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Invited Review

Formation of Microvascular Networks: Role of Stromal Interactions Directing Angiogenic Growth

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ABSTRACT

In the adult, angiogenesis leads to an expanded microvascular network as new vessel segments are added to an existing microcirculation. Necessarily, growing neovessels must navigate through tissue stroma as they locate and grow toward other vessel elements. We have a growing body of evidence demonstrating that angiogenic neovessels reciprocally interact with the interstitial matrix of the stroma resulting in directed neovascular growth during angiogenesis. Given the compliance and the viscoelastic properties of collagen, neovessel guidance by the stroma is likely due to compressive strain transverse to the direction of primary tensile forces present during active tissue deformation. Similar stromal strains control the final network topology of the new microcirculation, including the distribution of arterioles, capillaries, and venules. In this case, stromal-derived stimuli must be present during the post-angiogenesis remodeling and maturation phases of neovascularization to have this effect. Interestingly, the preexisting organization of vessels prior to the start of angiogenesis has no lasting influence on the final, new network architecture. Combined, the evidence describes interplay between angiogenic neovessels and stroma that is important in directed neovessel growth and invasion. This dynamic is also likely a mechanism by which global tissue forces influence vascular form and function.

Key words: angiogenesis, stroma, matrix, neovessel, remodeling

Abbreviation used: MMP, matrix metalloproteinase; SHG, second-harmonic generation; 3D, three-dimensional.

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INTRODUCTION

A number of challenges to tissue homeostasis result in the expansion of the microvasculature within the affected tissue. Either as an adaptation to long-term physiological changes or in response to pathological insult, vascular beds in affected tissues often expand, leading to increases in vessel density and changes in network topology. For example, in chronic exercise, skeletal muscle vasculatures expand to increase blood perfusion volumes and capillary surface area for exchange to accommodate new metabolic demands associated with elevated muscle workloads [12,13]. Following ischemic injury, expansion of the vascular beds adjacent to the ischemic zone provides additional blood flow to the insulted area to relieve the ischemia and to facilitate tissue repair [2,34,44]. However, uncoupling of new vasculature formation from tissue needs and function leads to nonhomeostatic changes, thereby exacerbating dysfunction and creating disease [5,17,46].

Effective expansion of a vasculature involves a complex interplay of vessel growth (i.e., angiogenesis) and vascular remodeling/adaptation that, in homeostatic processes, results in a vascular tree with a larger vascular volume and surface area that still retains effective perfusion potential [30]. Intimately coupled with remodeling processes such as arteriogenesis [51] and vessel diameter adaptation [45], angiogenesis, either by splitting or sprouting processes, is the primary means by which new vessel segments are added to the vascular tree. Decades of research have uncovered a vast array of cellular and molecular mechanisms underlying endothelial and perivascular cell activities related to angiogenesis, including recent discoveries of molecules orchestrating neovessel sprouting and elongation (see [1,6,9,12,16]). Despite these significant advances in our understanding of angiogenesis (sprouting angiogenesis in particular), much is still to be learned. One such area relates to the interplay between the intact, growing neovessel and the surrounding, 3-D tissue environment and how bulk tissue dynamics influences larger scale angiogenic behavior.

During angiogenesis, growing neovessels must move through the tissue stroma, navigating around and between parenchymal cells. Furthermore, to effectively integrate into

the existing vascular network, the growing neovessel must be able to locate and extend toward other vessels (likely other growing neovessels rather than existing, mature vessels). How these neovessels navigate through this three-dimensional stromal space is not clear, especially in the context of the adult tissue where there is a paucity of "global" patterning cues such as those in embryo (when body and organ plans are being established). Regardless of the mechanism, the stroma is playing a central role in neovessel navigation, acting directly or indirectly to influence neovessel behavior.

STROMA

While each tissue bed exhibits a specific and unique composition, most tissues generally contain some combination of parenchymal cells embedded within a stromal environment. This stroma is comprised of an extracellular matrix within which reside tissue support cells and the microvasculature. In general, the matrix environment is an interstitial gel consisting of collagens, glycosaminoglycans, and hyalurons all of which establish a fluid-rich, 3-D environment [18,31,35]. Matrix molecules also assemble into fibrils/fibers, which are integrated within and around the matrix gel creating a complex biochemical and mechanical milieu [29,31]. A key feature of most (if not all) stromal matrices is the viscoelastic behavior exhibited when mechanically loaded [28,39,42]. Acting simultaneously with the stretch and relaxation of the elastic elements of the matrix (typically the fibrous components) is a viscous drag created by the water trapped within the gel matrix. Thus, the stroma generally will undergo time-dependent, nonlinear changes in strain (e.g., change shape) when a stress is applied (e.g., as in stretching). This time-dependent dynamic is important for a number of tissue responses and functions [32,36,48]. Also, as will be discussed later, the viscoelasticity of the stromal matrix impacts angiogenesis and neovessel guidance.

As mentioned, embedded within the stromal matrix are a variety of tissue cell types. Generally thought to play critical roles in supporting parenchyma and tissue homeostasis, these cells synthesize and remodel matrix, and interact with parenchyma and the microvasculature (via paracrine and juxtacrine processes) to integrate tissue function [3,7,22,35,37]. While the stromal cells within a given tissue may have phenotypes unique to that tissue, the collection of stromal cell types across the many tissues in the body include fibroblasts, perivascular cells, mesenchymal pluripotent cells, and immune cells. No one cell appears to be more important in tissue health and function than another, although all are necessary as deficits in any one cell type may lead to dysfunction and disease. While virtually all cells synthesize and deposit matrix, the fibroblast has long been considered the primary driver cell type responsible for much of the stromal matrix deposition and remodeling, particularly in fibrotic diseases [23,49]. Only recently have the roles of other mesenchymal and immune cells in matrix deposition been uncovered [10,21,38,40,41]. Similarly, the stromal cells influence angiogenesis and vascular remodeling. Virtually all cells secrete factors that directly or indirectly regulate vascular cell activity and angiogenesis. Recently, there has been a renewed emphasis in the role the tissue macrophage plays in regulating angiogenesis either through the production of angiogenic factors and chemokines [19,33,43] or through direct interaction with the angiogenic vascular elements [14].

