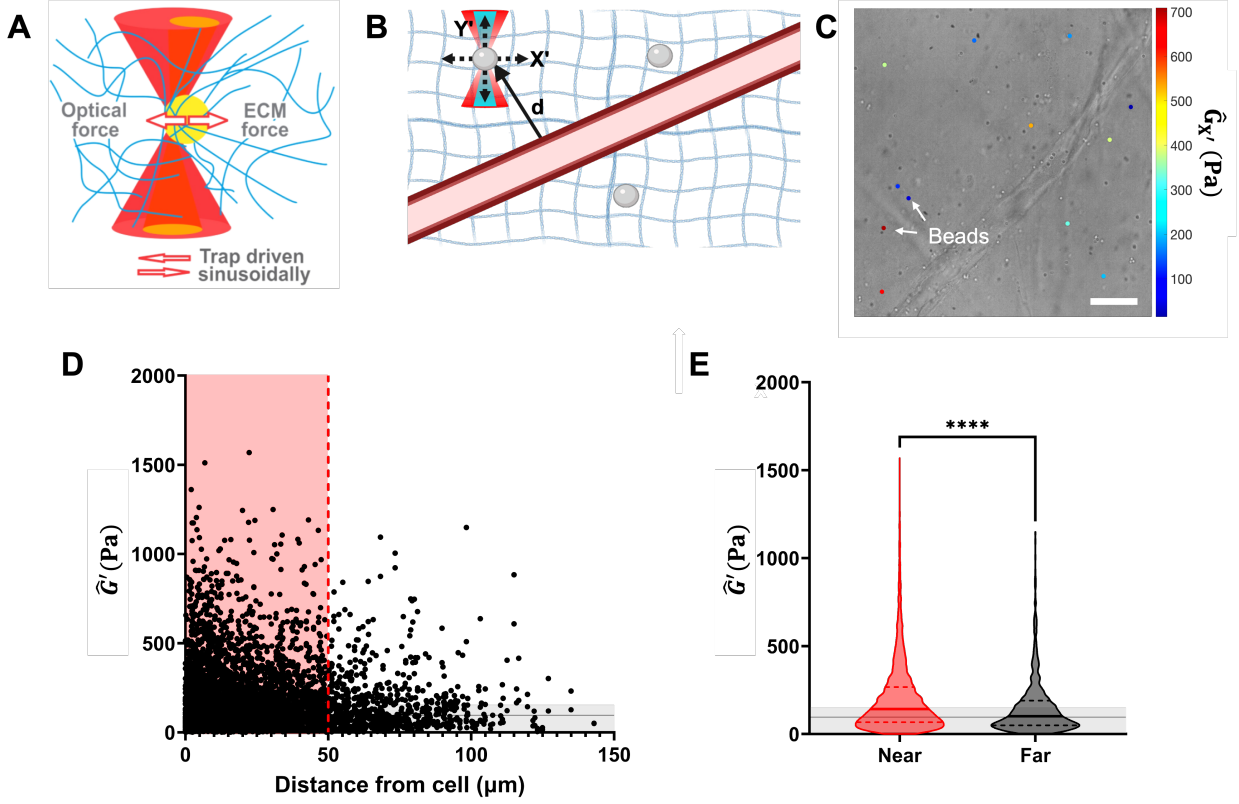


Figure 3.4: A) Schematic diagram of AMR method to measure microscale ECM mechanics; adapted from [72]. As the trapping beam is oscillated sinusoidally, the optical trapping force acting on the bead is opposed by ECM forces. B) Cartoon schematic of AMR performed around a growing capillary. The distance, d , is measured from each bead to the nearest cell. Each bead is oscillated in the X' and Y' direction. Created with Biorender.com. C) Brightfield image of beads surrounding a growing capillary (scale bar = $30\mu\text{m}$). Overlaid heat map of AMR-measured $\hat{G}'_x$ demonstrates micro-mechanical heterogeneity. D) $\hat{G}'$ of all co-cultures plotted as a function of distance to the nearest cell. Dashed line at $50\mu\text{m}$ indicates the separation point between “near” and “far” groups. Each data point is a single probe bead measurement along a single oscillation axis. The top of the gray-shaded bar represents the 75th percentile of $\hat{G}'$ of cell-free fibrin gels with the horizontal black line at the median, 95 Pa. E) AMR measurements from (D) separated into groups “near” and “far” based on proximity to the nearest cell ($\leq 50\mu\text{m}$ and $> 50\mu\text{m}$, respectively). Mann-Whitney U test: ****: $p < 0.0001$.

3.4.4 ECM stiffness changes across length scales are stromal cell and cell seeding density dependent, and accompany vascular development

Next, we evaluated the effects of stromal cell type and initial cell seeding densities on ECM stiffness dynamics using both bulk parallel-plate rheology and AMR. In EC-LF co-cultures, bulk G' trends over time differed considerably with increased initial cell seeding density. At the lower initial seeding density of 100 K/mL, mean G' in EC-LF co-cultures remained relatively constant over time, in contrast to the significant variation in G' with time in constructs seeded with higher cell densities (**Figs. 3.5A-B**). In the 100 K/mL condition, 94% of local $\hat{G}'$ measurements were within the 75th percentile of $\hat{G}'$ in acellular gels, indicating a low level of cell-mediated stiffening (**Fig. 3.5C**). A slight stiffening was detected ($p < 0.05$) between intermediate time points and day 14 (**Figs. 5A and C**). This difference was mainly driven by softening at these intermediate time points rather than stiffening over time, as day 14 $\hat{G}'$ rebounded to level just modestly above day 1 $\hat{G}'$. ($p < 0.1$)

In contrast, at the higher initial seeding density of 500 K/mL, EC-LF co-cultures exhibited a large increase in G' by day 14 ($p < 0.001$). Importantly, while assessing $\hat{G}'$ we found many regions of the ECM stiffened beyond the measurement limit of AMR. In these regions, the optical trap force was unable to overcome opposing ECM mechanical resistance and thus AMR bead movements were undetectable. As noted in the Materials and Methods, beads with undetectable movements were excluded from analysis, and **Fig. 3.5** and **3.6** only include the non-rejected beads. The percentage of rejected beads is a measure of stiffening, where local $\hat{G}'$ is at or above the maximum detectable stiffness $\hat{G}'_{max} = 1800$ Pa). The proportion of rejected beads increased over time, indicating significant local stiffening in the pericellular space (**Fig. S.3.10**). Additionally, only 42% of accepted AMR measurements were within the 75th percentile of $\hat{G}'$ in acellular fibrin constructs (**Fig. 3.5D**), further demonstrating

local stiffening over time, particularly at day 14, consistent with bulk observations (G') (Fig. 3.5B).

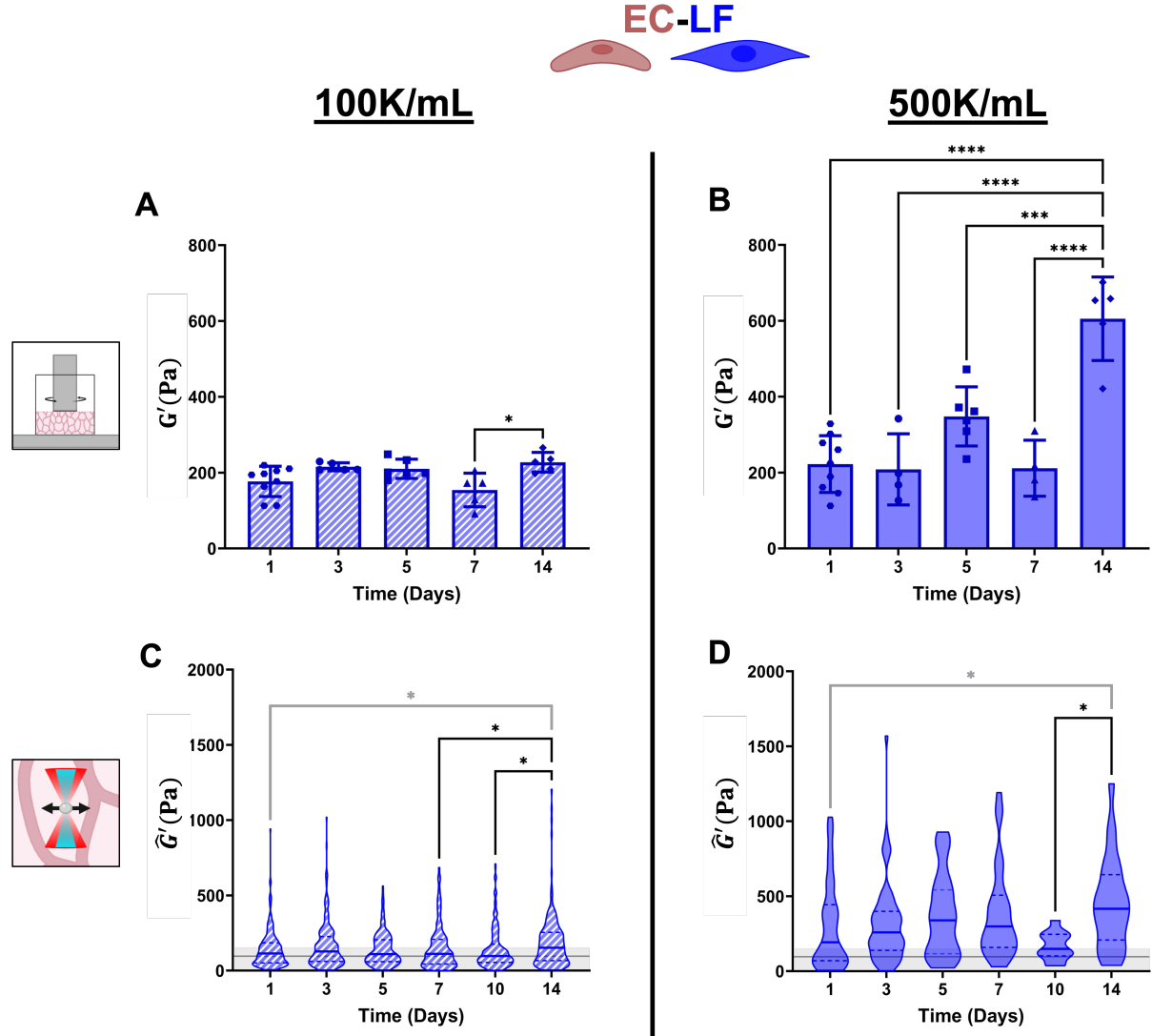


Figure 3.5: Bulk parallel-plate G' in EC-LF co-cultures with A) 100 K/mL and B) 500 K/mL seeding densities over 14 days. AMR-measured $\hat{G}'$ in EC-LF co-cultures with C) 100 K/mL and D) 500 K/mL seeding densities over 14 days. The top of the gray-shaded bar represents the 75th percentile of $\hat{G}'$ of cell-free fibrin gels with the horizontal black line at the median. For A) and B), one-way ANOVA with Tukey post-hoc testing: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. For C) and D), Kruskal-Wallis test with Dunn's multiple comparisons: gray-colored *: $p < 0.1$, *: $p < 0.06$; **: $p < 0.01$.

At low seeding density, ECM stiffness dynamics were similar between EC-MSC and EC-LF co-cultures (Figs. 3.6A,C, and S.3.11). For EC-MSC there was no bulk stiffening

between early time points and day 14 and a majority of $\hat{G}'$ measurements were within the 75th percentile of acellular $\hat{G}'$, indicating low levels of cell-mediated stiffening. Unlike EC-LF cultures, which soften at day 7 and “rebound” at day 14, EC-MSC did not stiffen their matrix at the two-week time point. Although both local and bulk softening were observed at intermediate time points in both SC co-cultures, no subsequent stiffening at day 14 was detected by either AMR or parallel-plate rheology in EC-MSC co-cultures seeded at low cell density, in contrast to EC-LF co-cultures.

Differences between EC-LF and EC-MSC co-cultures were evident at the higher seeding density of 500 K/mL (**Figs. 3.6B,D, and S.3.11**). No significant bulk stiffening was detected in EC-MSC co-cultures over time when seeded at 500 K/mL (**Fig. 3.6B**). Although an increase in median $\hat{G}'$ was detected from day 10 to day 14, the increase was lower in magnitude than was observed for EC-LF co-cultures (**Fig. 3.6D**). Further, day 14 median $\hat{G}'$ did not exceed that of day 1 and 74% of AMR measurements across the 14 days were within the 75th percentile of acellular $\hat{G}'$. This relatively static ECM stiffness was accompanied by lower vascular densities as compared to the EC-LF co-cultures, indicating that vascularization and ECM stiffness changes may be correlated.

To investigate a potential relationship between cell-mediated ECM stiffening and microvascular assembly, we treated EC-LF co-cultures with an initial seeding density of 500 K/mL with blebbistatin, a myosin II inhibitor, for either 1 hr on day 14 or overnight on day 13. Vascular densities were qualitatively similar +/- blebbistatin (**Fig. S.3.12C**), which was confirmed quantitatively across conditions (**Fig. S.3.12**). By contrast, there was a significant decrease in day 14 G' in both blebbistatin treated conditions (1 hr or overnight) as compared to the untreated control (**Fig. S.3.12B**). Interestingly, day 14 G' of these treated constructs were similar to G' of untreated constructs from day 1 to day 7 of culture (**Fig. S.3.12B**), indicating a role for active cell-mediated contractility in the development of stiffness over time.

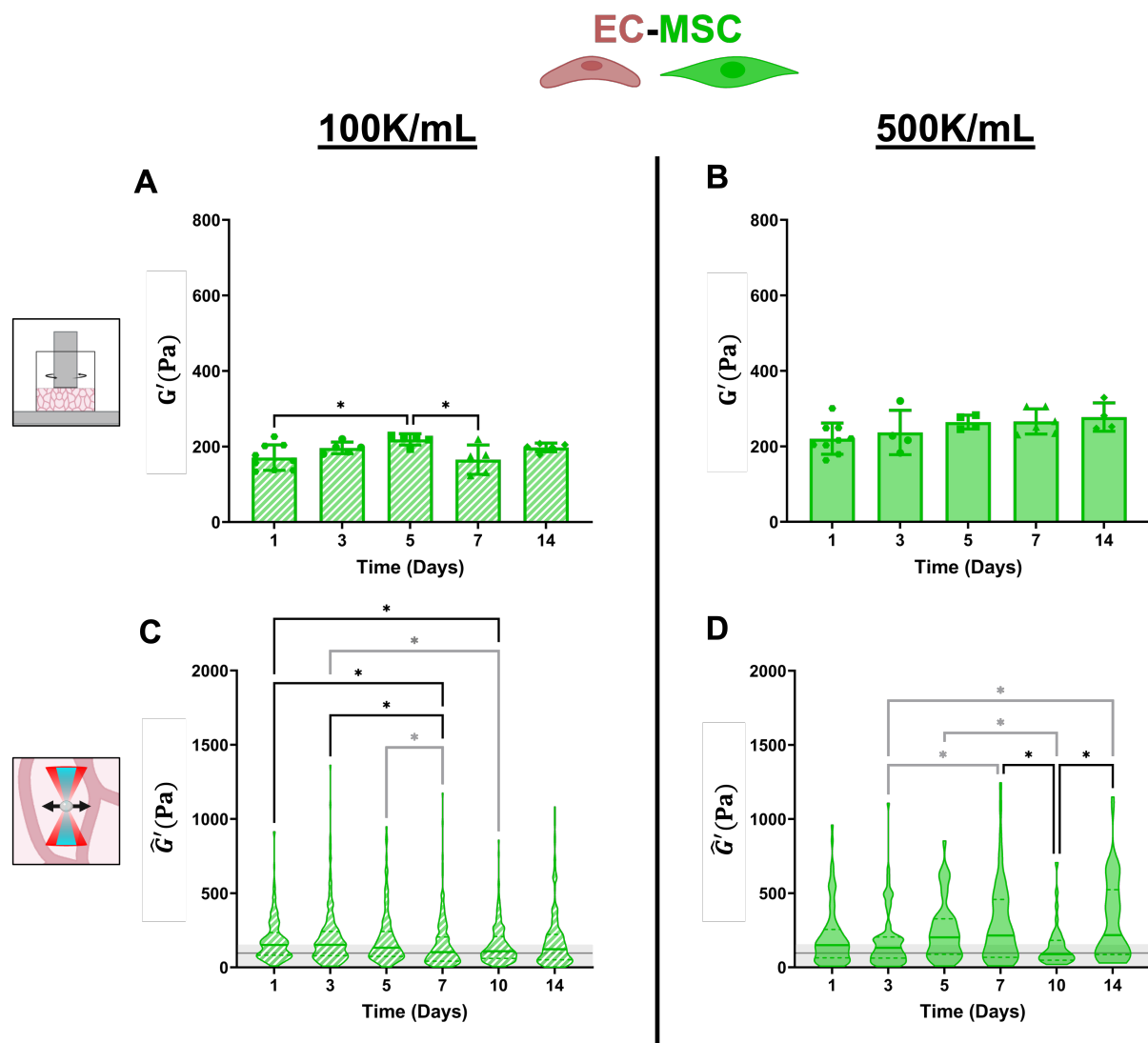


Figure 3.6: Bulk parallel-plate G' in EC-LF co-cultures with A) 100 K/mL and B) 500 K/mL seeding densities over 14 days. AMR-measured $\hat{G}'$ in EC-LF co-cultures with C) 100 K/mL and D) 500 K/mL seeding densities over 14 days. The top of the gray-shaded bar represents the 75th percentile of $\hat{G}'$ of cell-free fibrin gels with the horizontal black line at the median. For A) and B), one-way ANOVA with Tukey post-hoc testing: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. For C) and D), Kruskal-Wallis test with Dunn's multiple comparisons: gray-colored *: $p < 0.1$, *: $p < 0.06$; **: $p < 0.01$.

