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Mechanical Interaction of Angiogenic Microvessels With the Extracellular Matrix

Angiogenesis is the process by which new blood vessels sprout from existing blood vessels, enabling new vascular elements to be added to an existing vasculature. This review discusses our investigations into the role of cell-matrix mechanics in the mechanical regulation of angiogenesis. The experimental aspects of the research are based on in vitro experiments using an organ culture model of sprouting angiogenesis with the goal of developing new treatments and techniques to either promote or inhibit angiogenic outgrowth, depending on the application. Computational simulations were performed to simulate angiogenic growth coupled to matrix deformation, and live two-photon microscopy was used to obtain insight into the dynamic mechanical interaction between angiogenic neovessels and the extracellular matrix. In these studies, we characterized how angiogenic neovessels remodel the extracellular matrix (ECM) and how properties of the matrix such as density and boundary conditions influence vascular growth and alignment. Angiogenic neovessels extensively deform and remodel the matrix through a combination of applied traction, proteolytic activity, and generation of new cell-matrix adhesions. The angiogenic phenotype within endothelial cells is promoted by ECM deformation and remodeling. Sensitivity analysis using our finite element model of angiogenesis suggests that cell-generated traction during growth is the most important parameter controlling the deformation of the matrix and, therefore, angiogenic growth and remodeling. Live two-photon imaging has also revealed numerous neovessel behaviors during angiogenesis that are poorly understood such as episodic growth/regression, neovessel colocation, and anastomosis. Our research demonstrates that the topology of a resulting vascular network can be manipulated directly by modifying the mechanical interaction between angiogenic neovessels and the matrix. [DOI: 10.1115/1.4026471]

Keywords: angiogenesis, biomechanics, extracellular matrix, capillary, computational modeling, time series imaging

1 Introduction and Motivation

Angiogenesis is the process by which new blood vessels sprout from existing blood vessels, enabling the addition of new vascular elements to an existing vasculature. Angiogenesis is an important aspect associated with numerous issues and applications in medicine and bioengineering. Within the medical field, diseases including cancer and atherosclerosis involve uncontrolled blood vessel

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Fig. 1 An initial microvessel fragment within a collagen matrix imaged using two-photon microscopy. Endothelial cells and pericytes were imaged using autofluorescence (green), collagen fibrils were imaged using second-harmonic generation (red). Scale bar 20 μm .

growth, while scarring and poor wound healing involve insufficient vessel growth [1–3]. Bioengineers are also concerned with angiogenesis as a means to promote neovascularization of scaffolds for engineered tissue implants. Therefore, there has been increasing research interest into the regulatory mechanisms behind angiogenesis, with the hope of developing new treatments and techniques to either promote or inhibit angiogenic outgrowth depending on the application. Angiogenesis is highly sensitive to both the chemical and mechanical microenvironment [4–6]. Although extensive research has been performed regarding the chemical regulation of angiogenesis, the mechanical factors that regulate angiogenesis remain poorly understood. In particular, very little is known about how the mechanical properties of the EM, such as structure, composition, and boundary conditions, modulate angiogenic outgrowth toward a desired vascular solution.

During angiogenesis, sprouting endothelial cells within the parent vessel take on an invasive phenotype, releasing proteolytic enzymes to degrade the basement membrane, thus, allowing their expansion into the ECM. The motility, metabolism, proliferation, and differentiation of anchorage-dependent cells such as endothelial cells and pericytes are highly regulated by mechanical stimuli received by cells via mechanotransduction through the ECM [7–14]. This causes angiogenic neovessels to respond to the mechanical properties of the ECM. However, neovessels remodel the ECM during growth through a combination of applied traction forces, proteolytic activity, and generation of new cell-matrix adhesions, changing the properties that regulate their growth. This creates a dynamic feedback loop in which angiogenesis is coupled with deformation and remodeling of the matrix. This review discusses our recent efforts to investigate the role of cell-matrix mechanics as neovessels respond to the mechanical properties of the ECM, as well as how cells dynamically change matrix properties during growth.

It should be noted that we do not intend to review the entire body of current angiogenesis research (both experimental and computational modeling) or research into cell/matrix biomechanics and morphogenesis. The experimental aspects of this research effort involved the use of an in vitro organ culture model of angiogenesis [15]. To assist with testing specific hypotheses regarding the observed experimental results, in parallel we developed a computational framework to describe and predict angiogenic growth. Finally, we developed new live imaging techniques to elucidate the dynamics of angiogenic sprouting, elongation, branching, and anastomosis. The combined approach has allowed us to characterize many important mechanical factors during

angiogenesis and provides new insights for directing angiogenic growth by manipulating the mechanical interaction between cells and the ECM.

2 In Vitro Model

We utilized a three-dimensional organ culture model of microvessel fragments suspended within a type-I collagen gel during our investigation into the mechanical regulation of angiogenesis. Unlike angiogenesis models that use isolated endothelial cells, this model uses intact microvessel fragments, providing a more realistic representation of in vivo angiogenesis. “Parent” vessel fragments contain associated perivascular cells and retain their basement membrane after initial harvest and seeding in 3D collagen gels (Fig. 1). Sprouts elongate as patent tubes, branching and fusing with other vessels, and forming a new vascular network that ultimately fills the construct. Neovessel constructs form a functional vascular tree when implanted, rapidly inosculating with the recipient host circulation after implantation and carrying blood [16].

The methodology for creating these cultures was developed by Hoying et al. [15] (Fig. 2). Rat microvessel fragments were isolated from epididymal fat pads of retired breeder Sprague–Dawley rats (Fig. 2(a)). The pads were minced and subjected to limited digestion with 2.0 mg/ml of *Clostridium* collagenase (Worthington Biochemicals, Lakewood, NJ) and 2 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in Dulbecco’s cation-free phosphate buffered saline (DCF-PBS, pH 7.4) at 37 °C (Fig. 2(b)). The solution was centrifuged and the resulting pellet was washed twice and resuspended in DCF-PBS containing 0.1% BSA and then sequentially filtered through 350 μm and then 30 μm sterile nylon membrane filters (Fig. 2(c)). The first filtration step eliminates undigested particulate debris, and the second step retains larger vessel fragments of interest, while allowing single cells and smaller fragments to pass through. The 30 μm membrane was flushed with 0.1% BSA DCF-PBS, and the fragments were collected in a sterile Petri dish. Sterile rat tail collagen type-I (Discovery Labware, BD Biosciences, Bedford, MA) was mixed with filter-sterilized Dulbecco’s modified Eagle medium (DMEM, GIBCO-Invitrogen, Carlsbad, CA) to yield final concentrations of 3.0 mg/ml of collagen in $1 \times$ DMEM (Fig. 2(d)). Microvessels were suspended in the collagen solution at various seeding densities depending on the experiment. The collagen solution was then poured into custom rectangular Teflon molds polymerized at 37 °C and 95% humidity for 30 min (Fig. 2(e)). In certain experiments, a sterilized stainless steel mesh was inserted into the gel prior to polymerization to constrain displacement during growth.