As an integrated complex of viscoelastic matrix and various cell types, the stroma creates a dynamic environment entailing a vast array of biochemical/biomolecular stimuli and biomechanical influences. These input signals to both the parenchymal and stromal cells are the means by which tissue function and health are performed and maintained. It is in this complex environment that angiogenesis occurs, during which growing neovessels must integrate with each other to effectively expand the tissue microcirculation.

NEOVESSEL NAVIGATION DURING SPROUTING ANGIOGENESIS

Our understanding of the processes underlying the formation of a new angiogenic sprout from a parent microvessel and its growth have been well-described. Briefly, following an angiogenic stimulus, the microvessel wall is locally remodeled concomitant with the sprouting of an endothelial cell away from the microvessel. This endothelial cell establishes the growing tip of the angiogenic sprout and serves to lead the advancement of the forming neovessel during angiogenesis. Cross talk between the tip cell and the nascent stalk cells that make up the bulk of the growing neovessel (see [9,15,16]) maintains the long aspect ratio of the neovessel, keeping growth and migration directed. Thus, the tip cell serves to navigate through the stromal environment while coordinating the organization of the lagging, proliferating stalk cells.

Recently, we have observed in detail the behavior of a sprouting neovessel as it originates from the parent microvessel, advances through the stroma, and connects with a second advancing neovessel. Over a six-day period, time-lapse, two-photon video microscopy revealed that the behavior of the tip endothelial cell is dynamic, involving the extension of numerous, short filopodia during the entire neovessel growth period (Figure 1). Coordinately, the neovessel advances, retracts, and changes direction as the filopodia extend and retract. Interestingly, the stalk of the neovessel does not uniformly advance with the tip cell. Instead, the cell bodies of stalk endothelial cells move episodically along in the neovessel, first trailing behind and