3.4.5 Increased vascular development in EC-LF co-cultures is accompanied by anisotropic stiffening and extensive local ECM remodeling

AMR measurements taken along the probe axes (X' and Y') were projected along the axes of the nearest capillary (X and Y) to investigate cell orientation-dependent stiffening during vasculogenic self-assembly (**Fig. 3.7**). The difference between $\hat{G}'_x$ and $\hat{G}'_y$ for each bead was calculated as a measure of anisotropy where a difference of 0 indicates isotropic local ECM stiffness. ECM stiffness anisotropy was dependent on both SC type and seeding density. In EC-LF co-cultures with a seeding density of 100 K/mL, anisotropy was detected in the direction of the nearest capillary at day 5 alone (**Fig. 3.7B**). At high cell density (500 K/mL), ECM stiffness anisotropy emerged at day 5 orthogonally to the direction of the nearest capillary (**Fig. 3.7C**). Interestingly, the direction of anisotropy switched at day 14 when $\hat{G}'$ magnitude also largely increased (**Fig. 3.5D**). By contrast, EC-MSD co-culture stiffness anisotropy never developed in the direction of capillary growth (**Figs. 3.7D, E**). In the 100 K/mL condition, anisotropy in the direction orthogonal to the capillary was detected at early time points (before day 7) (**Fig. 3.7D**), while no significant anisotropy was detected in samples at the higher 500 K/mL seeding density (**Fig. 3.7E**).

In addition to stiffness changes of the ECM, we were also interested in investigating local ECM remodeling around the neovessels. To visualize the structure of the ECM, reflection confocal microscopy (RCM) images of fixed and stained co-cultures were acquired. Representative day 14 images demonstrate the differences in the local ECM architecture after vascular development (**Figs. S.3.8A, C**). In EC-LF co-cultures, the native fibrous matrix architecture, as seen in a cell-free gel, was significantly remodeled with large voids visible throughout the ECM (**Fig. S.3.8A**). Additionally, proximal to the growing vessel, the characteristic ECM mesh network was replaced by a diffuse reflected signal where the fibrous

structure is not able to be resolved. (Fig. 3.8B, indicated by arrows). This diffuse architecture is indicative of ECM remodeling as previously observed.[68] This same phenomenon was observed in the EC-MSC co-cultures but to a lesser extent (**Fig. 3.8C**). Less ECM degradation was observed proximal to a growing vessel and the fibrous network structure was more preserved throughout the ECM (**Fig. 3.8D**). Overall, these RCM images reveal that increased ECM matrix remodeling accompanied increased vasculogenesis and stiffening in the EC-LF conditions.

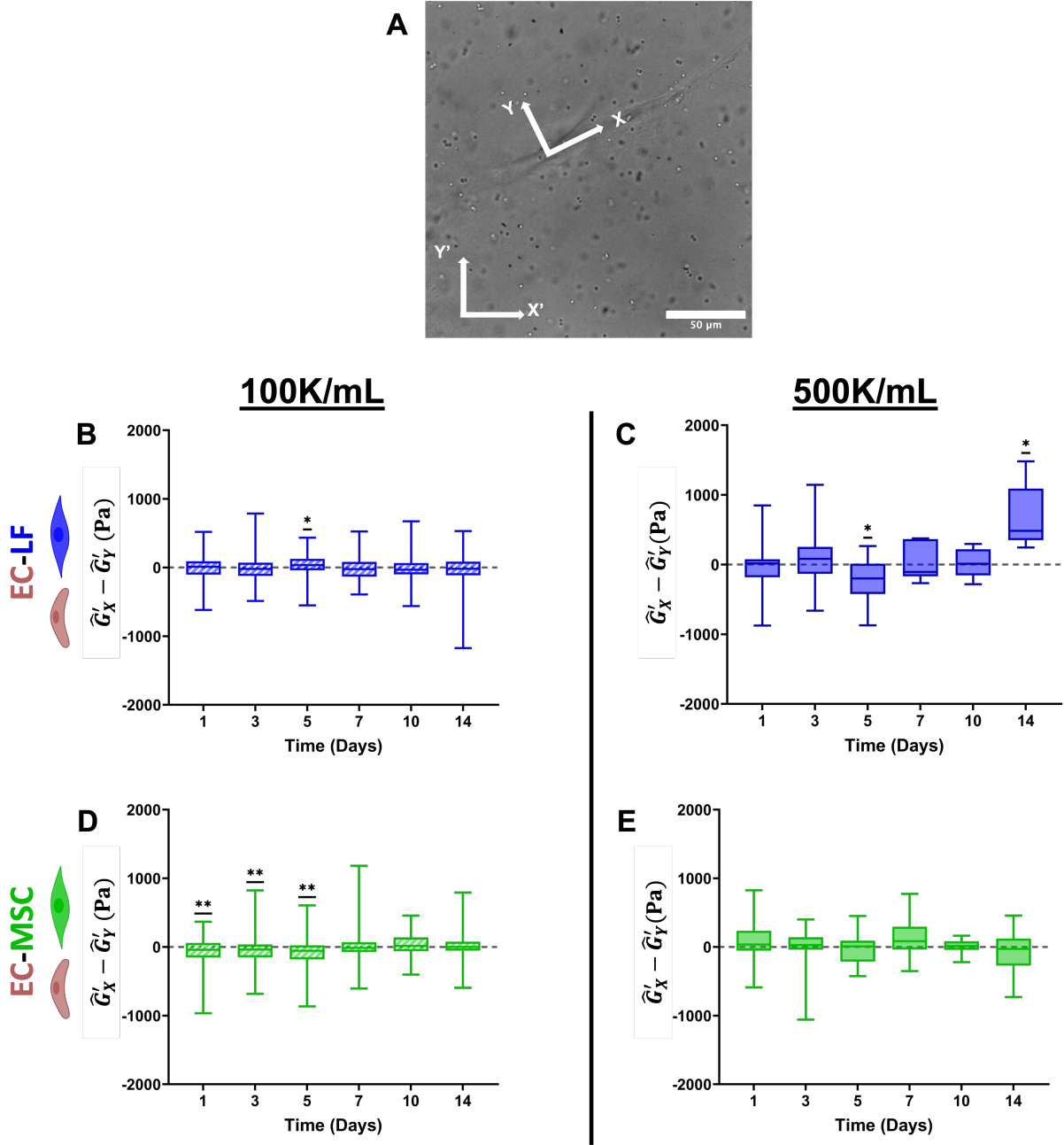


Figure 3.7: . A) Schematic diagram of AMR stiffness projection from global measurement axes (X' and Y') to the capillary axis (X and Y). Local stiffness anisotropy, calculated as the difference between AMR-measured $\hat{G}'_x$ and $\hat{G}'_y$ for EC-LF co-cultures seeded at B) 100 K/mL and C) 500 K/mL and EC-MSC co-cultures seeded at D) 100 K/mL and E) 500 K/mL. One sample Wilcoxon signed-rank test; $m_0=0$ indicating no anisotropy; *: $p < 0.05$; **: $p < 0.01$.

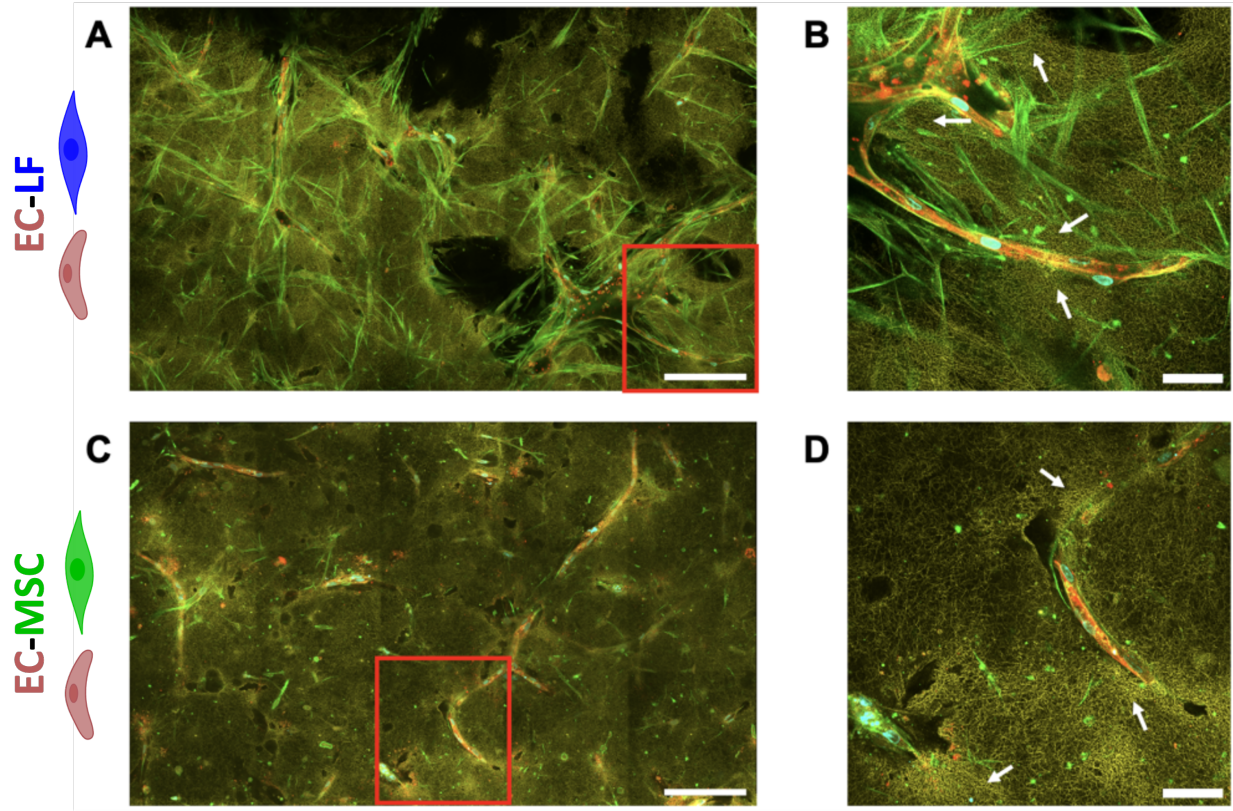


Figure 3.8: Representative confocal reflection images merged with confocal fluorescent images to visualize ECM reorganization surrounding growing vessels. Samples were stained with an endothelial-selective lectin, UEA (red), DAPI (cyan), and Phalloidin (green) to visualize the endothelial cells, cell nuclei, and F-actin respectively. Reflection images were taken with the 488nm laser line and are pseudocolored yellow. 15 individual 40x FOVs were stitched together to form a 3x5 grid covering a 1.519mm^2 area of an A) EC-LF co-culture after 14 days in culture. B) A single FOV taken from the EC-LF stitch (outlined in red). C) A representative stitched area of an EC-MSC co-culture after 14 days in culture with D) a single FOV taken from the EC-MSC stitch (outlined in red). Added white arrows highlight areas of ECM remodeling where fibrous structure can no longer be resolved. Scale bar = 200 μm for stitched grids and 50 μm for single FOVs.

3.5 Discussion

Development of functional vasculature is a complex multicellular process and is critical for tissue engineering and regenerative medicine.[115, 104] A common strategy for engineering microvasculature involves co-encapsulation of ECs and SCs in 3D matrices. SCs are instructive in vascular development, and a wide range of SC populations have been evaluated in prior studies.[158] Our prior research shows that SC identity influences microvascular function in fibrin hydrogels. Mesenchymal stem/stromal cells produce slower growing yet more stable, less permeable capillaries than fibroblasts, both in vitro and in vivo.[45, 46, 101] However, the mechanisms by which SC identity differentially regulates the process of vascular morphogenesis remain unclear. In this study, we investigated a potential mechanobiological link between SC identity, cell-mediated rheological dynamics, and vascularization. A fibrin model of vascular morphogenesis was used to compare lung fibroblasts (LFs) and mesenchymal stem cells (MSCs) as SC populations and evaluated for differences in the resulting density of the EC network over time with concurrent matrix stiffness changes both on the bulk scale (parallel-plate shear rheology) and microscale (AMR).

In both EC-LF and EC-MSC co-cultures in fibrin, microvascular self-assembly led to the formation of extensive, elongated networks, in comparison to EC monocultures. By day 3, vessel-like projections formed in all co-culture conditions, and subsequently elongated and branched over time in culture. Even at this early time point, the highest cell seeding density condition (500 K/mL) demonstrated a significant difference in vessel density between stromal cell co-cultures, where EC-LF exhibited significantly greater ($p < 0.05$) vessel densities than the EC-MSC condition (**Fig. 3.3A**). These results at an early time point suggest there is something intrinsically different about the mechanisms by which LFs and MSCs induce EC morphogenesis that cannot be explained by differences in SC number that may arise due to the differential proliferation of LFs vs. MSCs over longer time courses. By day 14, higher vessel densities and more extensive branching were observed in constructs fabricated

with increased initial seeding densities of 250 K/mL and 500 K/mL in both SC co-culture models, compared to the 100 K/mL constructs. Although both SC types supported vascular morphogenesis, EC-LF constructs resulted in significantly higher vessel densities than EC-MSK constructs after 14 days across all seeding densities (Fig. 3B). EC-LF constructs also exhibited more interconnected vascular networks, especially at higher seeding densities, with dense branching morphologies by day 7 compared to sparser tubule formation and a less dense network in EC-MSK constructs.

Consistent with recent studies by our group[35, 68] and others,[160] vascular morphogenesis was accompanied by stiffening of the fibrin matrix over time. Both AMR and bulk rheology demonstrated the magnitude of this cell-mediated ECM stiffening was dependent on SC identity and cell seeding density. In EC-LF co-cultures, initial cell density affected G' trends. In the 100 K/mL condition, both mean bulk G' and local $\hat{G}'$ remained relatively constant over the course of the 14-day experiment, where interestingly, 94% of $\hat{G}'$ values are within the 75th percentile of acellular gels, indicating low levels of cell-mediated stiffening. In contrast, in the 500 K/mL condition, bulk G' and local $\hat{G}'$ both increased markedly over 14 days, coincident with increased vascular development.

Notably, many of the $\hat{G}'$ values measured in the 500 K/mL EC-LF co-cultures were beyond the measurement limit of our AMR, which is approximately $\hat{G}'_{max} = 1.8$ kPa. Rather than simply excluding these beads from our analysis, we created a new AMR metric of stiffening by quantifying the proportion of beads surrounded by matrix with stiffnesses greater than or equal to $\hat{G}'_{max}$. Thus, two measured properties of matrix stiffness were analyzed. The first is the distribution of beads reporting stiffness greater than $\hat{G}'_{max}$. The marked increase in the proportion of beads reporting $\hat{G}'$ values beyond the $\hat{G}'_{max}$ threshold across 14 days indicates significant stiffening of the ECM (**Fig. S.3.10**). The second is the distribution of measurable $\hat{G}'$, which also demonstrates significant ECM stiffening by day 14 (**Fig. 3.5D**). Distributions of measurable $\hat{G}'$ extend beyond that of matched cell-free constructs, with only 42% of all

$G\hat{G}'$ values falling within the 75th percentile of these cell-free constructs. Together these assessments clearly demonstrate significant pericellular stiffening during vasculogenesis.