Microvessels were cultured in serum-free culture media [17] composed of a 1:1 mixture of $1 \times$ DMEM (low glucose) and F12 nutrient mixture with additional components (Insulin 5.0 mg/mL, Transferrin 100.0 mg/mL, Progesterone 20.0 nM, Selenium 30.0 nM, and Putrescine 100.0 mM), rhVEGF (10.0 ng/mL, Pepro-Tech, Rocky Hill, NJ), and Gentamycin (50.0 mg/mL). Growth media was changed every 2 days. Neovessel sprouting typically began around Day 3, and well-established vascular networks formed by Day 6 (Fig. 3). On the sixth day of culture, the constructs were fixed in 4% formaldehyde. Microvessels within the construct were washed several times with 0.1% Triton-X-100 in PBS. Endothelial cells were labeled with 2.0 mg/mL isolectin IB4-Alexa 488 conjugate (Invitrogen, Carlsbad, CA) and then imaged using confocal microscopy. Volumetric datasets were processed and skeletonized using Amira (FEI VSG, Burlington, MA), and morphometric data describing the vascular network was collected using a custom software application (WinFiber3D, <http://mrl.sci.utah.edu>). In some studies, confocal reflection microscopy was performed to visualize collagen fibril orientation within the construct.

Fig. 2 Methods for preparing organ cultures of microvessel fragments suspended within a type-I collagen gel originally proposed by Hoying et al. [15]. (a) Rat microvessel fragments were isolated from epididymal fat pads of retired breeder Sprague–Dawley rats. (b) The pads were minced and subjected to limited digestion. (c) The solution was then sequentially filtered through 350 µm and then 30 µm sterile nylon membrane filters to remove unminced chunks of tissue and single cells from the microvessel population. (d) Microvessels were then resuspended in liquid type-I collagen. (e) The collagen solution was poured into custom rectangular Teflon molds and polymerized at 37 °C and 95% humidity for 30 min to create the three-dimensional vascularized constructs.

3 Effects of Angiogenesis on the ECM

We found that angiogenic neovessels remodel the ECM extensively [18,19]. Neovessel sprouts from initial microvessel fragments extend into the matrix, applying traction and contracting the matrix inward. By Day 10 of culture, the cross-sectional area of rectangular vascularized constructs reached ~20% of avascular control constructs (Fig. 4(a)). The combined effects of matrilysis, cell adhesion, and the contraction and reorientation of ECM fibrils resulted in drastic changes to the material properties of the constructs. Messenger ribonucleic acid (mRNA) expression levels for matrix metalloproteases (MMPs) were increased at Day 6 of growth compared to Day 0 (Fig. 4(b)), and this increased proteolytic activity coincided with a decrease in the dynamic stiffness of the construct (Fig. 4(c)). The angiogenic phenotype in endothelial cells is marked by increased proteolytic activity, and a primary function of MMPs is collagen cleavage, a prerequisite for endothelial cells invasion and angiogenesis [20,21]. However, by Day 10, the dynamic stiffness was increased compared to the stiffness of initial cultures, even though proteolytic mRNA expression remained high (Figs. 4(b) and 4(c)). As neovessels applied traction and contracted the matrix, they compacted the collagen fibrils, condensing and reorienting fibrils toward the neovessel tip (Fig. 5(a)). This contraction resulted in exudation of water from the matrix and increased fibril density, increasing the stiffness of the vascularized constructs. Additionally, mRNA expression for matrix molecules that promote the angiogenic phenotype such as fibronectin (FN), decorin (DCN), and tenascin C (TNC) increased

during growth [22–27] while levels of the angiogenesis-inhibiting molecule hyaluronan (HAS) decreased (Fig. 5(b)) [28]. The creation of new cell-matrix adhesions through these matrix molecules likely contributed to the increased stiffness in the constructs. Neovessel and ECM fibril alignment induced by cell-generated contraction likely contributes to this stiffness increase as well. In summary, these experiments demonstrated that the process of angiogenic growth causes dramatic changes in the material properties of the ECM, suggesting a finely regulated relationship between angiogenesis, the forces generated by neovessels during growth, and the mechanical properties of the ECM.

4 Effects of Mechanical Boundary Conditions and Stretch on Vascular Alignment

Previous studies revealed a tight regulation between angiogenic growth and the mechanical properties of the ECM [19]. Based on these previous findings, we expanded these studies to examine the effects of mechanical stretch and boundary conditions on angiogenesis [29]. We found that boundary conditions and loading can affect the morphometry of the vascular network and induce alignment (Fig. 6). In these experiments, microvessels were cultured in rectangular gels with a long aspect ratio measuring 20.0 mm along the long axis, 5.0 mm along the short axis, and 3.0 mm through the thickness. Vessels within unconstrained constructs (i.e., shape controls) were randomly orientated with no preferred direction. When a 6% static or cyclic stretch was applied along the long-axis

Fig. 3 Angiogenic growth within unconstrained (top) and long axis constrained (bottom) vascularized constructs, showing images of different cultures at different time points of growth after initial seeding. Unconstrained gels were free-floating and allowed to contract in all directions. For the long axis constrained constructs, contraction along the long-axis was constrained by inserting a stainless steel mesh into the gel prior to polymerization. For both conditions, neovessel sprouting typically began at Day 3 of culture and well-established vascular networks formed by Day 6. Microvessels in the unconstrained constructs grew into a randomly oriented network, while vessels in the long axis constrained construct were highly aligned along the constrained axis, indicated by the black double arrow. Scale bar 200 μm .