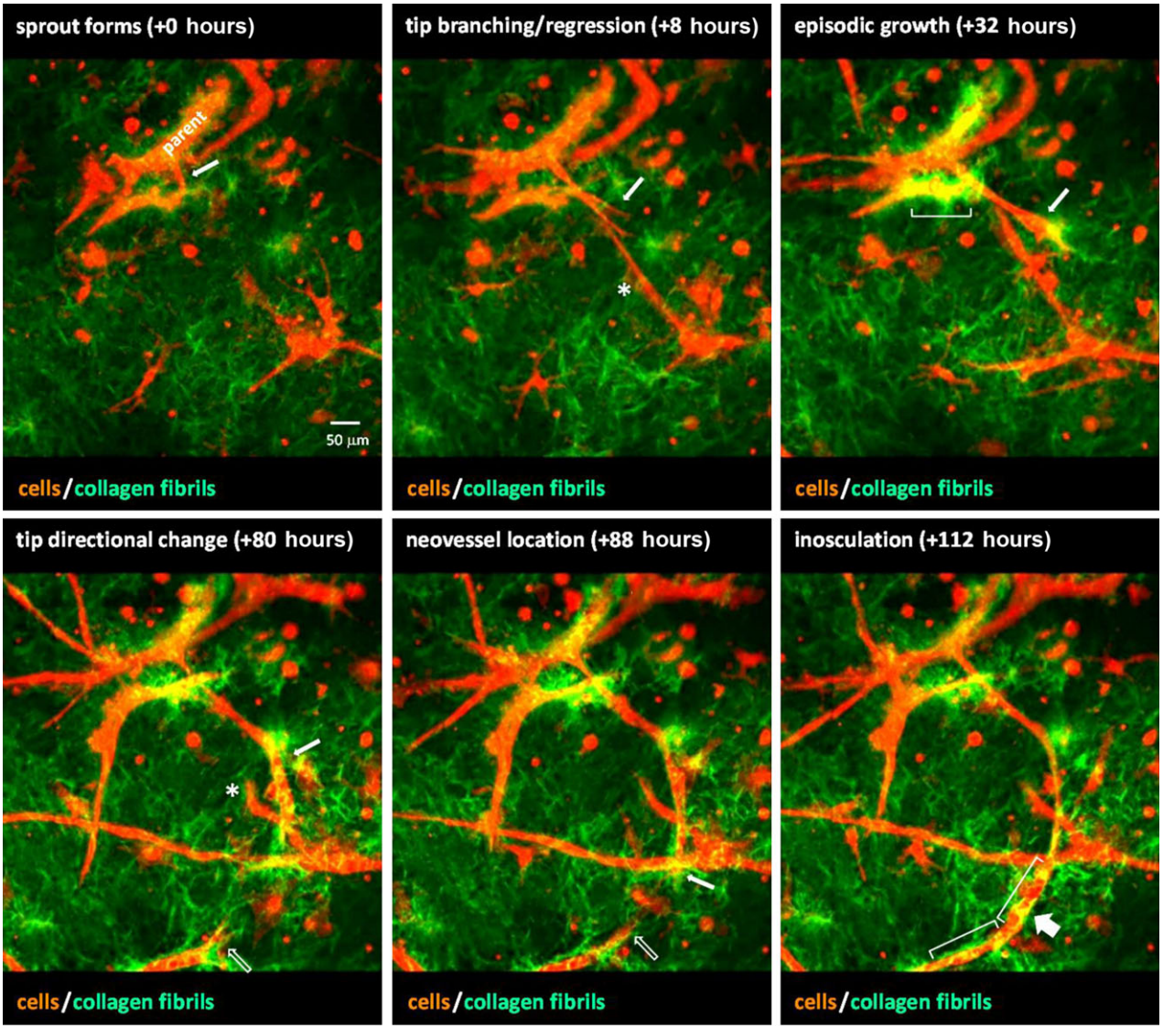

Figure 1. A sequence of still frames from time-lapse video of neovessel sprouting, growth, and inosculation within a collagen gel stroma. Microvessels (red) were imaged via confocal microscopy and collagen fibril structure (green) was visualized using SHG imaging. Over the course of ~4.5 days, a neovessel sprout (white arrow) forms, grows, changes direction to eventually inosculate (wide arrow) with a second neovessel (open arrow) that appears from out of the field of view. Brackets indicate areas of collagen condensation occurring at neovessel walls. The asterisk marks a neovessel sprout that forms and then regresses approximately three days later.

then quickly moving forward toward the tip. It is clear from the 3-D volume renderings of these time-lapse videos that growing neovessels are able to locate and advance toward other neovessels, some of which can be separated by hundreds of micrometers. Finally, collagen fibrils are actively remodeled, condensed, and deformed at the neovessel tip and along the neovessel stalk during the entire angiogenesis process. This latter observation highlights a question that our collaborative team and others have been addressing for many years related to the dynamics between the growing neovessel and the surrounding stromal matrix. Our focus has been on the role that biomechanical factors play in this dynamic. Specifically, we have been working to determine what happens to the matrix environment during angiogenesis from a stress–strain perspective and how this reciprocally influences neovessel behavior and overall microvascular outcomes.

GENERAL FORCES PRESENT DURING ANGIOGENESIS

As the neovessel advances through the stromal matrix, there is a combination of both pulling and pushing forces [50]. As shown by many experiments using isolated cells, the tip cell is

likely extending forward into the surrounding matrix, anchoring to the matrix, and then pulling itself forward [24]. Being attached to neighboring stalk cells, this effort will necessarily apply stress along the neovessel as the stalk (still attached to the parent vessel and comprised of a chain of cells) will not readily move forward with the tip cell. This stress, in part, may explain the episodic advancement of the stalk cell cytoplasm observed in the time-lapse video. It is likely that as the tip cell attempts to pull forward the adjacent stalk segment experiences tension. This tension may then pull stalk cells forward following subsequent release of downstream anchors resulting in a "sling-shot" type effect. Coordinately, there are cells within the stalk segment that are dividing, thereby providing the cellular building blocks for the growing neovessel. Given that proliferation must be constrained along the long axis of the neovessel during angiogenesis (otherwise the vessel would grow outward in diameter as opposed to lengthwise growth), the two daughter cells formed by cell division now occupy the space previously filled by the one parent cell. Either these two daughter cells are compressed and become smaller or they push outward along the neovessel length to create new space. These outward forces would then act to push forward the portion of the neovessel distal to the cell division event (conversely the stalk segment proximal to the cell division would be compressed against the parent microvessel). It is intriguing to think that perhaps the release event contributing to the rapid, episodic forward movement of cell bodies while under stress may be the result of a proliferative event and the addition of a new cell to the stalk, which is pulled forward by the tip before establishing new substrate anchors.

ANGIOGENESIS MODIFIES THE MATERIAL PROPERTIES OF THE STROMA

Regardless of the types of stresses generated by the neovessel, it is clear that the neovessel mechanically remodels the surrounding stromal matrix. Using a model of sprouting angiogenesis in which neovessels grow from isolated, intact microvessel segments within type I collagen gels, our collaborative team has shown that the overall stroma harboring the angiogenesis event becomes softer initially, and then later stiffer, as angiogenesis proceeds [26]. Initially, preceding angiogenesis, the simple stroma (microvessels + collagen) is slightly less stiff (more compliant) than collagen gels alone. The stroma becomes even less stiff early following angiogenic sprouting from the parent microvessels. However, in the presence of actively growing neovessels, the stroma stiffens to 1.5× that of empty collagen gels [26] (Figure 2). These changes are occurring as neovessel density increases. The period of lessened stiffening is associated with increased metalloproteinase activity [26]. However, MMP expression remains elevated during the entire angiogenesis period (Figure 3), indicating that MMPs are inhibited during the later stages of active neovessel growth (i.e., after the initial sprouting event) or some other process is occurring that counteracts matrix degradation to stiffen the matrix in later growth phases. While neovessel density is increasing in this system, it is unlikely that the addition of new neovessel segments to the gel is sufficient to explain the increases in stiffness observed later in angiogenesis. Neovessels contribute less than ~1% to the volume of the constructs, even in fully compacted gels (Weiss JA, personal observation). Also, inclusion of nylon fibers (as inert neovessel mimics) into collagen gels without microvessels does not appreciably change the collagen stiffness (Weiss JA, unpublished data). Interestingly, concomitant with the increase in neovessel density is the contraction of the collagen stroma. However, this contraction does not occur until the later phase when stroma stiffness increases so dramatically [26], suggesting a causal relationship. Certainly, compaction of the collagen fibrils due to the gel contraction would produce a stiffer system as fibril density increases. Empty collagen gels do not appreciably contract or change stiffness over time [28]. Yet, the addition of cytochalasin D to the microvessel system for disruption of the cytoskeleton, and, therefore, force generation by the cells, prevented gel contraction, even at high neovessel densities [52]. Thus, growing neovessels alter matrix material properties during angiogenesis via a combination of protease activity and force generation.