In contrast to the high-density EC-LF constructs, EC-MSD constructs exhibited modest mechanical changes. Both bulk G' and local $\hat{G}'$ trends show little to no stiffening over the 14 days, regardless of the initial seeding density. These relatively static ECM mechanics in all EC-MSD constructs as well as the low-density EC-LF constructs coincided with significantly lower levels of vascular development by day 14 compared to the high-density EC-LF constructs (**Fig. 3.3B**). The ECM in these constructs also remained relatively mechanically isotropic throughout the 14 days in culture. Thus, a correlation emerged between the amount of vascular development, the extent of ECM stiffening, and ECM stiffness anisotropy.

To better understand the potential mechanism driving this correlation, we investigated the effects of active cell contractility on bulk ECM stiffening in our model by applying blebbistatin for two different treatment times. In EC-LF co-cultures that had developed extensive vessel networks, blebbistatin treatment led to a significant drop in bulk ECM stiffness without compromising the integrity of the vascular network (**Fig. S.3.12**). This finding demonstrates that the ECM stiffening is largely due to active cell contractility, rather than mechanical reinforcement due to the vascular structures themselves. This agrees with a recent study that used a similar EC-LF self-assembly model of vascularization.[160]

Although the contractile machinery appears mostly responsible for ECM stiffening during vascular morphogenesis, it is unclear whether this contractility-dependent stiffening is directly guiding vascular development or simply accompanying it. It has been shown that EC-generated traction forces are required for capillary morphogenesis,[76] but the role of SC-generated traction forces is less understood. While paracrine signals from SCs to ECs may be one explanation for the observed differences in vascular development, SC-generated traction forces may also induce ECM mechanical changes to which ECs are responding. Elevated ECM stiffness[83] as well as pericyte contractility,[29] which can stiffen the ECM,

have been shown to induce EC sprouting. Additionally, it was recently shown that YAP-1 knockdown within LFs, affecting their active force generation,[90, 113] leads to reduced tissue stiffening and attenuated vascular development in an EC-LF vascular self-assembly model.[160] Thus, we hypothesize that SC-provided ECM stiffening, developed via their traction forces, can guide EC morphogenesis in the direction of force generation, and accelerate vascular development.

This hypothesis is supported by the observed differences in stiffening in each co-culture model. We have shown via AMR in other cell-laden hydrogel systems that pericellular ECM stiffening develops because of the synergy between proteolytic matrix remodeling and active cell contractility.[72, 62, 145] Thus, lower traction force capabilities of MSCs, as measured in 2D,[101] and differences in proteolytic mechanisms in EC-MSC co-cultures[41] may underlie the lack of significant stiffening in EC-MSC constructs, partially driving the observed differences in vascular morphogenesis. Reflection confocal microscopy (RCM) images further highlight clear differences in matrix degradation and ECM remodeling in EC-LF versus EC-MSC constructs. Proximity analysis of AMR-measured $\hat{G}'$ also revealed that LFs stiffen the ECM across longer ranges than MSCs (Fig. S.3.9). Together, these data demonstrate that SC-generated ECM stiffening and the extent of vascularization are correlated in these fibrin constructs and support that SC-mediated ECM stiffening regulates vascular development.

High cell density EC-LF co-cultures also exhibited distinct patterns in stiffness anisotropy compared to the other co-culture conditions. Interestingly, stiffness anisotropy in the direction of capillary growth emerged in samples that exhibited the most vascular development (Fig. 3.7). This anisotropic switch in ECM stiffness occurred at day 14 in these samples, coincident with an increase in vessel density as well as bulk and local ECM stiffness. The anisotropic nature of this emergent ECM stiffening may also be an important regulator of vascular development. Previously our group has shown that fibroblasts in monoculture can induce stiffness anisotropy within the pericellular ECM [147] and can respond to local stiff-

ness anisotropy introduced through selective photocrosslinking of the matrix.[62] Thus, it is plausible that the SCs are generating local stiffness anisotropy to which the ECs are responding. In a previous study, we applied uniaxial external strain to a microcarrier bead model of sprouting angiogenesis and found that EC orientation aligned to the direction of the externally applied strain. [18] The EC alignment was a transient effect that reversed after the strain was removed, with only modest fibrin fiber alignment, indicating that the EC alignment is not simply due to contact guidance, but may result in part due to local stiffness anisotropy. Additionally, a recent study implicated ECM stiffness anisotropy as an important regulator in muscle development,[138] where collective migration of myoblasts is similar to ECs during vascular morphogenesis.[122] Together these data implicate local stiffness anisotropy, induced by SCs in our model, as a potential regulator of EC morphogenesis.

One surprising observation in this study was an apparent softening effect in G' and $\hat{G}'$ after approximately one week in culture, observed for both the EC-MSC and EC-LF system. We have previously shown that cell-free fibrin gels at the same concentration do not exhibit this softening over 14 days, so the measured softening of the ECM is unlikely driven by intrinsic temporal degradation of the fibrin hydrogel itself.[68] This ECM softening is likely dependent on cell-mediated fibrinolysis. Further, it is the inclusion of stromal cells, and not the endothelial cells alone, driving this softening as evident when testing EC monocultures both at 250 K/mL, which is the same total cell density as in co-culture, and at 500 K/mL (**Fig. S.3.4**). This transient ECM softening is counter to the progressive stiffening that has been reported to accompany vascular development in similar vascular self-assembly models by us and others. A plausible explanation may be that the relatively low initial seeding density used here is more representative of early vascular development. In the case of angiogenic sprouting from parent microvessels within a type I collagen gel, MMP-mediated ECM softening was observed approximately one week into culture.[81] This ECM softening correlated with early angiogenic sprouting and elevated expression of various MMPs (2, 3, and 13). The ECM subsequently stiffened as neovessels grew and proliferated, consistent with our

observations here.

Lastly, an important aspect of this vasculogenic model that could affect the observed correlation between ECM stiffening, stiffness anisotropy, and vascular development is the fibrous nature of the fibrin-based ECM. The fibrillar nature of natural ECMs enables long-range cell-cell communication,[126, 15, 48, 137] contributes to tissue stiffening through strain-hardening,[98, 151] and may be important in the development of stiffness anisotropy.[44, 155] In future work, we plan to explore how the correlations between elevated ECM stiffness, stiffness anisotropy, and vascular development observed here may change in a non-fibrillar, synthetic hydrogel system.

3.6 Conclusions

In summary, we report stromal cell (SC)-dependent differences in vascular development that are accompanied with varying levels of ECM stiffening. Our findings build upon a growing body of work demonstrating a relationship between ECM stiffening and vascular development. The ECM remodeling capability of different SC populations, through both active cell contractility-dependent stiffening and degradation of the matrix, may underscore SC-dependent differences in vascular morphogenesis, highlighting the need to consider these effects in the development of vascularized engineered tissues. Additionally, we further demonstrated the importance of characterizing ECM mechanics across length scales. While AMR and bulk rheology trends generally echoed each other across time, AMR revealed spatial ECM stiffness heterogeneity and ECM stiffness anisotropy on the same length-scale as cells, which may be important local mediators of vascular development and are immeasurable by bulk rheology methods. Future work will investigate whether the SC-dependent development of ECM stiffening and stiffness anisotropy across length scales will translate to other, non-fibrillar materials.

3.7 Acknowledgements

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health (NIH) under Award Number R01-HL085339. ML was partially supported by the NHLBI Training Program in Cardiovascular Applied Research and Entrepreneurship at the University of California Irvine (T32-HL116270). IWZ was partially supported by the NIGMS Cellular Biotechnology Training Program at the University of Michigan (T32-GM145304). Confocal microscopy at the University of California Irvine was made possible by an NIH Office of the Director equipment grant 1S10OD025064. The content herein is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

3.8 Supplemental Information

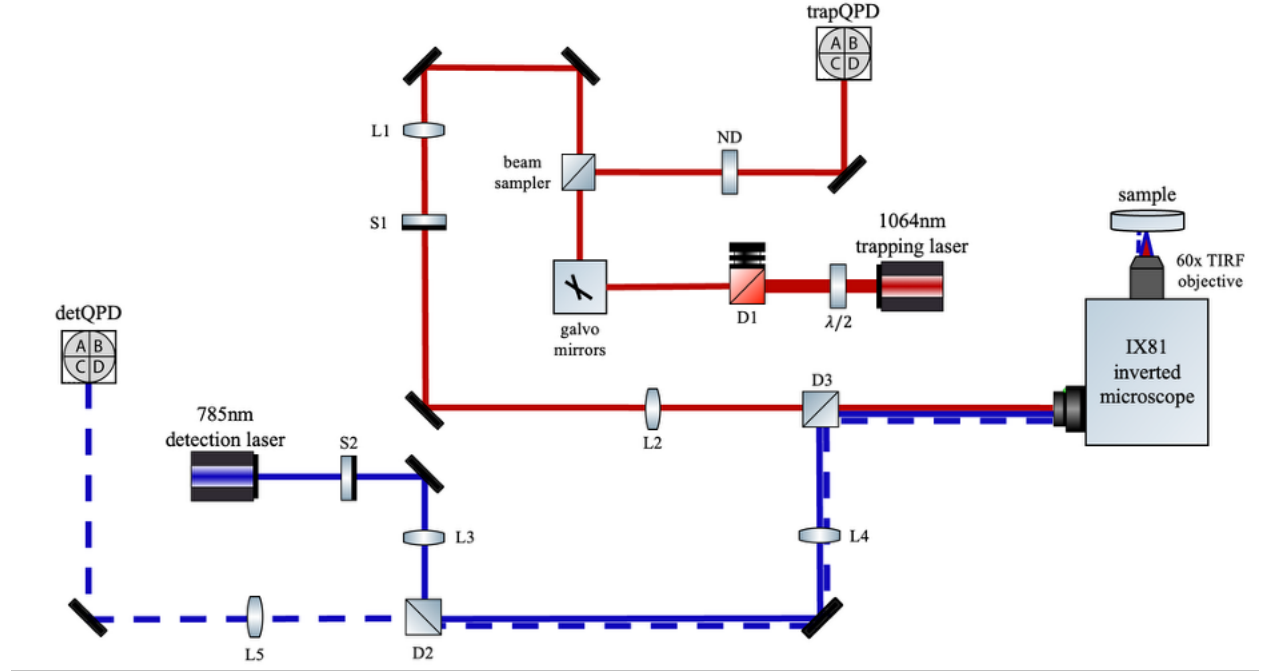


Figure S.3.1: Optical components: $\lambda/2$ – half-wave plate (WPH10M-1064, ThorLabs), ND – neutral density filter (NENIR20A-C, ThorLabs), D1 – polarizing beamsplitter with beam dump (CM1-PBS253, ThorLabs), D2 – dichroic beamsplitter (FF875-Di01, Semrock), D3 – dichroic beamsplitter (CM1-BP145B2, ThorLabs), L1-L5 – lenses, S1-S2 – shutters (SHB05T, ThorLabs).

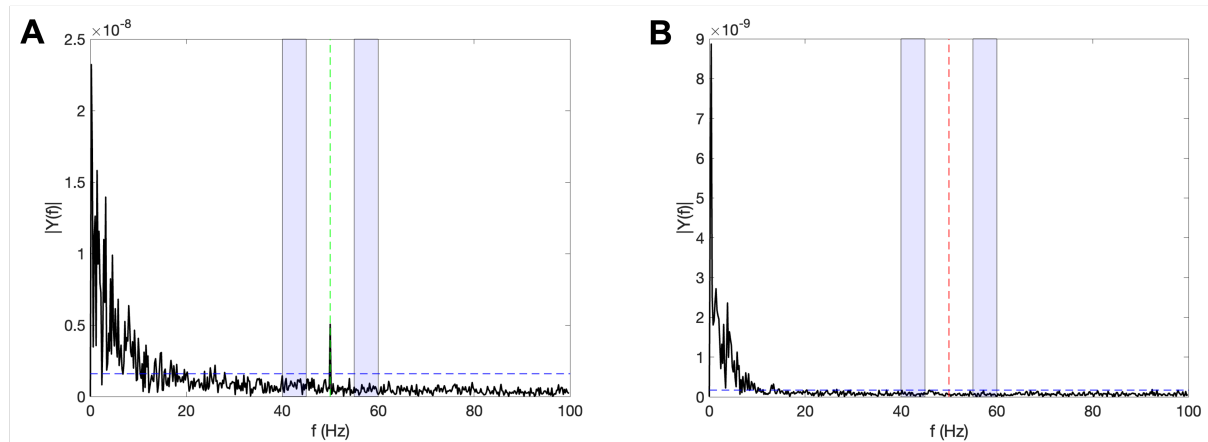


Figure S.3.2: AMR measurements were accepted or rejected based on the single-sided amplitude spectra of the detection beam QPD signal. Representative single-sided amplitude spectra from the FFT of the detection beam QPD signal in A) a successful AMR measurement and B) a rejected AMR measurement. The blue dashed horizontal line represents the noise floor and was calculated as 3σ above the average amplitude of the FFT at frequencies around the AMR driving frequency (blue shaded regions). The AMR driving frequency was 50 Hz. If $|Y(f)|$ at 50 Hz was below the noise floor threshold, the measurement was rejected.

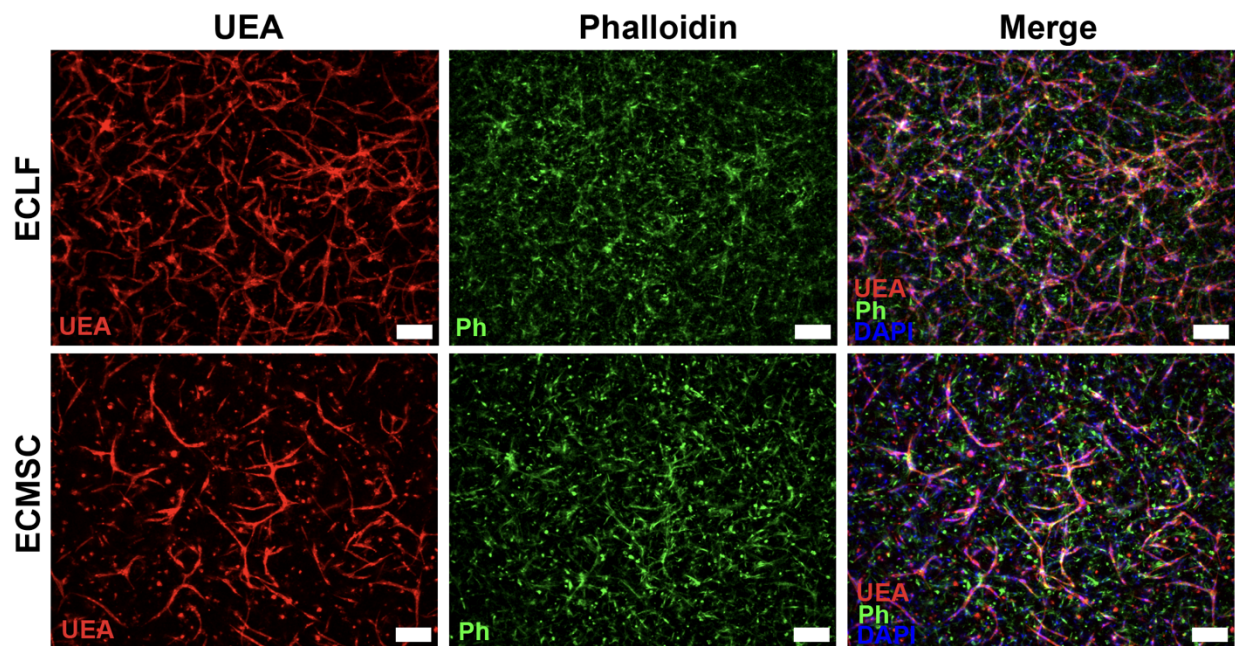


Figure S.3.3: Representative max intensity projection images of EC-LF and EC-MSC co-culture conditions after 7 days of culture at 500 K/mL seeding density and at 4X magnification (scale bar = 200 μ m) with endothelial cell (red), actin (green), and nuclei staining (blue).