of the rectangular constructs, microvessels were found highly aligned along the direction of applied stretch. However, simply constraining the long-axis of the construct and with no applied stretch resulted in the same amount of vascular alignment, and applying load did not produce additional alignment over simply anchoring the culture. Additionally, collagen fibers were aligned along the long-axis in both the constrained cultures (no stretch) as well as the static and cyclically stretched cultures [29]. When we replicated these loading scenarios using avascular constructs, fibers were only oriented in the static and cyclic stretching cases [29]. These results indicate that while externally applied mechanical loads are capable of aligning collagen fibrils, internal traction generated by neovessels in the constrained cultures were sufficient to induce fibril alignment. Neovessels align with these fibrils via contact guidance, causing a positive feedback loop, which results in the alignment of both microvessels and collagen fibrils along the constrained axis. Applying strain to the gels did not

Fig. 4 Angiogenic neovessels remodel the extracellular matrix during growth [18,19]. (a) Normalized cross-sectional area of the rectangular gels at Day 1, Day 6, and Day 10 of growth. The cross-sectional area of the gel significantly decreased over time as microvessels applied traction and contracted the matrix inward. (b) Normalized levels of mRNA expression for proteolytic matrix metalloproteases at Day 0, Day 1, Day 6, and Day 10. Expression of these proteolytic enzymes significantly increased over culture time. (c) Harmonic oscillation viscoelastic testing was used to measure the dynamic stiffness of the vascularized constructs at Day 1, Day 6, and Day 10. The cultures were tested at equilibrium strain levels of 2%, 4%, and 10% at frequencies of 0.05, 0.1, 1.0, and 5.0 Hz. Increased MMP expression during growth reduced the dynamic stiffness of the cultures by Day 6. However, by Day 10 the dynamic stiffness was increased compared to the stiffness of initial cultures, even though proteolytic mRNA expression remained high. This was likely due to compaction of the matrix, reducing the fluid phase of the constructs.

significantly increase alignment past the levels induced by the boundary constraint alone.

In a separate study, the implantation of prealigned vascularized constructs into immunocompromised mice was examined to determine the role of preexisting vascular organization on subsequent neovessel growth and alignment (Fig. 7) [30]. Rectangular constructs were grown in vitro within a stainless steel frame to constrain contraction along the long-axis. As a result, microvessels were highly aligned along the constrained long-axis prior to implantation at Day 7. During implantation, constructs were either cut away from the frame (unframed) or implanted with the frame (framed). At Day 30, microvessels within the unframed constructs had lost the initial prealignment and had no preferred directions while vessels within the framed constructs remained highly aligned along the constrained axis (Figs. 7(a) and 7(c)). These results demonstrate that prealignment of microvascular precursors do not significantly influence the final topology of the microvascular network. Rather, the displacement constraint in the framed implants caused these constructs to maintain their prepatterned orientation. Computational simulations demonstrated that the difference in the 3D deformation pattern between the unframed and framed constructs during growth and remodeling was sufficient to explain these results (Figs. 7(b) and 7(d)). Finite element (FE) models were used to simulate the contraction of a construct, and

Fig. 5 Angiogenic neovessels apply traction to the ECM and create new cell-matrix adhesions during growth [18,19]. (a) Two-photon image of a neovessel sprout, indicated by the yellow arrow. Vessels were imaged using autofluorescence (green) and the extracellular collagen matrix was imaged using second-harmonic generation (white). As neovessels applied traction and contracted the matrix, they drew collagen fibrils closer, condensing and reorientating ECM fibrils toward the neovessel tip. Scale bar 20 μm . (b) Normalized expression for matrix molecules fibronectin (FN), decorin (DCN), tenascin C (TNC), and hyaluronan (HAS) at Day 0, Day 1, Day 6, and Day 10 of growth. During angiogenesis, expression of matrix molecules that promote angiogenesis increased (FN, DCN, TNC) while expression for the angiogenesis-inhibiting molecule HAS decreased.

nodal displacement from the simulation was interpolated to a skeletonized collection of randomly-orientated microvessels. At Day 7 of the simulations, the unframed experiments were simulated by removing the boundary constraint representing the stainless steel mesh. A viscoelastic material model with stress dissipation was required in order to obtain realistic deformation of the construct prior to removal of the boundary constraint, as these cultures did not retract elastically after being cut away from the mesh, indicating a lack of significant tension along the constrained direction. Once the boundary condition was removed, the unframed constructs began to contract isotropically rather than anisotropically, and as a result, microvessels lost prealignment and became randomly oriented (Fig. 7(d)). Microvessels within simulations of the framed constructs not only maintained prealignment but became further aligned along the constrained axis (Fig. 7(d)). These

results demonstrate that constraining the ends of the culture to prevent contraction along the long axis is sufficient to cause and maintain vascular alignment, and preorganized microvasculature cannot maintain alignment without the displacement constraint.

These studies demonstrated the effects of deformation due to cell-generated traction forces and boundary conditions on vascular alignment, but the mechanisms behind these effects remained unclear. For example, do neovessels orient along tension along the constrained axis of the culture? Or, do microvessels become aligned along the direction of highest effective stiffness (i.e., the constrained axis) as the culture contracts laterally along the unconstrained directions? To further investigate the mechanisms by which boundary conditions induce vascular alignment, we performed experiments on rectangular vascularized constructs subjected to different mechanical conditions [31]. By changing the boundary conditions on the culture, we can control the directions of stress and strain during angiogenic growth. We investigated four different sets of boundary conditions on the vascularized constructs in this study (ordered from least to most constrained): unconstrained (UNC), long-axis constrained (LAC), short-axis constrained (SAC), and long-short-axis constrained (LSAC) (Fig. 8(a)). In these experiments, altering the boundary conditions of the construct had significant effects on the length, branching, and orientation of the vascular network (Figs. 8(b)–8(d)). Growth and branching decreased as the constructs became more constrained (Figs. 8(b) and 8(c)). The constrained directions can be thought of as presenting a very stiff resistance to traction forces applied by neovessels, and they are unable to condense the matrix and contract the construct along the constrained direction. As a result, tension (stress) develops along this direction. The trend of decreased growth and branching seen in the more constrained constructs suggests that raising the effective stiffness of the matrix through boundary constraint, thereby impeding the ability of neovessels to deform and remodel the matrix, reduces angiogenesis.