GROWING NEOVESSELS REORGANIZE COLLAGEN FIBRILS

The contraction of the collagen stroma by the growing neovessels suggests that the endothelial cells comprising the neovessel are engaging with the collagen and exerting force. While there are many different types of collagen, fibrous collagen (types I, II, and III) makes up the bulk of the interstitial collagen of the stroma [29,47]. Type I collagen polymerization is complex and can lead to multiple types of secondary and tertiary structure and organization [47]. Collagen is synthesized and assembled by the cell into a triple helix, which is further bundled via side-by-side interactions among many helices into fibrils which contribute to the larger fibers. Because of its highly ordered structure, the fibrils within a stroma are readily visualized via different microscopic approaches [4,53]. Using SHG and two-photon microscopy to visualize the fibrils, we determined that the interstitial collagen matrix is actively reorganized by the growing neovessel during angiogenesis [20]. This reorganization presented in two different configurations: fibril rearrangement and condensation. As the sprouting neovessel advanced away from the parent vessel out into the surrounding stroma, collagen fibrils at the leading tip were organized such that they radiated out from

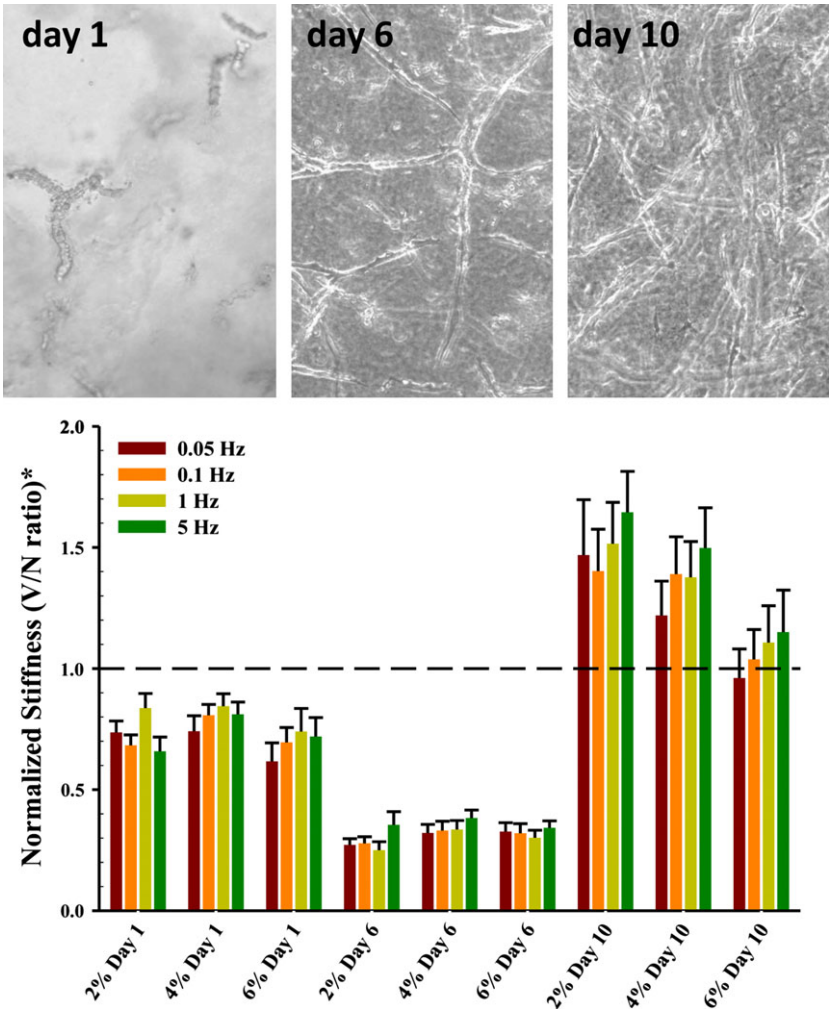

Figure 2. Phase microscopy images of neovessels growing from isolated parent microvessels in 3-D collagen gels over the course of 10 days. Given below is an analysis of the stiffness of the microvessel-collagen cultures over this same time course. Modified from [26].

the sprout and neovessel tip along the axis of neovessel growth (Figure 4). Concomitant with this nascent fibril reorganization at the sprout and neovessel tips is an SHG-bright (i.e., fibrous) layer of collagen accumulating at regions along the neovessel wall [20]. We have interpreted this condensation as a consequence of fibril recruitment and compaction by the neovessel endothelial cells; perhaps these are the fibrils first aligned by the tip cell, which are then compacted as the neovessel moves through that fibril region. However, the condensed collagen may be due to new collagen synthesis instead of or in addition to fibril recruitment. Ongoing experiments are addressing this. It is not yet clear what role fibril alignment and condensation play during neovessel growth and navigation through the stroma. While not specifically addressed in this previous study, the size of the region of fibrils influenced by the neovessel depends on the density of collagen in the matrix with smaller zones of fibril aligned at the neovessel tip in more dense collagen gels (Hoying JB, personal observation). Given that the density of collagen also influences neovessel density, length and branch points [52], it is likely that fibril alignment (and the ease at which these fibrils align) may influence neovessel guidance and growth persistence.