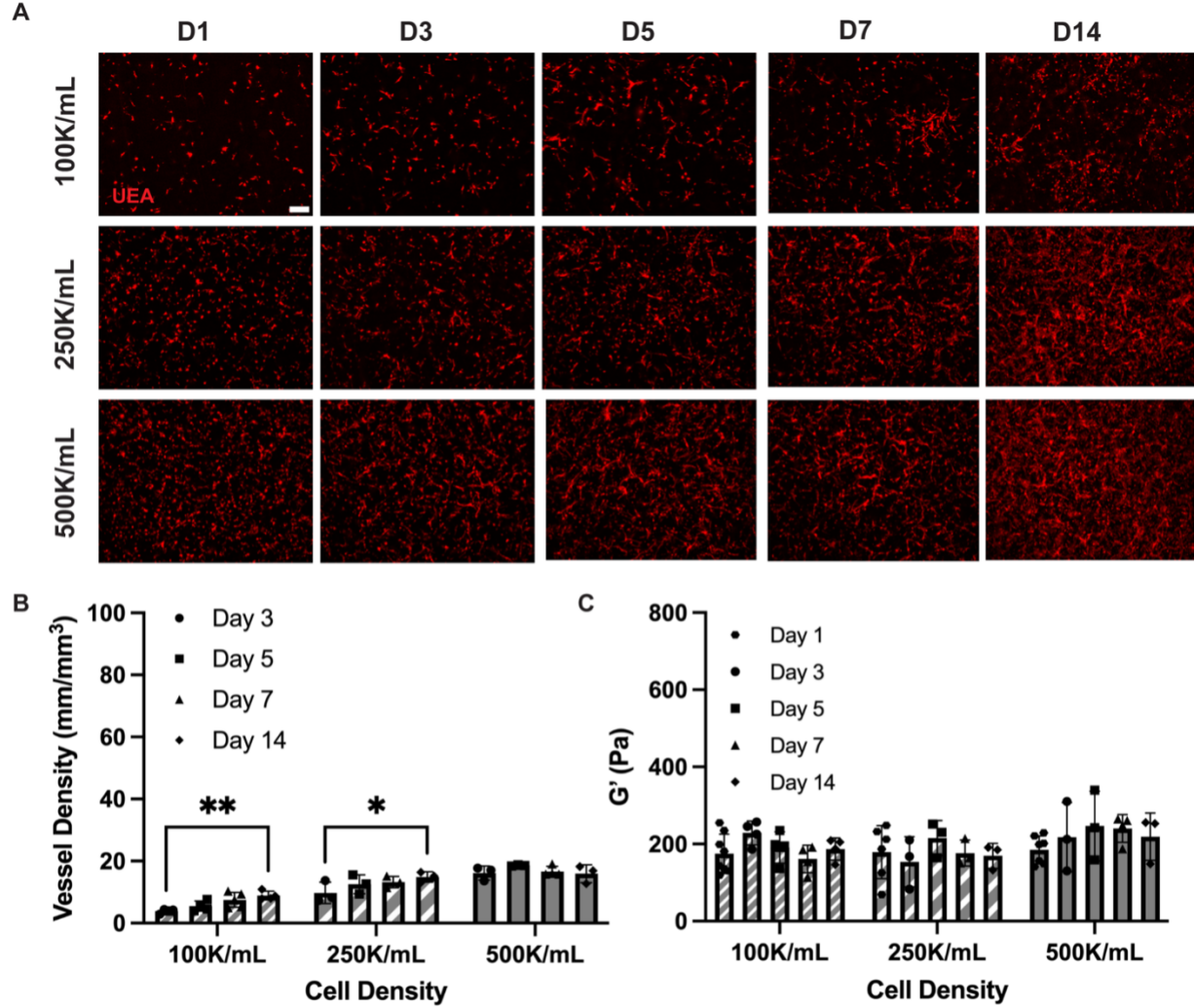


Figure S.3.4: A) Representative max intensity projection (z stack = 300 μm) images of vasculogenesis in fibrin constructs with EC monoculture at 100K/mL, 250K/mL, and 500K/mL total cell density, after 1, 3, 5, 7 and 14 days of culture, are shown stained with UEA (red; scale bar = 200 μm). B) Quantification of vascular density over culture time (days 3, 5, 7, and 14). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$ C) Shear storage modulus (G') in Pa measuring stiffness of EC monoculture vasculogenesis assays at varying culture timepoints (days 1, 3, 5, 7 and 14) for multiple initial cell density conditions (100K/mL, 250K/mL, 500K/mL).

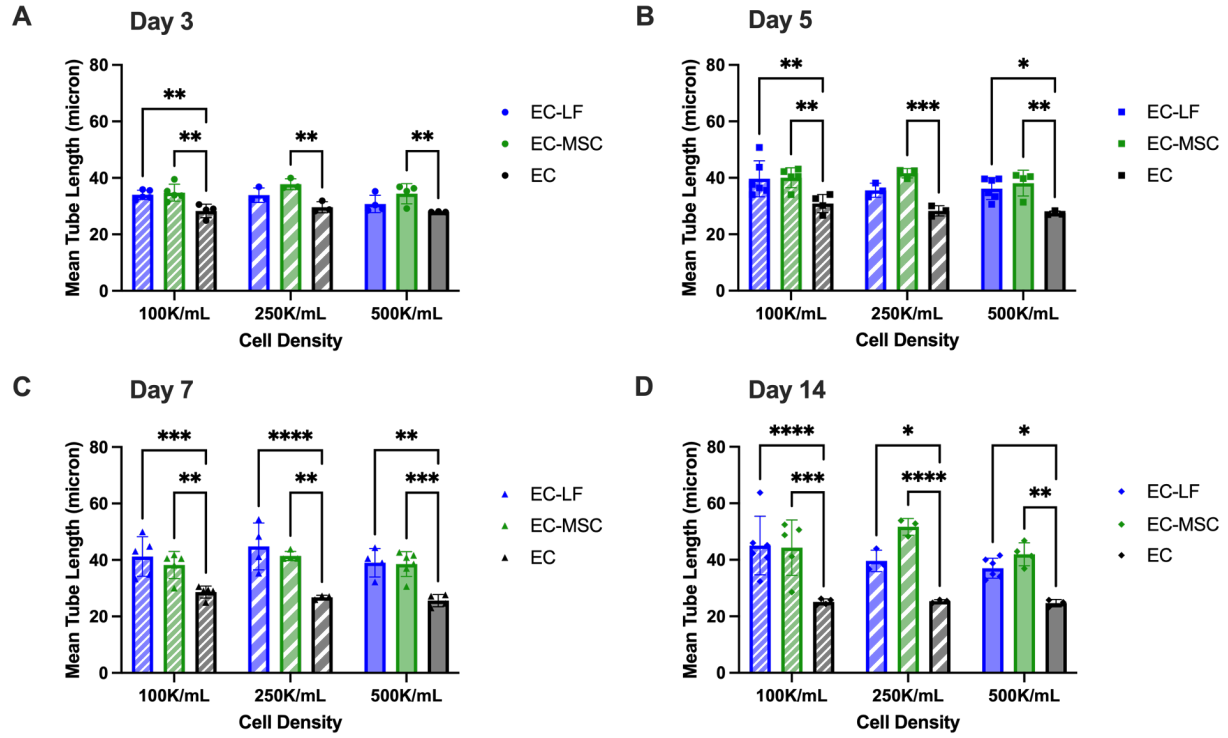


Figure S.3.5: Mean tube segment length of quantified vascular structures across co-culture (EC-LF, EC-MSC) and monoculture (EC only) conditions on A) day 3, B) day 5, C) day 7, and D) day 14 of culture. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$

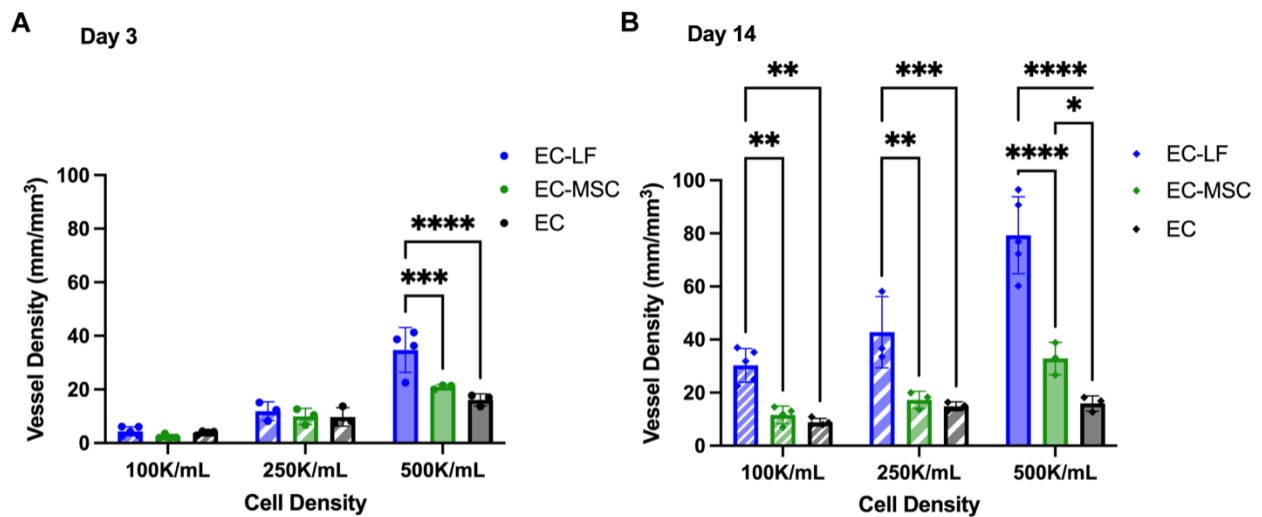


Figure S.3.6: Comparison of vessel densities quantified on (A) day 3, and (B) day 14, between co-culture (EC-LF and EC-MSC) conditions at varying initial cell seeding densities (100 K/mL, 250 K/mL, 500 K/mL). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$.

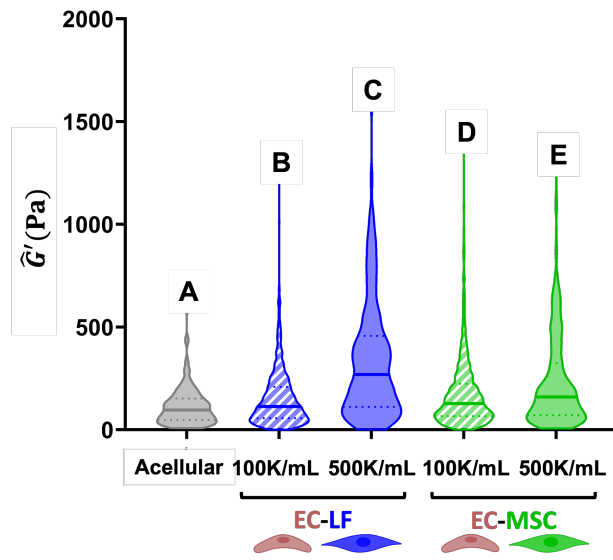


Figure S.3.7: AMR $\hat{G}'$ measurements in acellular fibrin gels ($t = 1$ Day) compared to aggregated AMR measured $\hat{G}'$ across all 14 days in cell-laden constructs. Kruskal-Wallis test with Dunn's multiple comparisons: groups not sharing any letters are significantly different ($p < 0.05$).

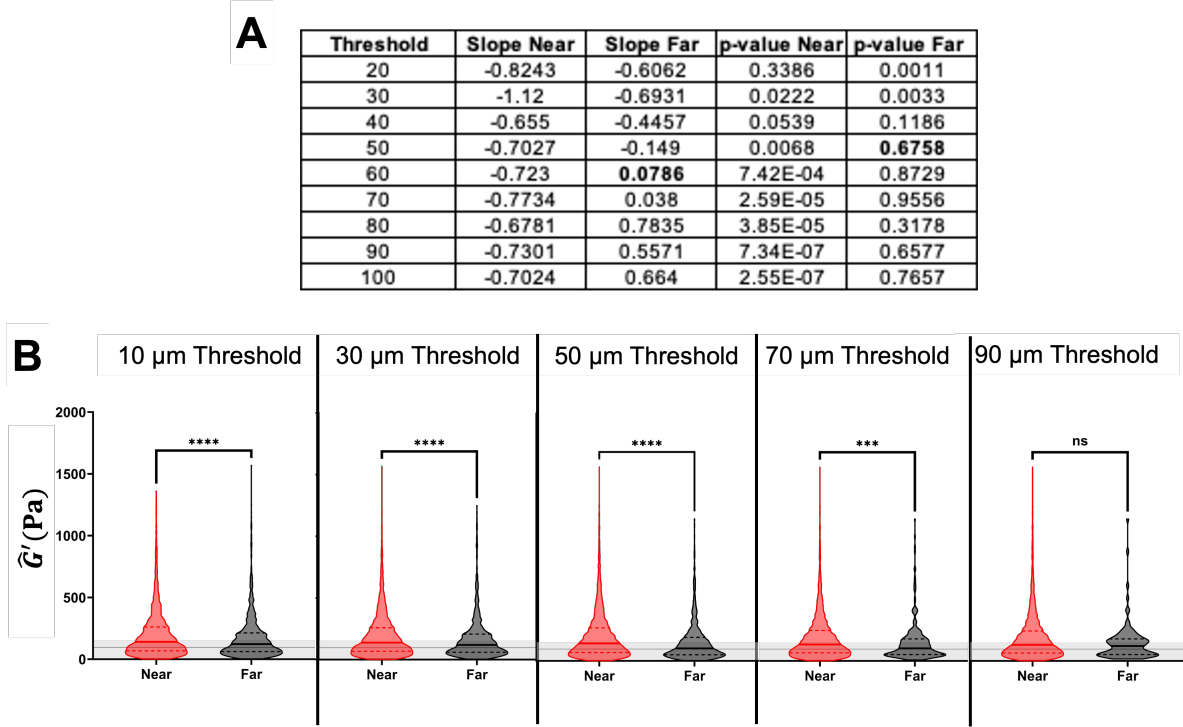


Figure S.3.8: (A) Linear regression was performed in near and far regions to evaluate the distance-dependence of $\hat{G}'$ as the proximity threshold was swept from 20 to 90μm. A drastic increase in the p-value of the slope farther than 50μm indicates that $\hat{G}'$ loses its distance-dependence around this distance from the cells. Additionally, there is an inflection point in the slope value as well, switching from negative to positive in this region. To further test this choice of threshold, (B) near and far regions were directly compared, and the proximity-dependent stiffening was robust as the threshold increased up to 90μm. (Mann-Whitney U-test; $p > 0.05$)

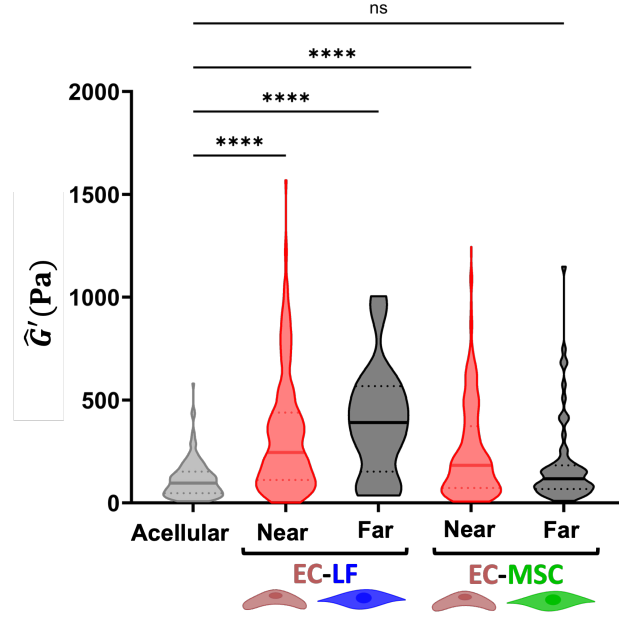


Figure S.3.9: Comparison of aggregate AMR $\hat{G}'$ measurements in acellular fibrin gels ($t=1$ day) compared to AMR measured $\hat{G}'$ across all 14 days in cell-laden constructs seeded at 500K/mL, separated into groups “near” and “far” based on proximity to the nearest cell ($\leq 50\mu\text{m}$ and $> 50\mu\text{m}$, respectively). Kruskal-Wallis test with Dunn’s multiple comparisons: ****: $p < 0.0001$.

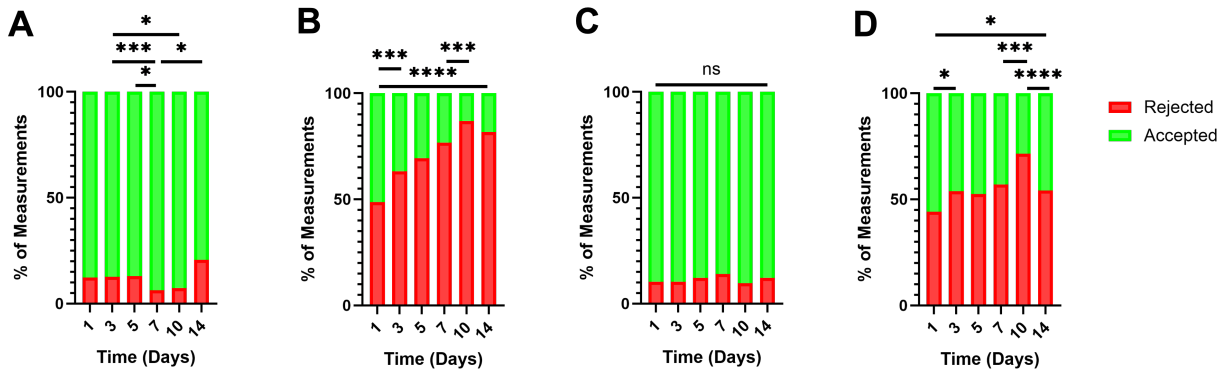


Figure S.3.10: Percentage of AMR measurements rejected due to lack of detectable peak in $—Y(f)—$ of the detection beam QPD signal at AMR driving frequency in EC-LF co-cultures seeded at A) 100 K/mL and B) 500k, and EC-MS co-culture seeded at C) 100 K/mL, D) 500 K/mL. Chi-squared Test: *: $p < 0.05$; ***: $p < 0.001$; ****: $p < 0.0001$.