Microvessels in the UNC constructs were oriented randomly, while vessels in the LAC constructs were highly aligned along the constrained long-axis (Fig. 8(d)). However, in the SAC and LSAC cases, microvessels did not orient with respect to either the long- or short-axis of the constructs. In the constrained construct experiments, tension developed along the constrained directions as neovessel attempt to contract the matrix. The finding that microvessels in SAC constructs did not orient along the short-axis suggests that neovessels do not align along tension during growth. Furthermore, independent experiments demonstrated that elastic stress does not accumulate within these constructs. In experiments involving LAC constructs treated with Cytochalasin D to prevent cellular force generation, we did not see any measureable retraction once the construct was cut away from the boundary constraint. These results suggest that significant elastic stresses do not accumulate within the matrix due to the viscoelastic nature of collagen hydrogels, as stress is dissipated at a rate faster than they accumulate. The constrained gel experiments demonstrate that the deformation of the gel in response to cell-generated forces and remodeling is sufficient to cause microvascular alignment. The LAC gels, the gel was free to deform lateral to the long-axis causing microvessels to align along the constrained axis. The SAC gels can be thought of as a strip biaxial test: the short-axis was constrained, and the long-axis had a high effective stiffness as well due the aspect ratio of the gel. This prevented contraction along both the long- and short-axes of the gel during the culture, and as a result neovessels in these gels had no preferred alignment within the long-short plane.

5 Computational Modeling of Angiogenic Growth

In the experimental model, it is difficult to study the changes in neovessel behavior that occur at the microscale in response to changes to the ECM at the larger macroscale, such as the addition of a boundary constraint. Computational simulations allow us to test and compare hypotheses in a systematic manner without

Fig. 6 Applied load and mechanical boundary conditions affect the topology of the vascular network and induce alignment [29]. (a) When vascularized constructs were free-floating and able to contract in all directions, microvessels grew into a randomly-oriented network (shape control). When a 6% static or cyclic stretch was applied along the long-axis, microvessels were highly aligned along the direction of applied stretch. However, simply constraining the long-axis of the construct (no stretch) resulted in the same amount of vascular alignment, and applying load did not produce additional alignment over the no stretch case (b) and (c). Microvessel orientation angles were measured between vessels and the long-axis (x -axis) φ and the vessels and the vertical direction (z -axis) δ . (b) Microvessels in shape control constructs exhibited no preference in φ , while vessels within the no stretch, static stretch, and cyclic stretch groups were significantly aligned along the long-axis (x -axis). (c) Microvessels in all the experimental conditions exhibited preferential alignment within the horizontal plane, as indicated by the large percentage of vessels oriented perpendicular to the vertical axis (z -axis).

performing the necessary experiments, which may be exceedingly difficult or impossible using available culture techniques. Therefore, we have utilized computational models to supplement and enhance the interpretation of our experiments. We developed a computational model of angiogenesis, named *angio3d*, in which neovessel growth is regulated by local properties of the ECM (Fig. 9). In *angio3d*, the ECM was represented using a regular hexahedral FE mesh. ECM properties that play a role in the regulations of angiogenic growth, such as fibril orientation [29] and matrix density [32], were prescribed at the nodes of the FE mesh. Initial microvessel fragments were represented as line segments and a random distribution was created throughout the mesh domain, representing the seeding density from the experiments. Growth was simulated by adding new line segments at the positions of all active growth tips. The direction of each new line segment was determined by local collagen fibril orientation at the sprout tip, and the length of these new segments, or the amount of growth that occurred over the time step, was determined by local matrix density. Local fibril orientation $\theta(x_s)$ and matrix density

$\rho(x_s)$ were calculated by interpolating nodal quantities to the sprout location x_s using trilinear shape functions,

$$\theta(x_s) = \sum_{j=1}^8 \theta_j N_j(x_s) \quad (1)$$

$$\rho(x_s) = \sum_{j=1}^8 \rho_j N_j(x_s) \quad (2)$$

where θ_j and ρ_j were the nodal values for fibril orientation and matrix density, and $N_j(x_s)$ was the value of the shape function for node j evaluated at the sprout position. Branching, the spontaneous formation of a new sprout along an existing vessel was modeled as a random process controlled by a branching probability parameter. Anastomosis, the fusing of two vessels, was also permitted for vessels that came within close proximity to one another.

Fig. 7 Implantation of prealigned vascularized constructs into immunocompromised mice demonstrates that preexisting vascular organization has no effect on subsequent vascular alignment [30]. (a) Confocal images of prealigned vascularized constructs at Day 7, prior to implantation (pre-implant, left). Constructs were prealigned by constraining the long-axis of the rectangular gels with a stainless steel mesh. Prior to implantation, constructs were either cut from the frame (unframed, middle) or left within the stainless steel frame (framed, right). Implants were removed and imaged at Day 30, revealing that microvessels within the unframed constructs had lost the initial prealignment and had no preferred direction while vessels within the framed constructs remained highly aligned. (b) The deformation field from the finite element models was interpolated to a skeletonized dataset of randomly-orientated microvessels in order to determine how the deformation would affect vascular alignment. (c) The anisotropy index measured using the fast Fourier transform was used to quantify alignment within the implants. An anisotropy index of 1.0 indicates high alignment, while an index of 0.0 indicates no preferred alignment. Anisotropy was measured prior to implantation at Day 7 (white bars) and after implantation at Day 30 (black bars). In the unframed implants, the anisotropy significantly decreased during implantation, indicating that vessels within these constructs lost alignment without the stainless steel boundary condition. Vessels within constructs implanted within the stainless steel frame not only maintained the original alignment but became more aligned during the implantation period. (d) Microvessel orientation predicted by the finite element models. The distribution of orientation angles with respect to the long-axis of the constructs was measured and collected in to 30 deg bins. An angle of 0 deg indicates alignment along the long-axis while an angle of 90 deg indicates alignment perpendicular to the long-axis. In the pre-implant simulation, microvessels were preferentially aligned along the long-axis of the construct. However, removal of the boundary condition in the unframed simulation caused microvessels to lose this alignment. Vessels in the framed simulation were subject to the boundary condition during the entire simulation and as a result were more highly aligned than vessels prior to implantation.