GROWING NEOVESSELS RESPOND TO DEFORMATION FIELDS AND NOT STRESS FIELDS

It is clear from these studies that the growing neovessel is actively remodeling the stromal matrix environment during angiogenesis: metalloproteinases mediate changes in collagen dynamic stiffness and neovessel endothelial cells pull and rearrange collagen fibrils. Together, these activities can lead to relatively large deformations of the stroma environment, unless the boundaries of the stroma are anchored [11,52]. Interestingly, the extent and direction of the stroma

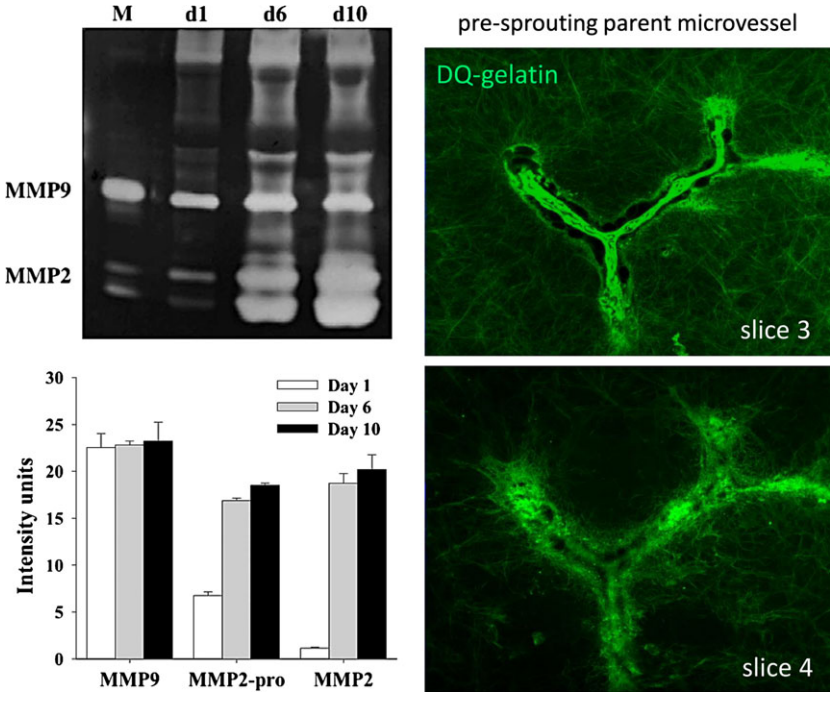

Figure 3. Expression of select MMPs by neovessels growing through collagen over the same time course as shown in Figure 2. Shown on the right are two adjacent images slices from a Z-stack of confocal images in which DQ-gelatin was used to localize gelatinase activity to a parent microvessel at a time when neovessel sprouting is just beginning. Modified from [26].

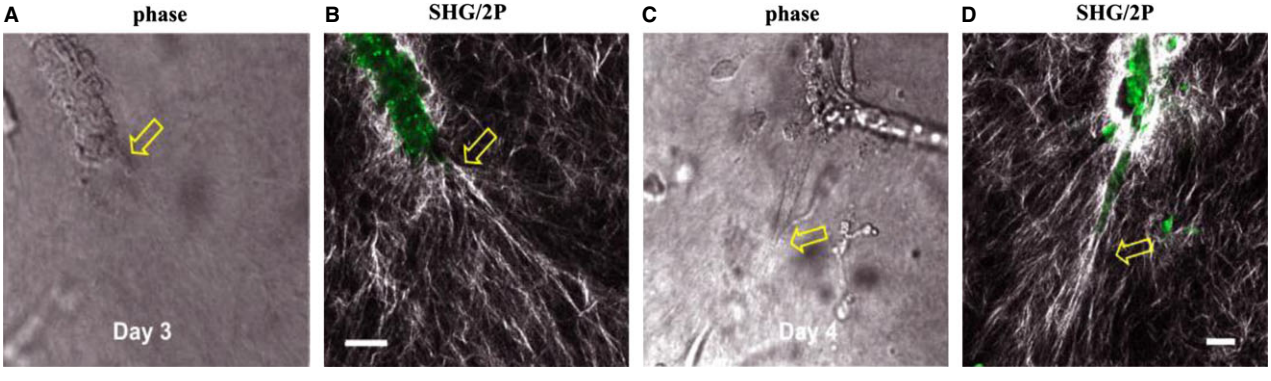

Figure 4. Two examples of collagen fibril reorientation by the tips of growing neovessels. Collagen fibrils (white) and endogenous endothelial cell fluorescence were visualized using SHG/two-photon microscopy. Yellow arrows point to new sprouts arising from the parent microvessel (green). Modified from [20].

deformation can have profound effects on the morphology and organization of neovessels [27]. Growing neovessels within round collagen gels of which all edges are anchored or free to deform uniformly randomly orient in the gel [27]. However, in a rectangular gel for which the two short sides of the gel are anchored while the two long sides are free to deform, the neovessels now orient parallel to the long axis of constraint (Figure 5). Conversely, anchoring the long sides but not the short sides has little impact on neovessel orientation (Figure 5). Importantly, unlike in the long-axis-constrained gels, little deformation occurs in these short-axis-constrained gels. Similar outcomes were observed if the gels were stretched (periodically or statically) along one axis allowing for deformation of the other axis [26]. Interestingly, in gels in which deformation did not occur, there was less angiogenesis and fewer neovessel branching [52].