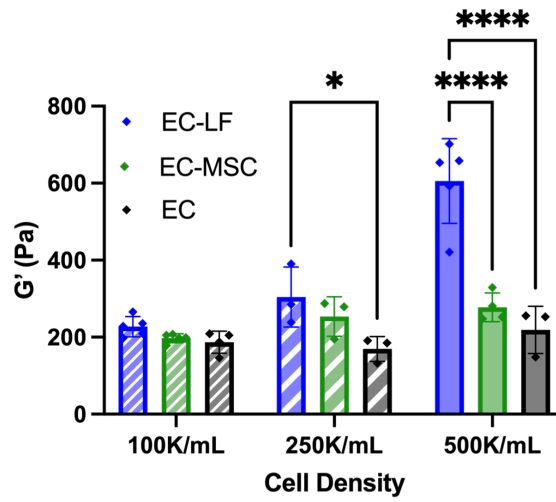


Figure S.3.11: Bulk parallel-plate G' in EC-LF, EC-MSC, and EC only constructs with 100 K/mL, 250 K/mL, and 500 K/mL seeding densities after 14 days of culture. : $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

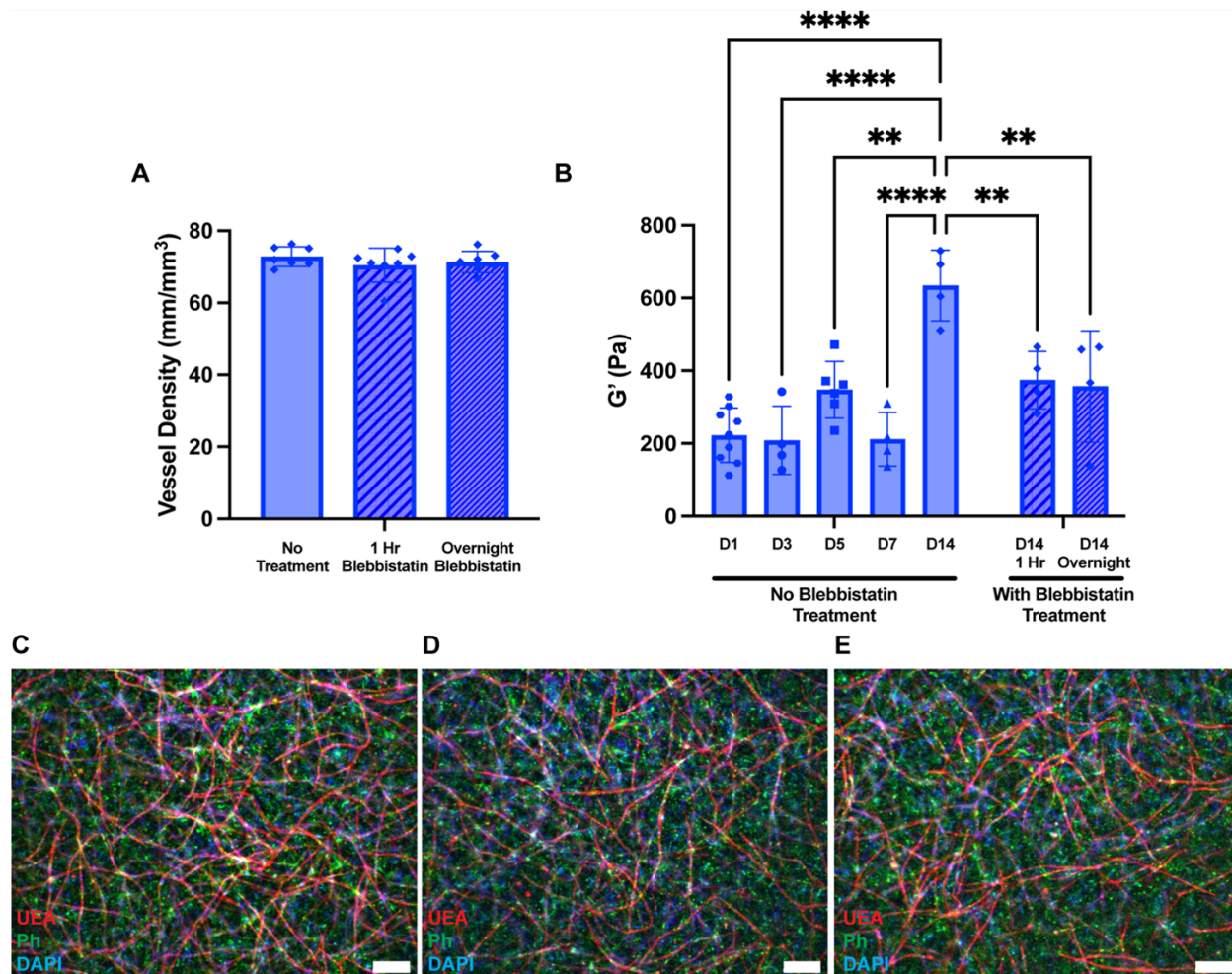


Figure S.3.12: 500 K/mL EC-LF co-culture constructs treated with blebbistatin after 14 days of culture. A) Quantified vascular density of treatment vs no treatment constructs, B) bulk G' data for no treatment versus treatment constructs, and representative max intensity projection (scale bar = 200 μ m; z stack = 300 μ m; UEA = red, phalloidin = green, DAPI = blue) images of C) vasculogenesis with no treatment, D) 1 hr treatment, and E) overnight treatment of blebbistatin. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, $N \geq 3$.

Chapter 4

Divergent Pericellular Stiffness and Anisotropy in Fibrous Fibrin and Amorphous GelMA Matrices

4.1 Abstract

Cells are continuously engaging in reciprocal interactions with the extracellular matrix (ECM), sensing the biophysical cues in their microenvironment while simultaneously remodeling the matrix through contractile forces and biochemical activity. Bulk rheological measurements fail to capture these dynamic cell-matrix interactions that can affect mechanical heterogeneity and anisotropy at the microscale. In this study, we used optical tweezers-based active microrheology (AMR) to measure local pericellular stiffness and stiffness anisotropy within two widely used hydrogel systems: fibrin, a naturally-derived fibrillar hydrogel, and gelatin methacryloyl (GelMA), a semi-synthetic amorphous hydrogel derived from collagen. We investigated how ECM type and cell type influence the local mechanical landscape by characterizing pericellular stiffness distributions and stiffness anisotropy around lung fibro-

lasts (LFs) and mesenchymal stem cells (MSCs) embedded in stiffness-matched hydrogels. Our results demonstrate that fibrin exhibits significantly greater stiffness heterogeneity and anisotropy than GelMA, consistent with its fibrillar structure. While cell-mediated ECM remodeling led to stiffening of fibrin that was cell-type dependent, cell contractility-dependent softening was observed in GelMA. Further, cell type-mediated directional stiffness anisotropy developed in fibrin gels, while ECM stiffness remained relatively isotropic in GelMA. These results highlight the importance of investigating pericellular stiffness at the microscale and offer new insight into how cells interact with fibrous versus amorphous ECM types.

4.2 Introduction

The extracellular matrix (ECM) gives structural and functional support to cells, providing critical biochemical and biophysical cues that regulate the development and homeostasis of tissues. [33, 70, 59, 95] Bulk matrix stiffness has been shown to be an important mechanical cue, affecting many biological process such as cell adhesion, migration, stem cell differentiation, and fibrosis. [165, 118, 31, 52] While cells sense and respond to the stiffness of the surrounding ECM, they simultaneously and reciprocally remodel the ECM as well. [26, 69] Cell-generated tractional forces have been shown to stiffen the surrounding ECM, while matrix metalloproteinase (MMP)-mediated degradation, additional matrix deposition, and rearrangement of ECM fibers can also modify the local ECM stiffness. [65, 151, 10, 91].

Local ECM remodeling can lead to spatial heterogeneity that requires techniques capable of resolving mechanical properties at the cellular length scale. Our group has previously shown that cell-mediated local ECM stiffness modulation can span orders of magnitude and vary spatially in both collagen and fibrin hydrogels. [72, 61] Further, cells can induce pericellular stiffening that is dependent on ECM type, cell line, cell contractility and MMP activity in fibrous ECM models. [72, 61, 63]

Under investigation is whether the fibrillar structure of the ECM mediates the transmission of cell-generated traction forces that induce ECM stiffening. Fibrous natural hydrogel models closely approximate the mechanical features of the native ECM as they are mechanically heterogeneous, anisotropic, macroporous and exhibit nonlinear strain-stiffening behavior. [87, 143, 91] Numerous experimental and computational modeling studies have shown that cells can sense each other across longer distances in fibrous natural protein matrices compared to amorphous synthetic polymer hydrogels [126, 15, 49, 137, 141] Long-range force transmission has also been shown to be dependent on the nonlinearly elastic, strain-stiffening behavior of fibrous ECMs. [48, 161] However it is still debated and not well understood if the nonlinear mechanics, or the fibrous architecture itself is responsible for amplified force transmission since these are difficult to decouple experimentally. Despite increasing recognition of fibrous ECM architecture as an important regulator of cell-generated force transmission, studies comparing local pericellular stiffness and directional anisotropy within 3D matrices with a fibrous or amorphous architecture remain limited.

In this study, we use multi-axis optical tweezers-based active microrheology (AMR) to measure local stiffness and stiffness anisotropy around cells in two stiffness-matched hydrogel systems, enabling us to isolate the effects of fibrillar ECM architecture and cell type on the pericellular mechanical landscape. Either lung fibroblasts (LFs) or mesenchymal stem cells (MSCs) were embedded inside fibrin and gelatin methacryloyl (GelMA) hydrogels and AMR measurements were taken 48 hours after sample preparation. These contractile cells have been shown to differ in their traction force generation, with fibroblast generating significantly more traction force in 2D. [101]

The role of cell contractility and matrix degradation in establishing pericellular stiffness distributions in each ECM type was further explored using inhibitor treatments. Y27632, a rho-kinase inhibitor that reduces cell contractility can affect migration of fibroblasts and the proliferation and differentiation of MSCs. [37, 111, 74] Batimastat (BB94) is a broad-

spectrum matrix metalloproteinase (MMP) inhibitor that works through competitive inhibition by directly binding to MMP active sites to reduce proteolytic ECM degradation. [12, 88] Past studies by our group have shown that Y27632 and BB94 can reduce pericellular stiffening in fibrous matrices, depending on cell and ECM type. [72, 61]

The results of this study highlight differences in the inherent mechanical heterogeneity and stiffness anisotropy in fibrous ECMs. Interestingly, ECM type critically regulated the cell-mediated changes in local ECM stiffness with cells stiffening fibrin gels, but softening GelMA. Cells also induced directional stiffness anisotropy in fibrin that was cell type-dependent, but not in GelMA. Changes in the stiffness landscape after inhibitor treatment suggest the role of cell contractility in mediating these ECM remodeling effects. ECM architecture is shown to affect cell-mediated stiffening and stiffness anisotropy regardless of initial ECM stiffness, building upon a growing body of evidence that fibers in the ECM regulate cell traction force transmission and must be considered in biomaterial design.

4.3 Materials and Methods

4.3.1 Cell Culture

Normal human lung fibroblasts (LFs, Lonza) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Bone marrow-derived mesenchymal stem cells (MSCs, RoosterBio) were cultured with fully supplemented Rooster Nourish media (RoosterBio). LFs were used by passage 14 and MSCs were used by passage 6 in this study. All cells were incubated at 37°C with 5% CO_2 and media exchanges were performed every other day.

4.3.2 Fibrin Hydrogel Preparation

Fibrin hydrogels were prepared at 2.5 mg/mL concentration as previously described. [62] A fibrinogen precursor solution was prepared by dissolving bovine stock fibrinogen (Sigma) into phosphate-buffered saline (PBS, Gibco). 0.08% (w/v) 2 μ m carboxylated silica microbeads (Bangs Laboratories) and 50 k cells/mL were added to the precursor. 20 μ L of bovine thrombin (Sigma) dissolved in PBS was added to the center of a 35mm glass bottom dish (MatTek) to achieve a final thrombin concentration of 1U/mL. A 1 mL hydrogel was polymerized by adding 980 μ L of the precursor solution and swirling the dish to evenly distribute the fibrinogen and thrombin solutions. Hydrogels sat at room temperature in the biosafety cabinet for 5min and were then incubated in a standard tissue culture incubator at 37 and 5% CO_2 for 25 min. Following polymerization, hydrogels were hydrated with 2 mL of culture media. 3 hydrogels were made per condition tested.

4.3.3 GelMA Hydrogel Preparation

Gelatin Methacryloyl (GelMA) hydrogels were prepared at 2.5% concentration. Prior to hydrogel preparation, a GelMA stock solution was prepared by dissolving PhotoGel® 50% DS (CELLINK), lyophilized methacrylated gelatin with 50% degree of methacrylation, in PBS at 2.5% concentration. The stock solution is warmed in an oven at 60°C prior to inclusion in a GelMA precursor solution which included 0.08% (w/v) 2 μ m carboxylated silica microbeads, 50k cells/mL, and additional PBS. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Sigma) was added to the precursor at 0.05%. 100 μ L of GelMA precursor solution was pipetted in the center of a 35mm glass bottom dish. UV crosslinking was carried by irradiation with a 365nm UV lamp at 50mW/cm² for 1min. Hydrogels were hydrated with 3mL of culture media following polymerization. Three hydrogels were made for each condition tested. For bead controls, 20 μ m polystyrene beads (Sigma) were added to the precursor solution at 50K beads/mL to mimic cell inclusion.

4.3.4 Inhibitor Treatments

Y27632 dihydrochloride (Y27632, MedChem Express) and Batimastat (BB94, MedChem Express) stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma) and filtered through a 0.22 μ m syringe filter for sterility. Stock solutions were prepared such that final DMSO concentration after each treatment was 0.2% (v/v). Y27632 or BB94 was added to media 1 hour or 48 hours before AMR measurements to achieve 20 μ M and 10 μ M, respectively. DMSO was added to media 48 hours before AMR measurements for carrier controls.

4.3.5 Active Microrheology

Active microrheology (AMR) was carried out using a dual-beam, multi-axis optical tweezers system built into an IX81 inverted microscope (Olympus) as previously described in **Chapter 2**. Hydrogel samples were mounted in a custom dish holder and on-stage incubator system (Tokai Hit) to maintain incubation at 37 and 5% CO_2 throughout experiments. The incubator system was placed inside a stage-top nanopositioning piezoelectric stage (P-545.xR8S PInano®XYPiezo System, PI) which sits atop a motorized XY stepper motor stage (MS-2000, Applied Scientific Instruments).

Silica probe microbeads within the hydrogels were oscillated by applying optical forces induced by a 1064 nm continuous-wave fiber laser (YLR-5-1064-LP, IPG Photonics) that was steered with a set of galvanometer mirrors (GVS012, ThorLabs) located conjugate to the back focal plane of the microscope objective lens (60x-oil PlanApo TIRFM 1.45NA, Olympus). The trapping laser was oscillated with sinusoidal waveforms with an amplitude of 150nm and frequency of 50Hz.

A 785nm single mode fiber-pigtailed laser diode (LP785-SF100, ThorLabs) was co-aligned to the trapping beam, and the back reflection of the detection laser was used to monitor bead displacement. The probe bead position and trapping beam position were recorded by

two quadrant photodiodes (2901 and 2903 respectively, Newport). Comparison of these two positional signals allows for the calculation of the complex material response by the relationship $\alpha^*(\omega) = X/F$, where X and F are the Fourier components of the bead displacement and optical force respectively. The local complex shear modulus $G^*(\omega)$ can be calculated from $\alpha^*(\omega)$ as previously shown by using the generalized Stokes-Einstein relationship in **Chapter 2**. In this study, we report the real component (G') of the complex shear modulus G^* , which we refer to as stiffness from here on.