The growth model was able to accurately simulate angiogenesis within different experimental conditions. When simulating growth within an aligned fibril field taken from confocal reflection imaging of LAC gels at Day 6, the model produced aligned vasculature similar to what was found in vitro [33] (Fig. 10(a)). The model also successfully simulated growth for different matrix densities [32] (Fig. 10(b)). Vascular networks grown in lower density collagen had greater overall length, greater branching, fewer free ends, and were more interconnected than networks grown in higher density collagen [32].

However, in these simulations, matrix properties were held constant and did not change after initialization. For simulation within an aligned collagen fibril field, this caused the model to overpredict alignment as vessels were grown in a prealigned fibril field rather than a field that was initially random and became aligned as neovessel remodel the ECM [18,29,33]. During experiments, the properties of the matrix are not constant and change due to forces applied by neovessels during growth and protease activity. This creates a dynamic feedback loop in which growing neovessels

respond to the properties of the ECM while simultaneously remodeling the ECM, changing these properties. Therefore, our next step was to expand our computational framework to couple angiogenic growth with the deformation/remodeling of the ECM.

6 Coupling Neovessel Growth With Matrix Deformation

In order to accurately update the properties of the ECM based on the deformation of the matrix caused by cell-generated forces, we coupled *angio3d* with the nonlinear FE software *FEBio* [34] through the plug-in file *AngioFE* [35]. The coupling in *AngioFE* was handled in an explicit manner, meaning information was passed from one component to another and that a component remained fixed while the other component was being resolved. First, *angio3d* simulated a growth step using fibril orientation and matrix density information in the ECM field at time step n (Fig. 11(a)). Then, sprout force representations were applied to the mesh (Fig. 11(b)). Next, *FEBio* was called to solve for the

Fig. 8 (a) Vascularized gel cultures subjected to various mechanical boundary conditions (in order from least to most constrained): unconstrained (top left), long-axis constrained (top right), short-axis constrained (bottom right), and long-short-axis constrained (bottom left). Altering the boundary conditions of the gel had significant effects on the length, branching, and orientation of the vascular network during angiogenesis [31]. (b) The total length of the vascular network per unit of gel volume was measured in each of the boundary condition cases. No significant difference in total vascular length could be detected between UNC and LAC cultures, but there was a trend of reduced vascular length in the SAC and LSAC cultures, with length in the LSAC experiments significantly reduced. (c) The number of branching points per unit of vessel length decreased as the constructs became more constrained. (d) Microvessel orientation was measured in each boundary condition case by collecting the distribution of vessel orientation angles with respect of the long-axis of the culture. Microvessels in the LAC cultures were highly aligned along the long-axis, while vessels within the UNC, SAC, and LSAC exhibited no preference.

deformation field using the cell-generated loading scenario (Fig. 11(c)). Finally, the deformation field predicted by FEBio was used to update fibril orientation and matrix density in the ECM field for the next time step $n + 1$ (Fig. 11(d)). The next growth step used this updated ECM field.

The forces generated by neovessel sprouts were represented using a body force field that was defined relative to the position of the sprout tip $\mathbf{x}_s$. At any position within the mesh, $\mathbf{x}$ the force due to a sprout $\mathbf{f}_s$ was calculated based on the vector $\mathbf{r}$ between $\mathbf{x}_s$ and $\mathbf{x}$.

$$\mathbf{r} = \mathbf{x}_s - \mathbf{x} = \|\mathbf{r}\|\hat{\mathbf{r}} \quad (3)$$

$$\mathbf{f}_s = a \cos^N\left(\frac{\psi}{2}\right) e^{-b\|\mathbf{r}\|} \hat{\mathbf{r}} \quad (4)$$

where a , b , and N were adjustable parameters. The angle ψ was defined between $\mathbf{r}$ and the orientation vector of the parent sprout $\mathbf{n}$ (Fig. 11(e)). The power cosine term dependent on ψ in this sprout force representation caused force to be directed towards a narrow region directly ahead of the sprout's growth path, and the

exponential term causes force to decrease as distances away from the sprout, localizing force around the sprout tip (Fig. 11(f)). Simultaneous two-photon (2P) imaging of vascularized constructs and the surrounding matrix has revealed that the ECM becomes highly condensed around the proximity of the sprout tip, creating a characteristic "fan shape" pattern in the ECM (Fig. 5(a)). The sprout force representation described in Eq. (4) was designed to create a similar deformation in the matrix as neovessels apply traction. Each sprout force field was mirrored across each symmetry plane in order to account for the influence of microvessels outside of the domain of the symmetry model. Additionally, this step ensures the conservation of momentum when considering the traction-free boundary conditions of the entire gel by producing equal and opposite force fields. Sprout forces were calculated at all material points within the FE mesh. Then, FEBio was used to solve for the resulting deformation field by enforcing the conservation of linear momentum under static equilibrium, during which sprout forces applied to the ECM were balanced by the divergence of the stress tensor [34]. The material behavior of the vascularized collagen constructs was represented with a composite constitutive model consisting of an ECM component and a microvessel

Fig. 9 Simulation of angiogenic microvessel fragments within a randomly-orientated collagen fibril network at Day 0, Day 3, and Day 6 of growth. The growth model, *angio3d*, uses local ECM properties to regulate neovessel elongation, branching, and anastomosis during growth.

component. Within each element of the mesh, the total Cauchy stress σ was calculated by taking the weighted average of the ECM stress response σ_{ECM} and the microvessel stress response σ_v ,

$$\sigma = \phi \sigma_v + (1 - \phi) \sigma_{ECM} \quad (5)$$

where ϕ was the fraction of the element volume that was occupied by microvessels. A hyperelastic constitutive model based on a uniform continuous fiber distribution was used to represent the

solid phase of the ECM [36]. This model captures the major features of the material properties of collagen gels, including nonlinear elasticity, tension-compression nonlinearity, and strain-dependent Poisson's ratio [36,37]. An isotropic neo-Hookean constitutive model was used to represent the stress contribution from the microvessels. The total stress within the composite was assumed to be dissipative given the large water content and viscoelastic behavior of collagen hydrogels [38,39]. Therefore, we implemented Maxwell model viscoelasticity within our constitutive model, causing deformation in response to cell-generated traction forces to become permanent [40]. The time-varying second Piola–Kirchhoff stress $S(t)$ was defined by the convolution integral

$$S(t) = \int_0^t G(t-s) \frac{dS^e}{ds} ds, \quad (6)$$

$$G(t) = e^{-\frac{t}{\tau}}, \quad (7)$$

where the elastic stress S^e is the pull-back of the Cauchy stress σ in Eq. (5), and $G(t)$ is the reduced relaxation function with a time constant τ .