Modeling of collagen and neovessel dynamics indicate that, due to the viscoelastic nature of fibrillar collagen gels [28], cellular traction forces applied by angiogenic neovessels to the collagen fibril lattice do not accumulate and are rapidly dissipated in this simple stroma system [11,52]. Experiments in which one end of the anchored

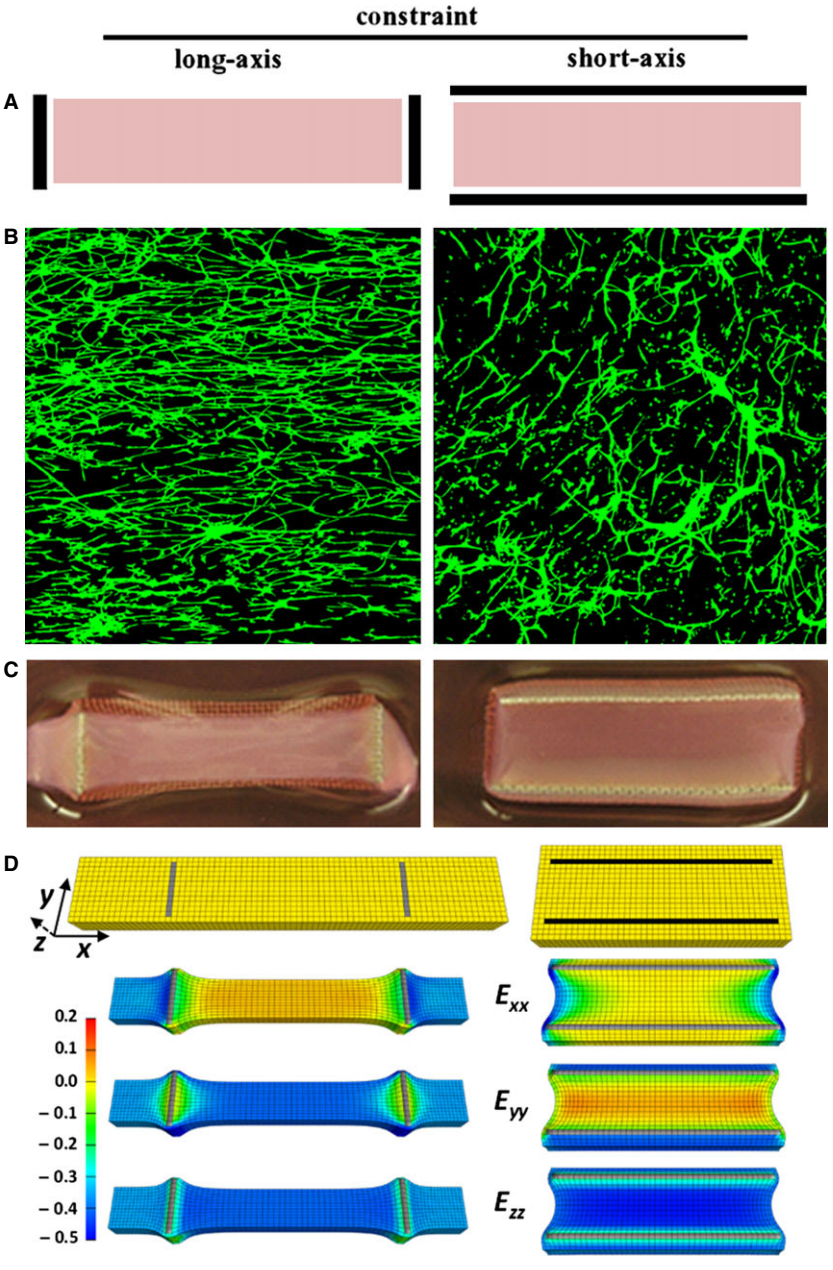

Figure 5. Neovessel alignment in 3-D angiogenesis cultures in constrained conditions. (**A**) Schematic of long-axis and short-axis-constrained gels (pink) by anchors (black bars). Gels are free to contract perpendicular to the constrained axis. (**B**) Projections of confocal image Z-stacks of neovessels (green) within these constrained cultures. (**C**) Top views of constrained cultures showing the extent and direction of gel deformation. (**D**) Finite element simulations of constrained gels showing relative strain fields in the long (E_{xx}), short (E_{yy}), and vertical (E_{zz}) axes. Scale bar indicates the magnitude of the strains, which is a ratio of $(L - L_o)/L_o$. Negative values indicate compressive strain (contraction). Modified from [11,52].

long-axis-constrained culture was released in the presence of cytochalasin indicated that there is effectively no stress on these gels that is maintained longer than seconds [52]. In addition, as mentioned, neovessels do not form an aligned network in collagen gels that are fully constrained around the edges (as in the fully constrained round and short-axis-constrained configurations described above). Finally, the density of collagen fibrils and, therefore, the compliance (or stiffness) of the collagen matrix influences the degree of matrix contraction and subsequently neovessel alignment and behavior [11]. Thus, given the absence of a persistent tension in the matrix and the coordinate changes in neovessel alignment with changes in the matrix strain field, it appears that during angiogenesis, cellular traction forces produced by growing neovessels result in stromal deformation (i.e., compressive strain), the extent of which is determined by

the effective compliance and boundary conditions of the matrix. When the boundary constraints allow directionality in the resulting strain field, neovessels align perpendicular to the primary direction of compressive strain and not along directions of tensile stress [52]. Interestingly, the collagen fibrils within the gel also align along the axis of constraint (i.e., perpendicular to the compressive strain axis) regardless of whether there are vessels present or not [52], suggesting that perhaps neovessels are following collagen fibril paths via contact guidance [25]. It is not yet clear whether fibril alignment precedes neovessel alignment or *vice versa*. But, if neovessel alignment lags behind fibril alignment, it would suggest that fibril orientation within a stromal matrix can influence neovessel guidance and orientation.

If true, perhaps then the active local reorientation of collagen fibrils by the neovessels described earlier may in fact be a means by which neovessel growth is mechanically directed. If neovessels preferentially grow along zones of parallel fibrils (as might be indicated in the constrained gels) and the neovessel tip is actively aligning fibrils in one direction (i.e., parallel), then it seems reasonable that this dynamic might maintain a persistent direction of neovessel growth, the path of which would be influenced by the deformability of the fibril lattice. How spatial gradients of growth factors likely present within the stroma influence directional behavior in the context of these guiding mechanical stimuli has yet to be determined.