AMR measurements in hydrogels were taken after 48hr at approximately 35 μ m above the cover glass. Prior to AMR measurements, the system was aligned and calibrated in water as previously described in **Chapter 2.3**. Each probe bead was oscillated in both the horizontal and vertical axes (X and Y respectively) of the camera image field of view (FOV).

4.3.6 Cell Orientation Assessment

Brightfield images were taken of each FOV where AMR measurements were performed using a monochrome USB camera (EO-4010, Edmund Optics) mounted to the camera port of the IX81 microscope. The location of each probe bead measured using AMR was registered for each FOV using Fiji and individual FOVs surrounding each cell were stitched together. In MATLAB (MathWorks) using the image processing toolbox, cells were manually traced and the cell centroid position, long and short axes, of the cells, and cell orientation were computed using *regionprops*. The minimum distance between each probe bead and the cell was computed and AMR measurements taken in the X' and Y' axes representing the reference from of the camera FOV were projected onto the cell orientation axis which we called X and Y.

4.3.7 Immunostaining

After completion of AMR measurement, cells inside hydrogels were fixed in zinc formalin (Z-fix, Anatech) for 15min at room temperature (RT) then washed with PBS three times for 5min. Samples were permeabilized using 0.1% Triton X-100 (Sigma) diluted in PBS and washed with PBS three times for 5min. Blocking was performed for 1hr at RT with 2% bovine serum albumin (BSA, Thermo) diluted in PBS. Next, samples were incubated with anti-fibronectin antibody (Thermo) for 1hr at RT and then washed three times in BSA prior to incubation with secondary Cyanine5 Goat anti-Rabbit IgG antibody (Thermo) for 1hr at RT. After washing three times for 10min with BSA, Nuclei and F-actin were stained using DAPI (Thermo) and Alexa Fluor 488 Phalloidin (Thermo) diluted in BSA for 30min at RT. Lastly, hydrogels were washed three times with PBS and covered with PBS to remain hydrated during storage at 4°C prior to imaging.

4.3.8 Confocal Microscopy

Reflection confocal microscopy (RCM) images of samples were taken during AMR experiments using the 488 laser line on the Fluoview 1200 laser scanning confocal microscope (Olympus) using the same microscope objective lens used for AMR.

After fixing and staining the samples, fluorescent images were taken using a Fluoview3000 laser scanning microscope equipped with a 10x UPlanXApo 0.40NA objective lens (Olympus). Z-stack imaging was performed with a step size of 3 μm across a 50 μm depth. Each scan was acquired with a scanning speed of 2 μs /pixel and a scan resolution of 2048 pixels x 2048 pixels across the FOV. 30 cells across the 3 samples in each group were imaged. First, the maximum intensity projection (MIP) across all image planes was computed in Fiji.[131] MIP images were then processed in MATLAB to mask the F-actin regions. Cell circularity was calculated as $(4 * Area * \pi) / (Perimeter)^2$ and solidity was computed as $(Area / ConvexArea)$ using the *regionprops* function.[56]

4.3.9 Statistical Analysis

Brown-Forsythe testing was performed using Matlab to compare the variance of AMR stiffness and stiffness anisotropy between ECM types. All other statistical tests were performed in Prism (GraphPAD). AMR data was analyzed using non-parametric tests because distributions are non-normal. Data are represented as median $\pm$ IQR. Comparisons between two AMR distributions were performed using the Mann-Whitney U test. Comparisons between three or more AMR stiffness or stiffness anisotropy distributions were performed using Kruskal-Wallis test followed by Dunn’s post-hoc test for multiple comparisons. Stiffness anisotropy was compared to 0 using the Wilcoxon signed-rank test. For cell morphological comparisons, one-way ANOVA with Tukey post-hoc testing was performed.

4.4 Results

The fibrous ECM architecture has been implicated as an important mediator in the transmission of cell-generated traction forces. In previous studies by our group, cell contractility-mediated stiffening has been shown to generate pericellular ECM stiffening and stiffness anisotropy in fibrous matrices that depends on ECM type, initial stiffness, and cell line. [72, 62, 61] In order to investigate how the attenuation of cell tractional forces in amorphous matrices lacking fiber structure may lead to different pericellular stiffness landscapes, two distinct cell types, lung fibroblast (LFs) or mesenchymal stem cells (MSCs), were embedded in either 2.5mg/mL fibrin hydrogels or 2.5% gelatin methacryloyl (GelMA) hydrogels with a 50% degree of methacrylation. After 48 hours in culture, the local ECM stiffness was measured using optical tweezers-based AMR by oscillating probe beads in both the horizontal (X’) and vertical (Y’) axes of the camera in the AMR system. (**Figure 4.1A-B**). Measurements in cell-laden gels were projected onto the long axis (X) of the cell where G'_x and G'_y indicate local stiffness in the direction of the long axis of the cell and the short axis respectively. (**Figure 4.1B**)

4.4.1 Pericellular Stiffness Landscapes Differ in Fibrin and GelMA Hydrogels

Prior to cell experiments, fibrin and GelMA formulations were optimized to ensure initial stiffness was comparable, even though the ECM structures are distinct. (**Figure 4.1E, S.4.1**) While fibrin has a macroporous, fibrous architecture, GelMA has an amorphous and more homogenous structure at the length scale of cells. These structural differences are apparent when visualizing cells embedded in each gel using reflection confocal microscopy (RCM). (**Figure 4.1C-D**). Differences in the microstructure are also reflected in the heterogeneity in local stiffness measured by AMR in the cell-free gels. (**Figure 4.1E**). Although cell-free controls do not differ significantly in their local median stiffness ($p > 0.99$) (**Figure S.4.1**), the heterogeneity of local stiffness differed significantly between cell-free fibrin and GelMA hydrogels (Brown-Forsythe; $p=0.0023$). Fibrin exhibited significantly higher stiffness heterogeneity at the microscale than GelMA, consistent with the known differences in ECM structure.

Local ECM stiffness (G') distributions measured by AMR around 6 individual cells in fibrin and GelMA are aggregated and shown in **Figure 4.1 E-F**. G' is aggregated across both probe axes, X' and Y' , to compare pericellular stiffness distributions independent of cell orientation. Summarized G' values are found in **Figure S.4.3**. Cell type-dependent pericellular stiffening above cell-free stiffness levels was observed in fibrin hydrogels. Median local ECM stiffness was significantly higher around MSCs in Fibrin than LFs. ($p < 0.0001$) In contrast, pericellular stiffness was reduced in GelMA hydrogels after 48hrs. Level of softening were equivalent across cell types, with equivalent median G' around LFs and MSCs in GelMA. ($p=0.63$)

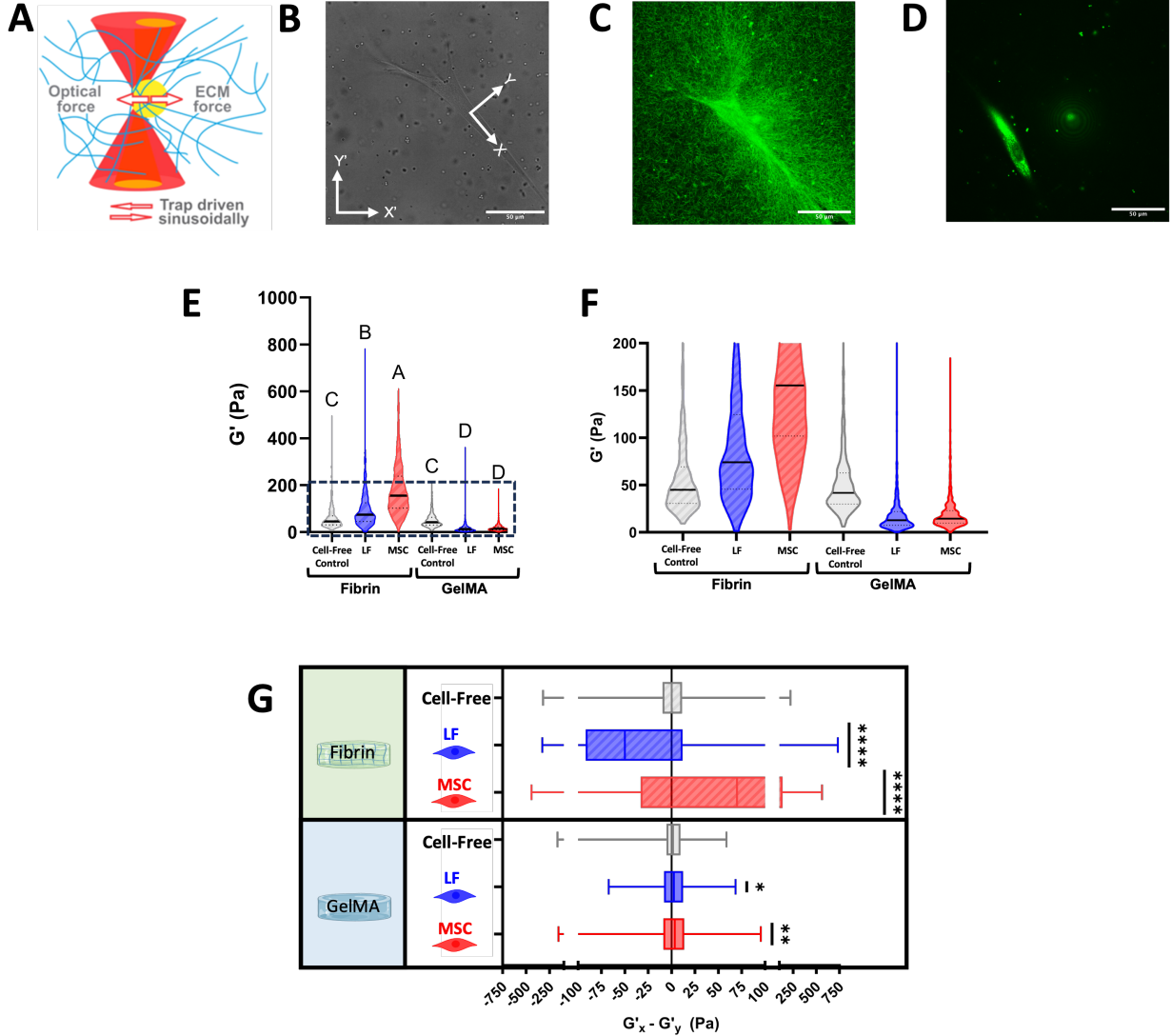


Figure 4.1: (A) Cartoon schematic of optical tweezers-based AMR. The trapping beam is oscillated sinusoidally, and the optical forces of the trapping beam are opposed by local ECM forces. (B) Representative brightfield (BF) image of an LF embedded in a fibrin hydrogel. (Scale bar= 50μm) AMR measurements were taken in the X' and Y' reference frame of our AMR system and then projected on the cell axes (X and Y). Representative reflection confocal microscopy (RCM) images of (C) an LF embedded in fibrin and (D) an LF embedded in GelMA after 48hrs. RCM images demonstrate distinct differences in ECM architecture. Bright, anisotropic fibers are visible in fibrin gels, while no structures are visible in GelMA. (E) Local G' measurements taken in the X' and Y' axes were aggregated and compared. (Kruskal-Wallis with Dunn's multiple comparisons: Compact letter display is used to denote statistical differences between groups) (F) A zoomed in view of the dashed box in panel (E). Local stiffness anisotropy was calculated for each bead as $G'_x - G'_y$. Significant deviation from zero indicates direction anisotropy (Wilcoxon Signed-Rank: *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$)

Next, AMR measurements were projected along the axes of each cell, and a measure of local stiffness anisotropy was computed for each bead as the difference between G'_x and G'_y . Distributions of stiffness anisotropy are shown in **Figure 4.1G** and summarized in Figure S.4.4. Stiffness anisotropy distributions were compared to 0 using the Wilcoxon signed-rank test, where any significant deviation of the median from 0 is considered significant directional anisotropy. While both cell-free gels exhibit no direction anisotropy, comparison of the variance in local stiffness anisotropy in cell-free gels further illustrates the differences in the mechanical landscapes within each gel type. The spread in local stiffness anisotropy values within fibrin is significantly greater than in GelMA (Brown-Forsythe; $p=0.0027$), demonstrating that the fibrous ECM environment is highly anisotropic throughout fibrin hydrogels and relatively isotropic in GelMA.

4.4.2 Distinct Cell Morphologies in Stiffness-Matched Fibrin and GelMA

Cells embedded in GelMA took on distinct morphologies compared to cells embedded in Fibrin. Brightfield (BF) and RCM images of representative cells around which AMR measurements were taken are shown in **Figure 4.2**. Qualitatively, while cell spread and elongate more in fibrin, they are smaller and less spread in GelMA. RCM images of embedded cells show clear distinctions in ECM structure. Around both LFs and MSCs in fibrin, there is a visual densification of ECM fibers, indicative of local ECM remodeling and potential new matrix deposition.

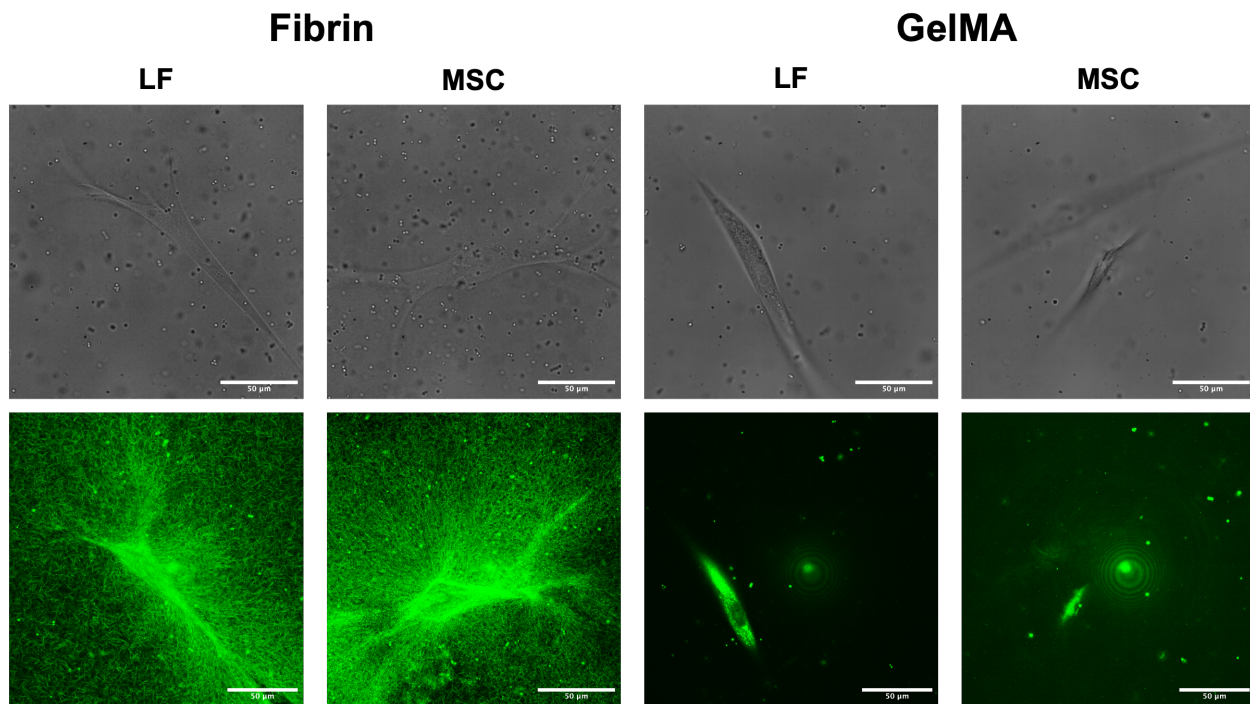


Figure 4.2: Brightfield and reflection images of cells embedded in either fibrin or GelMA. (Scale bar =50µm)

To further explore morphological differences in embedded cells, as well as investigate differences in matrix deposition, confocal microscopy was conducted. Cell-laden samples were fixed and stained to identify the cell nucleus DAPI, F-actin, and fibronectin. Representative max intensity Z projection images of cell within a volume in each gel qualitatively illustrate potential differences in proliferation, morphology, and fibronectin deposition. (**Figure 4.3A**) In fibrin, a higher density of MSCs is detected than LFs in the same gel volume. Both cell types exhibit significant fibronectin in the intracellular and pericellular regions (blue). In contrast, LFs and MSCs were present at a lower density and exhibited significantly less fibronectin abundance. In agreement with BF and RCM images presented in 4.2, both LFs and MSCs take on a more spread and elongated morphology in fibrin than in GelMA. To quantify these morphological differences, cell solidity, a measure of cell protrusions, and circularity were calculated based on the actin channel for 30 cells in each group. Consistent with qualitative observations, cell solidity and circularity differed significantly between cells

embedded in fibrin and GelMA. (Figure 4.3 B-C) MSCs and LFs had lower solidity in Fibrin, indicating more cell protrusions compared to cells in GelMA. Both cell types in GelMA also were more round compared to cells in fibrin. LFs and MSCs proliferated more, elongated more, and deposited more fibronectin in fibrin than in GelMA.