Once the FE problem was solved using FEBio, the deformation field was used to update microvessel positions, ECM fibril orientation, and matrix density to the new deformed configuration. In this model, the microvessels and ECM experienced the same deformation, i.e., the deformation of the two components was compatible. Microvessel geometry was updated by interpolating nodal displacement to the microvessels using the shape functions within the mesh. ECM fibril orientation vectors were treated as free vectors and updated using the deformation gradient tensor F ,

$$\theta_j^{n+1} = F_j^n \theta_j^0 \quad (8)$$

where θ_j^{n+1} is the fibril orientation vectors for node j and time step $n+1$, θ_j^0 is the initial fibril orientation for node j , and F_j^n is the deformation gradient tensor for node j and time step n . Matrix density was updated based on conservation of mass, such that

$$\rho_j^{n+1} = J_j^n \rho_j^0 = \det F_j^n \rho_j^0 \quad (9)$$

where ρ_j^{n+1} is the matrix density for node j at time step $n+1$, J_j^n is the Jacobian for node j at time n , and ρ_j^0 is the initial matrix density at node j . The Jacobian is the determinant of the deformation gradient and provides the change in volume that occurred over the deformation.

The modeling framework was validated by simulating angiogenesis within an LAC construct and comparing predictions from the model to measurements taken during the experiments (Figs. 12 and 13) [35]. When constructing the mesh for this simulation, we took advantage of symmetry of in order to construct a 1/8th symmetry problem. Sprout forces were mirrored across the symmetry planes to account for force fields due to vessels in the unmeshed portions of the gel. During the simulation of growth, the sprout forces applied to the ECM caused the mesh to contract inward toward the center. The boundary constraint caused the mesh to deform anisotropically, resulting in a “neck” shape with the most contraction occurring at the center of the gel, the furthest distance away from the boundary condition (Figs. 12(a) and 12(b)). The shape of the deformed gel predicted by the FE mesh closely resembled the shape of LAC gels at Day 6 (Figs. 12(c) and 12(d)). Gel contraction was measured by calculating contractile engineering strain along the unconstrained axes at the geometric center of the gel (E_{yy} , E_{zz}). Predictions of gel contraction in the LAC simulations were within a single standard deviation of the experimental data (Fig. 13(a)). Microvessel alignment was measured at the geometric center of the vascularized gels in both the simulations and experiments as well. Microvessels in the LAC simulations were highly aligned along the long-axis of the gel at

Fig. 10 Our angiogenic growth model, *angio3d*, accurately simulates angiogenesis within numerous gel experiment conditions [32,33]. (a) (Top) Simulation of growth within an aligned fibril field [33]. Collagen fiber information was collection from long-axis constrained gel at Day 6 and used to seed a fibril field aligned along the constrained direction (horizontal). Since microvessels used local fibril orientation to determine the direction of growth, vessels grew along the constrained direction. (Bottom) The distribution of vessel orientation angle with respect to the horizontal demonstrates that vessels in these simulations were similar to vessels in long-axis constrained experiments. Scale bar 400 μm. (b) Angiogenesis is highly sensitive to the density of the matrix. Vascular networks grown in low density had more overall length, more branching, less free ends, and more interconnectivity compared networks grown in high density matrix [32]. Our growth model used local matrix density to determine the rate of growth and branching during angiogenesis and successfully simulated growth at different levels of matrix density (2.0, 3.0, and 4.0 mg/ml). Scale bar 350 μm.

Day 6, similar to what was seen in the gel experiments (Figs. 13(b) and 13(c)). However, alignment was not as pronounced in the simulations as seen in vitro.

These simulations included two alignment mechanisms, which occurred at different scales. At the macroscale, microvessels aligned passively due to the anisotropic contraction of the surrounding matrix. The strain tensor at the geometric center of the gel was essentially diagonal, with $E_{xx} \approx 0$ due to the effective stiffness along this direction caused by the boundary constraint and $E_{yy} \approx E_{zz}$ (Fig. 13(a)). As the matrix contracted along the y and z directions, vessels became aligned along the constrained x -axis. The second alignment mechanism occurred on the micro-scale as microvessels actively aligned along the x -axis by preferentially growing along collagen fibrils that oriented along the x -axis due to contraction of the matrix. Our modeling framework provides the unique advantage of allowing the isolation and separation of passive and active alignment mechanisms. For example, the passive alignment can be disabled while active alignment is maintained by updating ECM fibril direction via the deformation field but not applying the deformation field to microvessel geometry. In this case, simulations produced much less alignment, indicating that passive alignment due to the contraction of the gel is the predominant mechanism producing alignment in LAC cultures. Parameter sensitivity analysis revealed that this contraction was most sensitive to the parameter b , which controls the range over which sprouts can apply force and deform their local surroundings [35]. In our simulations, we were able to prescribe this parameter based on observations made using two-photon imaging of angiogenic neovessels within collagen gels, revealing that neovessels were capable of deforming the matrix up to 1000 μm away from the sprout [18]. Although our modeling framework has proven useful in uncovering mechanical mechanisms for alignment during angiogenesis, nondeterministic mechanisms were used to simulate branching and anastomosis within our modeling

framework and details into the mechanical mechanisms behind these angiogenic behaviors remain unclear. Therefore, we ventured into live imaging of the angiogenesis in order to obtain better information into the mechanical interactions between neovessels and the ECM and to study how these interactions influence these poorly understood phenomena exhibited by angiogenic neovessels.