MICROVASCULAR NETWORK TOPOLOGY IS ALSO INFLUENCED BY STROMAL DEFORMATION

As mentioned, angiogenesis in the adult results in the addition of new vessel segments to an existing microcirculation. Thus, the neovessels generated via angiogenesis must inosculate with other microvessels to form a provisional network consisting of new and existing microvessel segments eventually leading to a mature network of defined topology [30]. Our previous research has shown that stroma deformation influences angiogenesis outcomes with respect to neovessel orientation and character of neovessel growth. It appears that these same biomechanical stimuli can act to determine the final topology of a new microcirculation as well [8]. While constraining the long axis of a collagen gel containing angiogenic neovessels results in anisotropic neovessel alignment, removal of this constraint during progression to a mature network disrupts this organization, resulting in randomly oriented segments within the final network (Figure 6) [8]. However, maintaining the long-axis constraint during post-angiogenesis network remodeling and maturation resulted in arrays of parallel, mature microvessels within the functional microcirculation (Figure 6). Interestingly, as seen during angiogenesis, this biomechanical stimulus correlated with changes in the segment makeup of the network as the mechanically constrained networks had a higher proportion of capillaries (Figure 6). Despite this change in vessel composition, the constrained, ordered networks were as equally perfused as the unconstrained, disordered networks [8]. As observed in the other studies, there was substantial uniaxial stromal deformation associated with the parallel orientation of the vessel segments in the constrained microvascular networks and uniform deformation in the unconstrained networks.

DISCUSSION

During angiogenesis, the growing neovessel responds to a broad spectrum of inputs from the stroma and parenchyma [6]. Within the stroma, nonvascular cells produce a variety of soluble and insoluble paracrine signals modulating and directing neovessel growth. Molecular signals from the extracellular matrix similarly mediate neovessel stability, growth, and morphology. Growing evidence now demonstrates that the physical character of the stromal matrix also strongly influences neovessel dynamics. Furthermore, there is a reciprocal interplay between the neovessel and the matrix (Figure 7) such that the neovessel actively remodels the matrix, which in turn leads to matrix deformation, which in turn influences neovessel growth and morphology. Thus, the matrix, as a biomechanical environment, is not simply a passive structure that the neovessel must move through but instead is an indirect determinant of neovessel growth and navigation. We envision the following working model of how neovessel-matrix interactions contributes to neovessel navigation through the stromal environment during angiogenesis. At the single neovessel level, as the sprouting neovessel extends forward, the preceding matrix fibrils are pulled by the neovessel tip thereby aligning the fibrils in advance of the neovessel. Because a neovessel appears to preferentially grow in the direction of aligned fibrils, it will extend into this freshly aligned zone simultaneously aligning the fibrils in the next forward zone and so on. Coordinately, matrix condensation along the growing neovessel significantly reduces adjacent fibril deformability thereby retarding changes in direction without a more active remodeling event (i.e., metalloproteinase degradation). As many neovessels (tens of thousands in our experiments) are simultaneously pulling on the matrix fibrils, there is a global contraction of the stromal environment. In the absence of any boundary constraints (or complete boundary constraint), only local fibril remodeling will occur resulting in local control of neovessel navigation. However, if the stroma is not fully constrained, it will deform in bulk resulting in global changes to fibril organization. Depending on the nature of the deformation (uniaxial in our experiments), this larger fibril organization, the extent of which is determined by the matrix