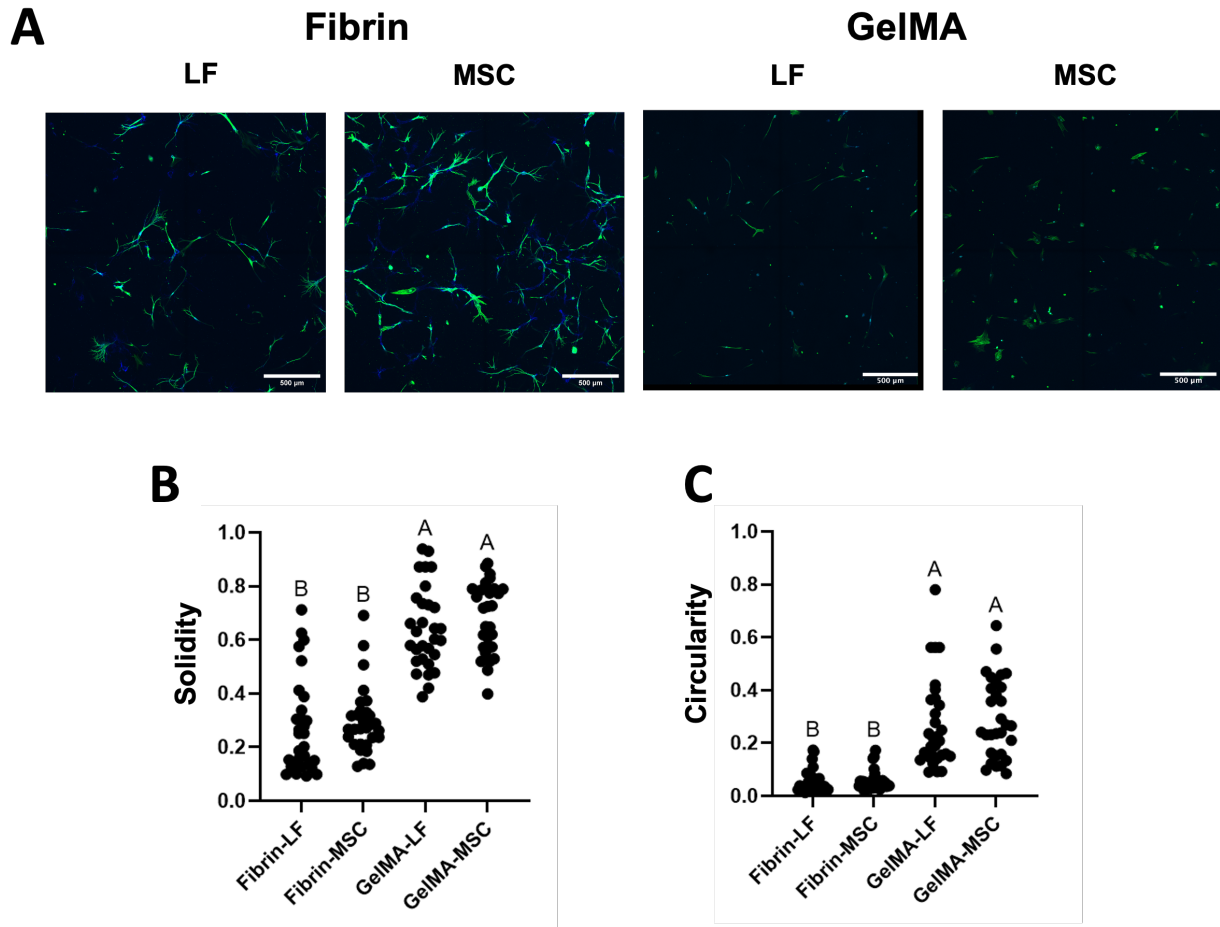


Figure 4.3: Cells embedded in fibrin and GelMA differ in morphology. (A) Confocal images of representative MIP Z-stacks in cell-aladen hydrogels. Nuclei (DAPI, cyan), F-actin (Phalloidin, green), and Fibronectin (blue) are visualized. (Scale bar = 500 μm) Comparisons of cell (B) solidity and (C) circularity quantitatively demonstrate differences. (One-way ANOVA with Tukey Post-Hoc: Compact letter display shows groups that are statistically different at $\alpha < 0.5$.)

4.4.3 Cell-mediated ECM Stiffness Changes are Contractility Dependent

Next, to investigate potential mechanisms of the divergent pericellular stiffness landscapes in fibrin and GelMA, AMR was conducted around cells that were treated with either Y27632 or BB94 to inhibit cell contractility or MMP activity respectively. For BB94 treated cells, 10 μ M BB94 was added to media immediately after gelation and followed with 48hr incubation. For Y27632 treatment, 20 μ M Y27632 was added to one hour prior to AMR measurements. DMSO was used as the solvent vehicle for inhibitor treatments and was applied at a constant 0.2% (v/v) concentration across treatments. Consistent with previous studies, BB94 and Y27632 lead to modification in cell morphology. (**Figure 4.4**)

Prior to AMR studies with cells, the effect of the inhibitor treatments was evaluated in cell-free hydrogels. (Figure S.4.1) Both inhibitors did not affect pericellular stiffness in cell-free fibrin and GelMA hydrogels. In fibrin, treatment effects differed between cell lines. (Figure S.4.3) Around LFs, median pericellular G' did not change significantly with the addition of either BB94 or Y27632. (**Figure 4.5A-B**; $p > 0.99$) Around MSCs, local pericellular stiffening was reduced after treatment of both BB94 and Y27632. ($p < 0.001$; $p = 0.0031$) The DMSO carrier control also showed a decrease in pericellular stiffening around MSCs ($p < 0.001$), indicating that MSCs are sensitive to this low DMSO concentration. Interestingly, stiffness anisotropy distributions showed that while there was no apparent change in median G' around DMSO treated LFs, there were significant changes in local stiffness anisotropy. (**Figure 4.5C**) For both LFs and MSCs, DMSO, BB94, and Y27 all led to a reversal in directional stiffness anisotropy.

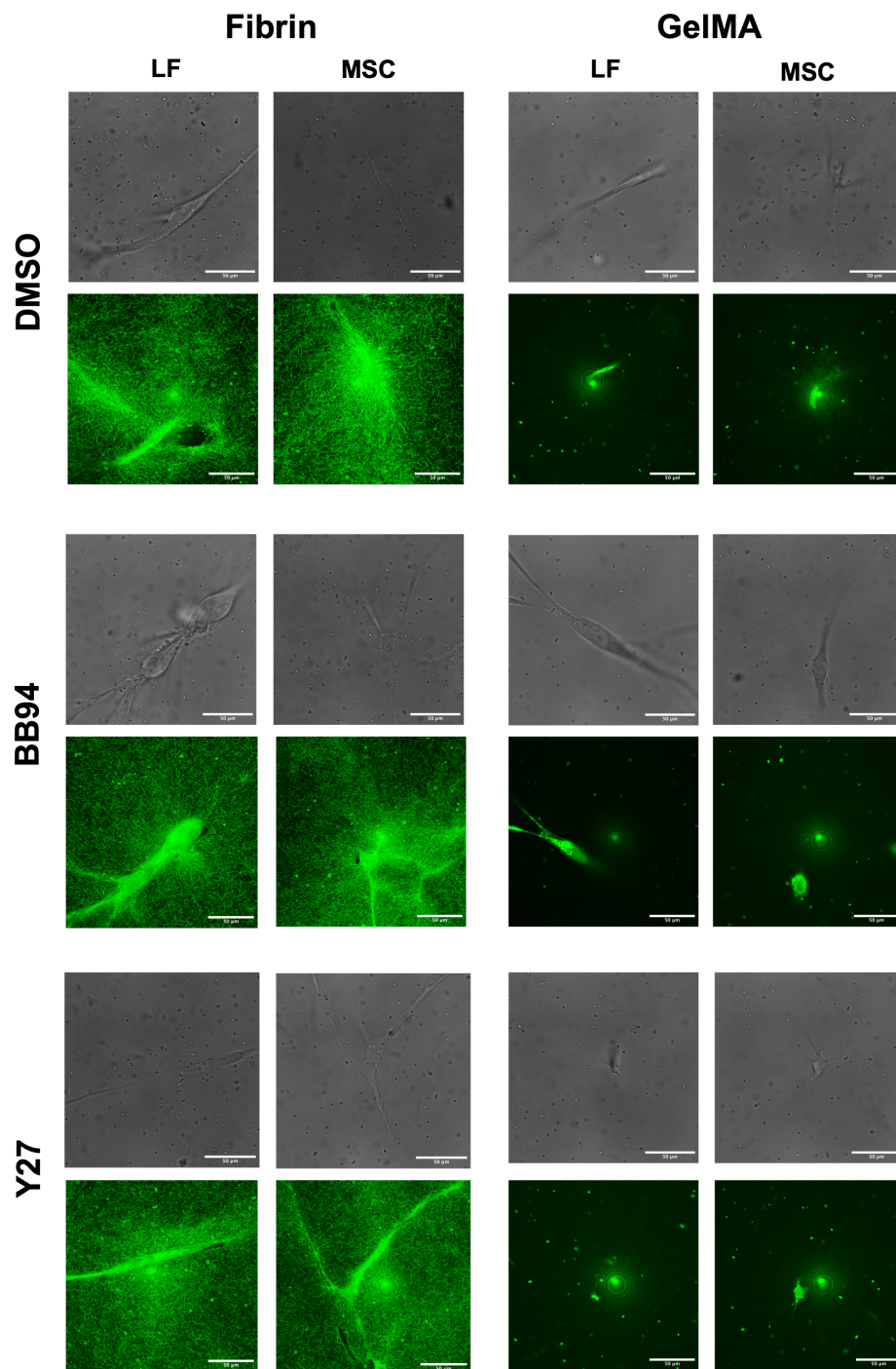


Figure 4.4: Treatments affect cell morphology. Brightfield and reflection confocal microscopy (RCM) images of cells with the DMSO vehicle control, MMP inhibitor BB94, and contractility inhibitor Y27632. (Scale bar = 50 μm)

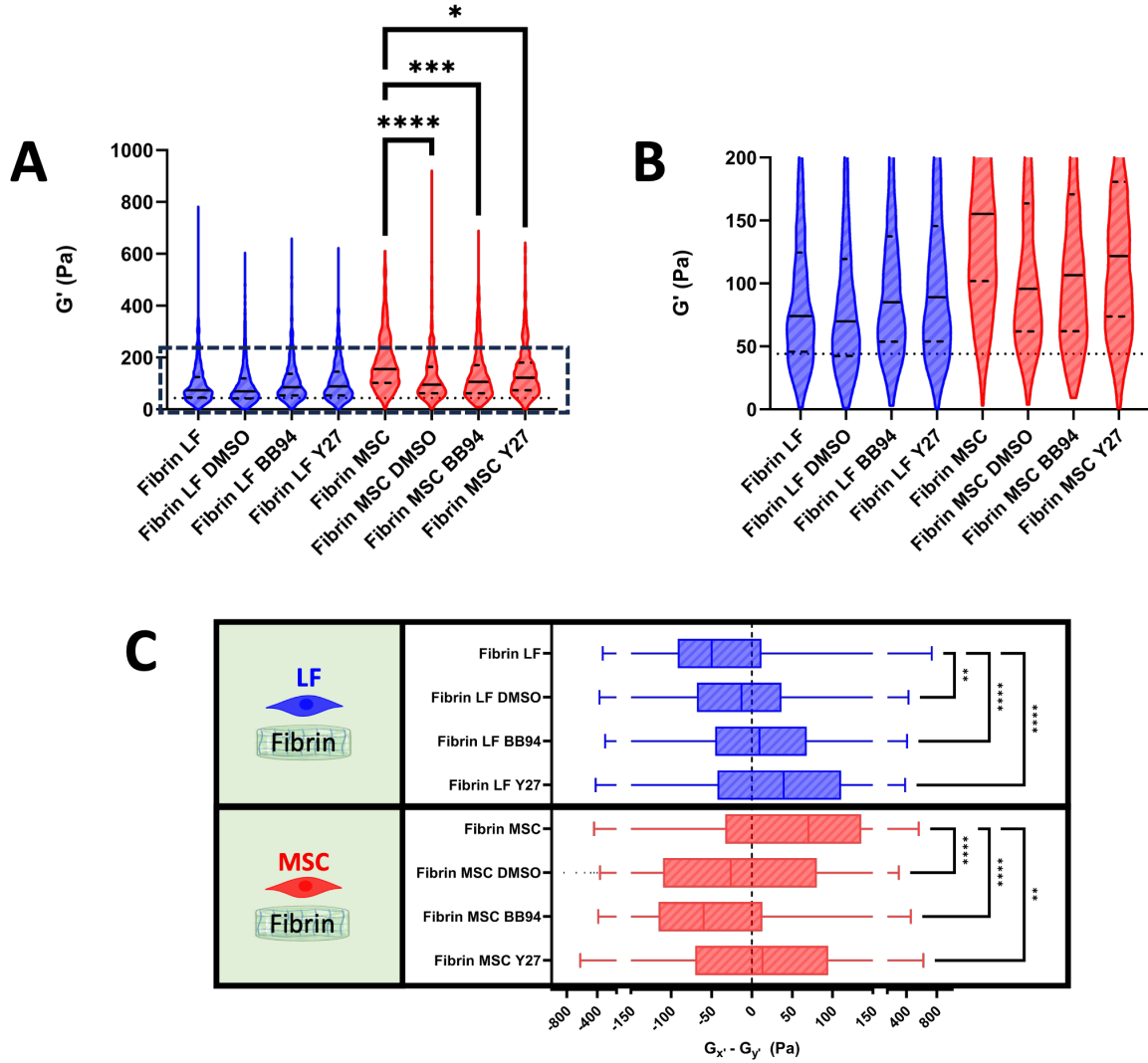


Figure 4.5: Treatment Response of LFs and MSCs embedded in Fibrin. Comparisons of G' distributions in cell-laden fibrin after treatment with Y27632 or BB94 shown at (A) full scale and (B) zoomed. Pairwise comparisons of local stiffness anisotropy in treated samples compared to untreated. (Kruskal-Wallis and Dunn's post hoc: : *: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

Prior to exploring the contractility and MMP dependent mechanisms, we investigated whether the inclusion of cell bodies within GelMA could result in the softening observed due to disruption of the crosslinking network. Inclusion of 20 μ m polystyrene bead to mimic cell bodies did not alter median G' in cell-free GelMA samples. (**Figure S.4.1**) In GelMA, inhibitor treatments implicate cell contractility rather than MMP activity as the predominate media-

tor of ECM softening. (**Figure 4.6A-B**) In GelMA, treatment with the DMSO vehicle and BB94 had no effect on ECM softening observed in untreated gels. However, treatment with Y27632 lead to a significant reduction in pericellular softening around LFs. ($p < 0.0001$) In MSC-laden GelMA samples, pericellular softening was also reduced in Y27632 treated gels compared to untreated samples. ($p=0.0002$) DMSO treated MSC samples also exhibited a reduction in pericellular softening, further demonstrating that MSCs are sensitive to the DMSO. ($p=0.0036$) Treatment of MSC samples with BB94 resulted in no change to pericellular softening, despite equivalent DMSO concentration indicating that contractility-mediated softening of GelMA may also depend on MMP activity for MSCs. Stiffness in cell-laden GelMA hydrogels remained largely isotropic, regardless of treatment condition. (**Figure 4.6C**) Only a small change in stiffness anisotropy was detected in the LF samples treated with the DMSO vehicle. ($p=0.035$)