7 Time Series Imaging

Our most recent efforts to investigate the mechanical regulation of angiogenesis involved the development of a novel imaging system that enables live visualization of angiogenic neovessels on a gigavoxel scale using two-photon 4D microscopy [41]. Live 2P imaging provides insight into the dynamics of angiogenesis by allowing direct observation of neovessel sprouting, elongation and anastomosis in real time. 2P microscopy allows us to capture the large spatial scale over which angiogenic network formation occurs while maintaining the viability of exposed angiogenic microvessels over the long time period required. Additionally, the utilization of second-harmonic generation (SHG) allows 4D imaging of the collagen matrix structure during angiogenesis as well, providing valuable information about how the matrix is deformed and remodeled during growth.

Microvessel fragments were obtained from rats expressing green fluorescent protein (GFP) and cultured in a type-I collagen gel. After 48 h, cultures were moved into an incubation chamber assembled on the stage of a 2P microscope. Perfusion tubes allowed for daily media flushing and removal. The circular gel was circumferentially constrained by a stainless steel mesh to keep the gel from floating away during culture. A $2.0 \times 2.0 \times 0.9$ mm domain at the center of the gel was imaged using mosaicking every 2 h. Autofluorescence signal from GFP was collected to image the cellular portion of the constructs, and

Fig. 11 Our growth model was coupled to the nonlinear finite element software FEBio in order to simulate neovessel growth and matrix deformation [34,35]. (a) First, angio3d simulates a growth step using fibril orientation and matrix density information in the ECM field at time step n . (b) Then, a local directional sprout force field was applied to the mesh at each active sprout location. (c) FEBio was then called to solve for the deformation using the cell-generating loading scenario. (d) Lastly, the kinematics predicted by FEBio was used to update fibril orientation and matrix density in the ECM field for the next time step $n + 1$. This step involved displacing microvessels, reorienting ECM fibrils, and updating matrix density. The next growth step takes place in this updated ECM field. (e) Schematic of sprout force representation. To calculate force at a point inside the finite element mesh, a vector r was drawn from that point to the sprout location as described in Eq. (3). The force at that location due to the sprout was calculated using Eq. (4). This sprout force field included two terms: (1) a power cosine term that depends on the angle ψ between r and the sprout direction n , functioning to direct the force field in front of the sprout and (2) an exponential term that depends on the magnitude of r and localizes force around the sprout tip. (f) Fringe plot of a local directional sprout force field found in Eq. (4) in 2D. The parameters were $a = 1.0$, $b = 1/250 \mu\text{m}^{-1}$, and $N = 2$. The force field is at its maximum directly in front of the sprout tip and force drops to zero away from the sprout.

SHG data was collected to provide information about the collagen matrix. Since there is no way of predicting the location of spontaneous sprout formation and vessel anastomosis, a large spatial domain had to be imaged. As a result, the 4D dataset for a single experiment was extremely large, consisting of several gigavoxels of volumetric data at each of the 60 time points. After processing, the 4D volumetric-temporal dataset was visualized using a workstation with a graphics processing unit and the software FluoRender [42].

Live imaging of angiogenic neovessels demonstrated that the ECM is extensively deformed and remodeled during growth. SHG data from the 4D datasets reveal that mature neovessels are surrounded in a condensed layer of collagen (Fig. 14). Additionally, collagen was condensed at the sprout tips as tip cells apply traction to the matrix (Fig. 15). The live 2P imaging also allowed us to observe many interesting neovessel behaviors that could not be seen in the previous static culture experiments. We observed neovessel growth to be episodic, meaning neovessels spontaneously switch between elongation and regression behavior (Figs. 16(a)–16(c)). Elongation typically occurred at a slower rate than regression and usually involved several filopodia within the sprout tip sampling the ECM and probing in multiple directions. It was common to see neovessels regress by up to $100 \mu\text{m}$ within a few hours, and this regression was usually followed by elongation along a new direction (Fig. 16(d)). Nearby neovessel sprouts also seemed to be able to locate one another, leading to an anastomosis

event and the expansion of the vascular network (Figs. 16(e) and 16(f)). Each of these neovessel behaviors is accompanied by condensation and remodeling of the ECM structure as seen in the SHG channel. Although still early in development, we believe that these live imaging techniques will allow us to gain further insight into the deterministic mechanisms that cause episodic growth, regression and direction change, and local neovessel location in angiogenic neovessels interacting with the ECM.

8 Discussion and Future Directions

Our research has characterized the mechanical regulation of angiogenesis. We performed these studies with a combination of experiments using an organ culture model of angiogenesis and computational simulations using an FE model with angiogenic growth coupled with matrix deformation. During angiogenesis, neovessels sprout from parent fragments, apply traction along components of the ECM, and extend into to matrix. This cell-generated traction, in combination with proteolytic MMPs, extensively deforms and remodels the matrix during growth. Neovessels use this traction to pull ECM fibrils towards the sprout, expunging water from between the fibrils and condensing the matrix. Neovessel sprouts also create new cell-matrix adhesions, remodeling the ECM structure and increasing the stiffness of the vascularized constructs. Imposing a boundary constraint during these cultures prevents the gels from contracting along the

Fig. 12 Simulation of a long-axis constrained gel using AngioFE. (a) and (b) A 1/8th symmetry model was used to simulate the vascularized construct. This figure shows the mesh viewed in the xy -plane at Day 0 (a) and Day 6 (b). The fully constrained edge of the mesh is on the right, while the geometric center of the gel where symmetry was applied is on the left. The presence of the boundary constraint causes the mesh to deform anisotropically as neovessel contract the ECM, resulting in “necking,” with the most contraction occurring at the center of the gel, the furthest distance away from the boundary condition. (c) Full geometry reconstruction of a long-axis constrained gel at Day 6 using the results from the 1/8th symmetry mesh. The shape of the deformed gel predicted by the simulation closely resembled the shape of long-axis constrained gel culture at Day 6 (d).

Fig. 13 Simulations of long-axis constrained gels using AngioFE were able to make accurate predictions of gel contraction and microvessel alignment compared to experimental data. (a) Gel contraction was measured by calculating contractile engineering strain along the unconstrained axes at the geometric center of the gel (E_{yy} , E_{zz}). Predictions of gel contraction in the long-axis constrained simulations (gray bars) were within a single standard deviation of the experimental data (black bars). (b) and (c) Microvessel alignment was measured at the geometric center of the vascularized gels in both the simulations and experiments. The distribution of microvessel alignment angles indicate that vessels were highly aligned along the long-axis of the gel in both the experiments (triangular markers) and the simulations (square markers) (c). However, alignment predicted in the simulations was not as pronounced as seen in the experiments.