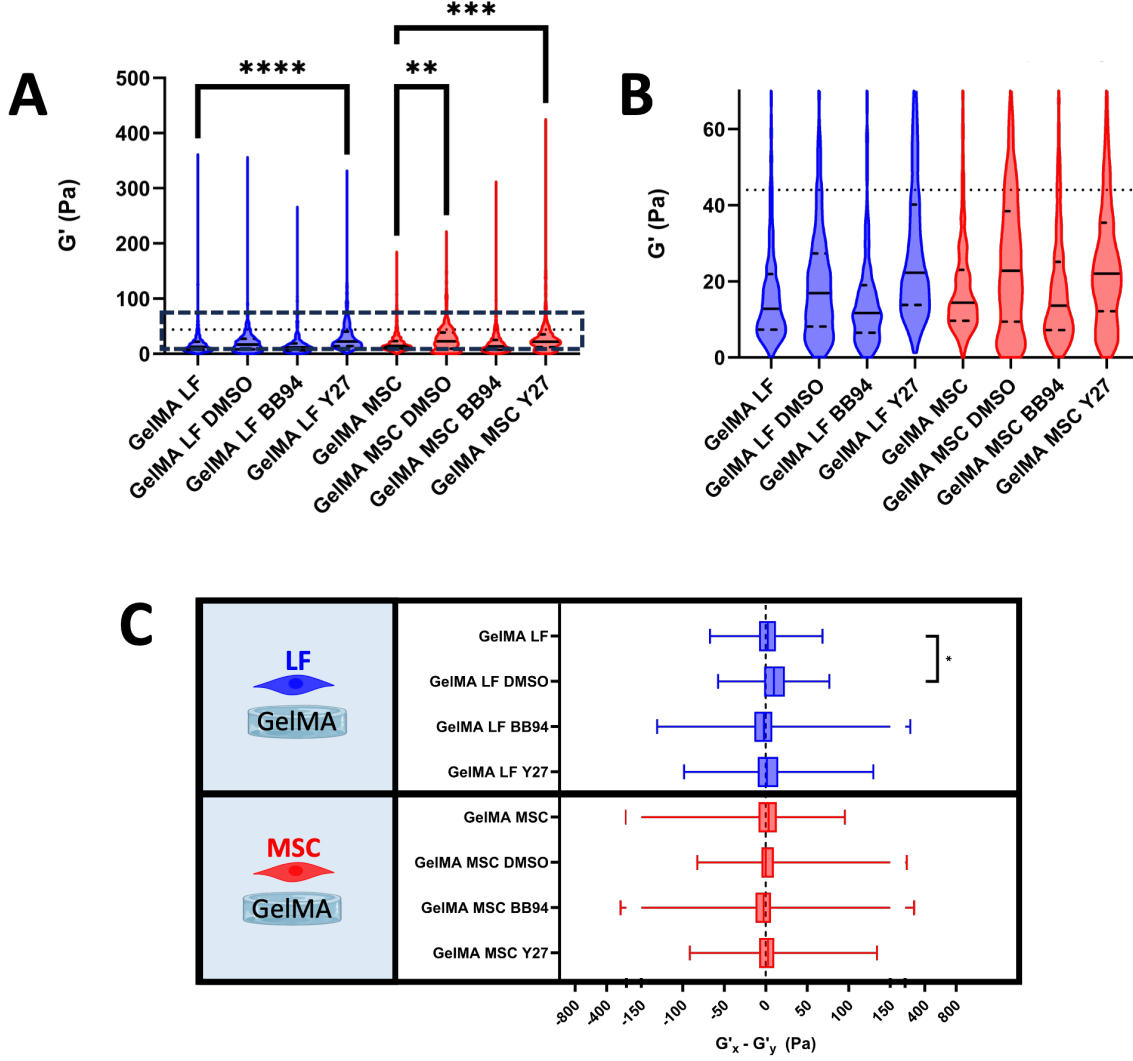


Figure 4.6: Treatment Response of LFs and MSCs embedded in GelMA. Comparisons of G' distributions in cell-laden GelMA after treatment with Y27632 or BB94 shown at (A) full scale and (B) zoomed. Pairwise comparisons of local stiffness anisotropy in treated samples compared to untreated. (Kruskal-Wallis and Dunn's post hoc: *: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$; **** $p < 0.001$)

4.5 Discussion

Pericellular stiffness distribution and stiffness anisotropy have previously been shown by our group to depend on ECM type, ECM density, and cell type within fibrous matrices. [63, 61] Here we expand upon this work, by adding new information about pericellular stiffening and

anisotropy in amorphous hydrogels that lack fibrillar ECM architecture and by comparing two different cell types, lung fibroblasts (LFs) and mesenchymal stem cells (MSCs).

Stiffness-matched fibrin and gelatin methacrylol (GelMA) hydrogels were used in this study to explore pericellular stiffness landscapes in ECMs with distinct architecture. Fibrin is a macroporous ECM model that exhibits nonlinear stain-stiffening behavior and consists of a 3D connected network of long semiflexible fibers which have been shown to be critical to cell-generated force transmission. [64, 30] In contrast, GelMA is an amorphous network of randomly crosslinked gelatin chains that does not exhibit strain-stiffening. [4, 92] Structural differences between these ECM types are highlighted using reflection confocal microscopy to visualize the fibrous architecture of fibrin and lack of microscale architecture in GelMA. **(Figure 4.2)** Differences in microscale structure were correlated to differences in mechanical inherent heterogeneity and anisotropy. **(Figure S.4.1)** Consistent with prior studies in fibrous matrices, heterogeneity in pericellular stiffness spanned orders of magnitude in fibrin. **(Figure 4.1)**[72, 62, 61] In contrast, the heterogeneity in local stiffness and stiffness anisotropy was significantly lower in GelMA than in fibrin.**(Figure 4.1)**

While we have previously shown that cells embedded in fibrous matrices such as fibrin and collagen stiffen the surrounding ECM, we had yet to explore how these cell-mediated changes to pericellular stiffness are altered in an amorphous ECM. In this study, we observed highly divergent cell-mediated stiffness changes in fibrin and GelMA hydrogels. In fibrin, LFs and MSCs both stiffened the pericellular space. **(Figure 4.1)** These results are consistent with prior observations of cell contractility-mediated pericellular stiffening in fibrous hydrogels.[161, 65, 72, 62, 61]

Somewhat surprising was that MSCs stiffened fibrin more than LFs. Although LFs and MSCs are morphologically similar, secrete ECM proteins similarly, and exhibit similar cell surface markers, [28] they were chosen for comparison in this study due to differences in traction force generation. [101] Given the aforementioned importance of cell tractional forces on

ECM stiffening, we would have expected LFs to stiffen fibrin more than MSCs. However, one explanation is that MSCs are simply proliferating more (**Figure 4.3**), leading to more global traction force generation across the gel as compared to LFs. Another alternative explanation is that the LFs are modulating the initial mechanics of their ECM to a preferred stiffness setpoint, which may be lower than what MSCs prefer, as we’ve seen previously with DFs in collagen. [62]

In GelMA, cells caused ECM softening compared to the ECM stiffening observed in fibrin. (**Figure 4.1**) Both LFs and MSCs softened GelMA significantly after 48hrs with no cell type-dependent differences in ECM softening observed. (**Figure 4.1**) These results are consistent with evidence of matrix softening within GelMA hydrogel at similar concentrations embedded with MSCs and adipose-derived stromal cells. [106, 103] This softening may be attributed to plastic deformation of the polymer network, induced by cell-generated forces, or the degradation of the matrix via MMPs. [162, 88]

Y-27632, a ROCK inhibitor that affects actomyosin-mediated contractility and batimastat (BB94), a broad-spectrum matrix metalloproteinase (MMP) inhibitor, were used to investigate the impact of contractility and MMP activity on pericellular stiffness changes in these ECM models. [85, 12] Previously, we have shown that the development of pericellular stiffening and stiffness anisotropy in collagen is dependent on the cooperation of cell contractility and MMP activity. [72] Here, we observed a reduction in pericellular ECM stiffness around MSCs in fibrin after Y27632 treatment, as well as BB94 and DMSO vehicle control treatments. (**Figure 4.5**) Given the similar effects of the vehicle control and both inhibitors, it is difficult to draw conclusions regarding the role of MMP activity and MSC contractility on pericellular stiffening in fibrin.

In GelMA, a reduction in pericellular softening was observed in both LF and MSC-laden samples after treatment with Y27632, implicating cell contractility as an important mediator of the softening effects in GelMA. (**Figure 4.6**) BB94 treatment in LF samples did not affect

ECM softening. These data support that the mechanism of softening in these amorphous GelMA matrices may be predominantly due to plastic deformation rather than direct proteolytic degradation [103, 162] However, BB94 treatment in MSC samples also did not affect ECM softening, despite DMSO carrier treatment reducing the ECM softening at the same effect level of Y27632 treatment.

Although MSCs seem to be more sensitive to DMSO given modification in G' landscapes across ECM types, stiffness anisotropy changes across cell and ECM types after DMSO suggest that DMSO can affect LF behavior as well in a subtle way. Despite the DMSO concentration used in this study being low (2%v/v) and within the range commonly used for DMSO vehicles, prior studies have demonstrated that even 0.1% (v/v) DMSO can subtly modulate cytoskeletal organization. [163] Additionally, DMSO at low concentrations between 1-5% (v/v) more broadly has been shown to potentially alter membrane lipids, cell cycle progression, and DNA and protein structure which can alter a variety of cell processes. [150] Since cytoskeletal contractility is central to the generation of traction forces, reduction of pericellular stiffness changes and changes in stiffness anisotropy in DMSO vehicle controls across ECM types may be due to perturbations in the cytoskeleton. Together, these data underscore the importance of including vehicle controls in mechanobiology experiments.

In conclusion, we investigated the relationship between pericellular stiffness landscapes developed around lung fibroblasts and mesenchymal stem cells in hydrogels with distinct ECM architectures. While LFs and MSCs differentially stiffened fibrin, both cells mediated ECM softening within GelMA. Differences in pericellular stiffness after treatment with MMP and contractility inhibitors implicate cell contractility as a predominant mediator of pericellular stiffness changes. However, cooperation between matrix degradation and cell contractility may also be needed to induce ECM stiffness changes. Although we cannot directly decouple the nonlinear strain-stiffening mechanics and fibrous structure itself, these results highlight the differences in the development of pericellular stiffening and stiffness anisotropy in fibrous

versus amorphous matrices. This study builds upon our growing body of work demonstrating that bulk stiffness may not reflect the local mechanical environment experienced by individual cells, and local pericellular stiffness should be considered in mechanobiological studies.

4.6 Acknowledgements

ML was partially supported by the NHLBI Training Program in Cardiovascular Applied Research and Entrepreneurship at the University of California Irvine (T32-HL116270). Confocal microscopy was made possible by an NIH Office of the Director equipment grant 1S10OD025064. The content herein is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

4.7 Supplementary Material

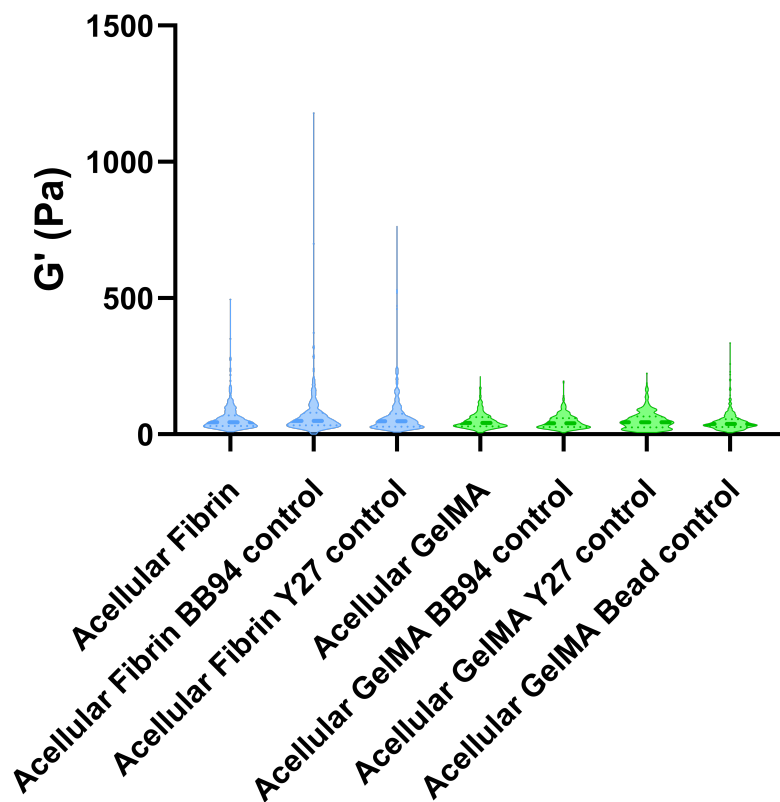


Figure S.4.1: Comparison of cell-free fibrin and GelMA controls. Aggregated AMR-measured G' in cell-free fibrin and GelMA (Kruskal-Wallis Test with Dunn's Multiple Comparisons; $p > 0.05$)

	Acellular GelMA	GelMA BB94 control	GelMA Y27 control	GelMA Bead control	Acellular Fibrin	Fibrin BB94 control	Fibrin Y27 control
Median G'	41.74	40.4	44.69	37.74	44.89	49.95	47.67
IQR	32.75	32.94	39.32	27.85	38.77	45.47	46.91
Number of values	352	360	357	345	307	340	333

Figure S.4.2: Table of AMR measured G' in acellular controls.

	Fibrin LF	Fibrin LF BB94	Fibrin LF DMSO	Fibrin LF Y27
Median G' (Pa)	74.02	85.12	69.95	89.04
IQR	78.9	83.69	77.06	91.61
Number of values	609	638	627	664
	Fibrin MSC	Fibrin MSC BB94	Fibrin MSC DMSO	Fibrin MSC Y27
Median G' (Pa)	155.3	106.5	95.68	121.8
IQR	136.7	108.63	101.54	107.03
Number of values	505	528	581	632
	GeIMA LF	GeIMA LF BB94	GeIMA LF DMSO	GeIMA LF Y27
Median G' (Pa)	12.8	11.71	16.89	22.26
IQR	14.558	12.455	19.199	26.37
Number of values	681	632	658	711
	GeIMA MSC	GeIMA MSC BB94	GeIMA MSC DMSO	GeIMA MSC Y27
Median G' (Pa)	14.4	13.6	22.77	22.05
IQR	13.364	17.89	28.997	23.22
Number of values	694	686	672	663

Figure S.4.3: Table of AMR measured G' in cell laden hydrogels.

	Fibrin LF	Fibrin LF BB94	Fibrin LF DMSO	Fibrin LF Y27
Median G' _x -G' _y (Pa)	-49.9	9.263	-12.84	39.42
Number of values	268	300	287	314
	Fibrin MSC	Fibrin MSC BB94	Fibrin MSC DMSO	Fibrin MSC Y27
Median G' _x -G' _y (Pa)	-23.4	-59.77	-26.16	13.1
Number of values	222	201	240	291
	GeIMA LF	GeIMA LF BB94	GeIMA LF DMSO	GeIMA LF Y27
Median G' _x -G' _y (Pa)	2.513	-2.174	10.01	1.399
Number of values	325	285	304	351
	GeIMA MSC	GeIMA MSC BB94	GeIMA MSC DMSO	GeIMA MSC Y27
Median G' _x -G' _y (Pa)	3.486	-2.675	2.373	2.85
Number of values	340	332	233	307

Figure S.4.4: Table of AMR measured Stiffness Anisotropy in cell laden hydrogels.

Chapter 5

Conclusions

Complex, reciprocal cell-matrix interactions are critical in understanding mechanobiological processes. The impact of bulk ECM stiffness as an important mediator of cell processes like migration, differentiation and proliferation has long been appreciated. [33] Cells have also been shown to reciprocally modify the mechanics of the ECM through degradation, new matrix deposition, and remodeling. [91] Local cell-mediated changes to the ECM can alter the behavior of neighboring cells. Thus, dynamic local ECM mechanics must be investigated at a length-scale relevant to individual cells.

My thesis work builds upon our lab’s previous research investigating pericellular stiffness dynamics in fibrous ECMs using optical tweezers-based AMR. In **Chapter 2**, I describe the multi-axis optical-tweezers based AMR system we developed to enable measurement of local stiffness on the micron length-scale. With my work, modifications were made to the optical path, calibration scheme, imaging modalities , and data processing workflow to improve the reliability of measuring local stiffness and stiffness anisotropy. In **Chapter 3**, I used the modified multi-axis AMR system to investigate stromal cell-dependent ECM stiffening and stiffness anisotropy in a fibrin-based vascular morphogenesis model. An important and

novel finding of this work was the development of long-range and anisotropic stiffening of the ECM concurrent with vascular development in the direction of capillary growth. This work built upon our standing collaboration with the Putnam Lab, demonstrating the importance of characterizing dynamic stiffness changes during vascular development at the microscale, rather than solely at the bulk level. Finally, in **Chapter 4**, I explored the role of fibrillar ECM architecture on pericellular stiffness landscapes. Growing evidence demonstrates the importance of ECM fibers for the transmission of cell-generated forces.[[2]] However, the role of ECM fibers in pericellular stiffening is not well understood. Using AMR in fibrin and GelMA hydrogels, we showed that pericellular stiffness dynamics and stiffness anisotropy are dependent on the architecture of the ECM, extended our work from fibrous to amorphous hydrogels.

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