Fig. 14 Live imaging of angiogenic neovessels and the extracellular matrix using two-photon microscopy. Vessels were imaged using autofluorescence from green-fluorescent protein, shown in red. The extracellular collagen matrix was imaged using second-harmonic generation, shown in green. Live imaging revealed that collagen is condensed into a layer surrounding the angiogenic microvessels, as demonstrated by the high intensity of SHG signal localized around the vessels. Scale bar 100 μm .

constrained direction. Neovessels displace with the deformation of the matrix, causing them to align along the directions of greatest effective stiffness (i.e., less contraction) and perpendicular to the directions of least stiffness (i.e., more contraction). In the LAC experiments, the matrix was constrained along the long-axis (high stiffness) and had low effective stiffness along the other two directions due to the narrow aspect ratio of the gel, causing microvessels to become aligned along the long-axis. However, in the SAC experiments, both the short- and long-axes had high effective stiffness due to the imposed boundary constraint and gel geometry, respectively. As a result, microvessels did not significantly align with respect to either of these axes. We were able to demonstrate this alignment mechanism using the computa-

tional model of angiogenesis, in which angiogenic growth was coupled to the matrix deformation using the FE method. In simulations of an LAC gel using this model, alignment developed as the mesh deformed anisotropically under cell-generated loading. Microvessels were updated by interpolating mesh displacement to the vessels, causing vessels to convect with the deformation of the mesh and align according to this deformation.

The effective stiffness and ability cells have to deform the matrix regulate angiogenesis in more ways than just determining vascular alignment. There is substantial evidence demonstrating that the angiogenic phenotype within endothelial cells is promoted by ECM deformation and remodeling. We found trends of reduced neovessels outgrowth and branching as we increased the effective stiffness of the matrix, either by imposing boundary constraints or increasing the density of the matrix. Numerous studies utilizing other various models of angiogenesis have found a similar relationship between angiogenesis and the effective stiffness of the ECM [43–46]. Additionally, sensitivity analysis using our FE model of angiogenesis suggest that the range of cell-generated traction during growth is the most important parameter controlling the deformation of the matrix and, therefore, angiogenic growth and remodeling. Analyses of our studies suggest two priority targets for utilizing the mechanical regulation of angiogenesis to influence growth: the structure and composition of the ECM and the traction forces applied by neovessel sprouts. The structure and composition, including boundary conditions, determine the stiffness of the matrix and how cell-generated forces transmit through and deform the fibrillar structure. Local matrix deformation is an important promoter of angiogenic growth at the microscale, and the structure/composition of the matrix can also act on the macroscale to determine vascular alignment in the case of the constrained gel experiments. The other potential way to regulate angiogenesis through the mechanics of growth is to change the way cells remodel the matrix, including their ability to generate force and apply traction, synthesize and release MMPs, and generate new cell-matrix adhesions. Promoting these cellular behaviors promotes matrix remodeling and growth while inhibiting these activates growth. Together, our collection of research offers the mechanical regulation of angiogenesis as another tool for engineering angiogenic growth towards desired vascular solutions, whether the intention is to promote or inhibit neovascularization.

Fig. 15 Traction applied by neovessel sprouts remodels and condenses collagen during growth. This figure shows two-photon imaging of two neovessel sprouts, indicated by the white arrows, at culture times of 98 h (a) and 108 h (b). Neovessels were imaged via autofluorescence from green-fluorescent protein, shown in red, the extracellular collagen matrix was imaged via second harmonic generation and is shown in green. Collagen was condensed at the sprouts as neovessels applied traction and deformed the matrix as indicated by the higher collagen signal around the sprouts as time increased. Additionally, this figure demonstrates how most sprouts tended to produce multiple filopodia tips, but only elongated along a single direction. Scale bar 25 μm .

Fig. 16 Live two-photon imaging reveals neovessel behavior that would be difficult to investigate using traditional static culture experiments. Neovessels imaged using autofluorescence are shown in red; collagen matrix imaged using second-harmonic generation is shown in green. (a) This figure tracks the behavior of a particular neovessel sprout, indicated by the white arrow. The sprout initially branched from a parent vessel fragment. (b) The sprout elongated and extended into the matrix, its new position indicated by the arrow. However, sprouts also exhibited regression, during which the neovessel collapsed back toward the original sprouting location. A regressed sprout can be seen in this image, indicated by the asterisk. The sprout can be viewed in the previous image prior to the regression (a). (c) Sprouts exhibited both elongation and regression, and this spontaneous switching between elongation and regression caused episodic neovessel growth. In this image, the sprout has regressed back and has begun to elongate in a new direction. (d) Regression event were usually followed by elongation along a new direction, causing the neovessel to change its direction. In this image, a second neovessel sprout appeared, indicated by the second white arrow (point to the right). (e, f) Nearby neovessels seemed able to locate one another, leading to an anastomosis event indicated by the white dashed outline (f). Each of these neovessel behaviors was accompanied by condensation and remodeling of the ECM structure as seen in the second-harmonic generation data. Scale bar 50 μm . [Supplemental material](#) to be linked here April 2014 for video file of time series data.

There are still aspects of the role of mechanics during angiogenesis that remain poorly understood. For example, our growth model represented neovessel branching as a random process, as the deterministic mechanical mechanisms for branching remain unclear. It is difficult to gain significant insight into the mechanical regulation of this dynamic process using traditional experimental techniques. However, the new methodology for live 2P imaging provides the opportunity to investigate the dynamics of angiogenic growth and the associated matrix remodeling in ways not permitted using static culture techniques. Live 2P imaging has also revealed numerous neovessel behaviors during angiogenesis that are poorly understood such as episodic growth/regression, neovessel colocation, and anastomosis. SHG data reveals significant matrix deformation and remodeling during these events, but the mechanisms regulating these behaviors is unknown. The next step in our research efforts will be to perform new experiments using live 2P imaging and expand our existing computational framework in order to provide details into the mechanical regula-

tion of these complex aspects of angiogenesis that occur through the interaction between neovessels and the ECM.

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