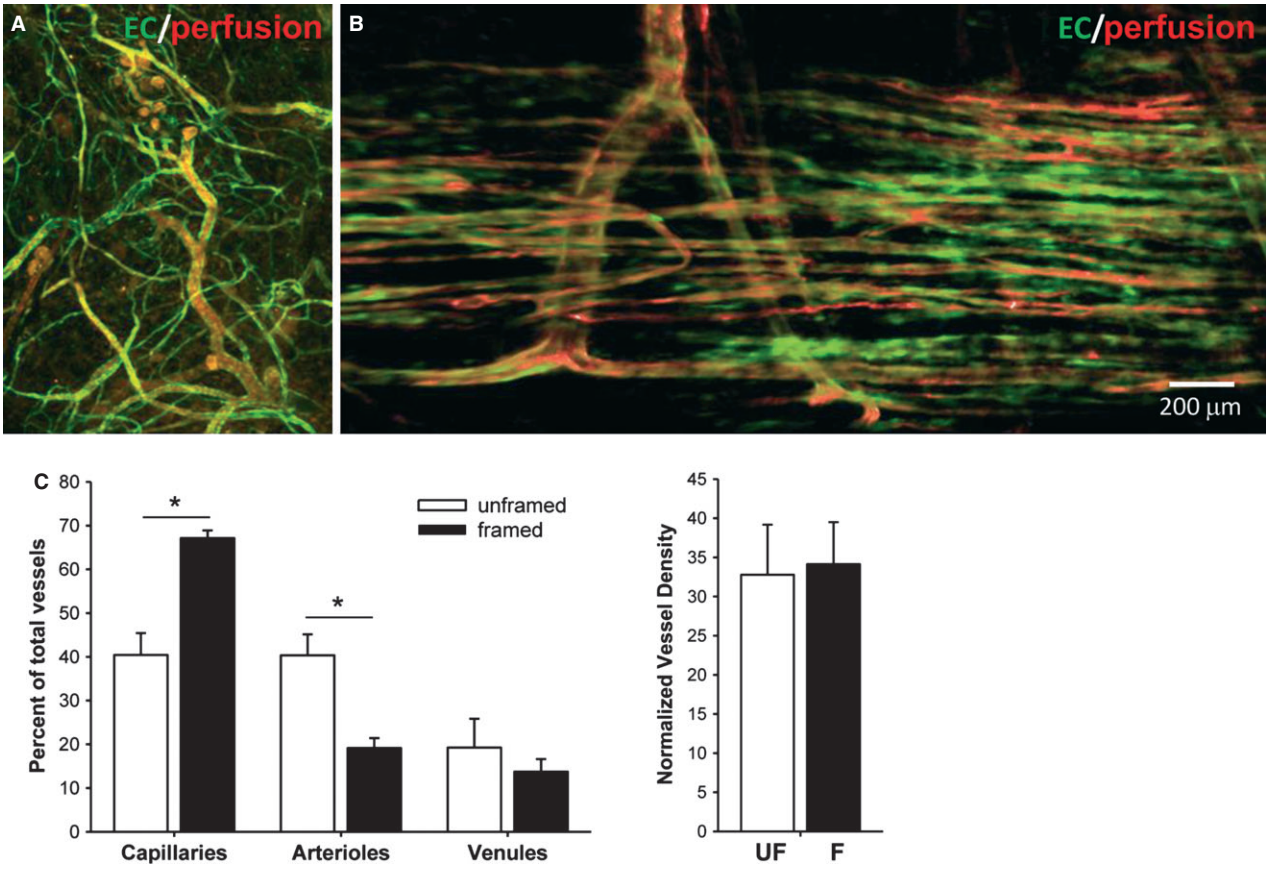

Figure 6. Architecture of and vessel type distribution in microvascular networks formed in unconstrained (**A**) or long-axis-constrained (**B**) stromal environments. The axis of constraint in (**B**) is from left to right. (**C**) Distribution of microvessel types and vessel density within the unconstrained (UF) and constrained (F) networks. Modified from [8].

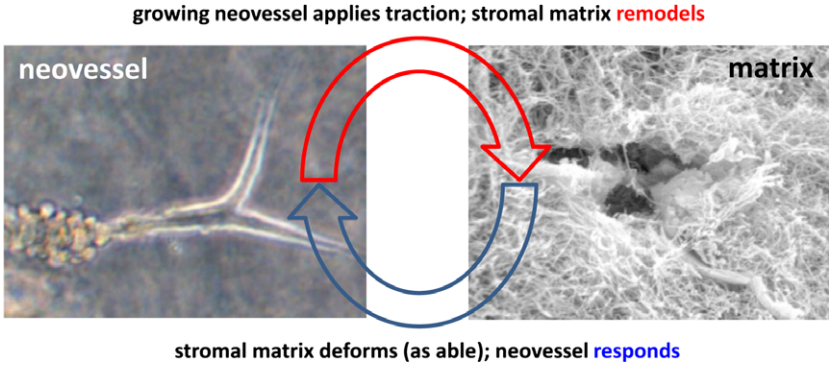

Figure 7. Schematic highlighting the interplay between the growing neovessel and the surrounding matrix structure.

deformability, will possibly establish a larger scale neovessel organization as they grow within this prestructured fibril environment. Differences in stromal compliance and physical constraints may explain, in part, how the different vascular topologies arise specific to different tissues (compare the topology of the mesentery to that of skeletal muscle).

While there are a number of implications in this model, deformability of the fibrils, which can be modulated via cross-linking, fibril density, other matrix elements, and stromal cells, is a central feature. The extent at which a neovessel can deform the fibrillar matrix, considering both the forces needed to do so, and the size of the affected fibril zone extending from the neovessel tip, determines the direction of the growing neovessel tip and, perhaps, even the generation of new branch points. In stiffer, less deformable matrices, the neovessel can align a smaller preceding

zone of fibrils effectively retarding neovessel activity. Conversely, a neovessel can create large zones of aligned fibrils in a highly deformable matrix, which might enable directional changes and/or branching. Changes to the matrix deformability via cross-linking or additional matrix elements that entangle and interconnect fibril networks would alter the deformability of matrix elements and fibril networks, which would in turn regulate neovessel growth direction and character. This may explain why neovessel activity is limited in collagen gels prepared with higher concentrations of collagen and therefore higher densities of fibrils [52]. Similarly, some tissues have specifically organized vasculatures, which may reflect, in part, the composition of the stromal matrix and, therefore, the matrix deformability.

It is difficult to resist speculating on the role matrix (and specifically fibril) deformation might play in neovessel navigation. It is generally considered that neovessels grow toward higher sources of angiogenic factors or away from repulsive factors [1]. However, growth factor gradients alone do not explain how one active neovessel is able to locate and grow toward another active neovessel resulting in the inosculation between the two neovessels. Besides the fact that two neovessels might incidentally grow toward each other as they grow to the top of a growth factor gradient, similar gradients do not adequately explain how the two neovessels can locate each other so effectively (as we see in the time-lapse video of angiogenesis). If, for example, the tip cell was to generate a paracrine gradient to attract a nearby neovessel, there would need to be many different gradient molecules such that each neovessel is responding to a separate signaling gradient and not its own (Figure 8). While there may indeed be growth factor signals that direct neovessel growth over a large scale (most likely from nonvascular point sources), deformation of nascent fibrils extending from the growing neovessel tip may act to track neovessels to each other in the final stages of interconnection. In this scenario, the fibril "fan" of one neovessel might overlap with that of another, thereby creating a shared fibril track that both neovessels can follow necessarily "meeting in the middle" (Figure 8). An implication is that larger zones of fibril alignment in front of neovessels, perhaps due to greater matrix deformability, would enable more zones to overlap thereby promoting more neovessel connections. Indeed, in our angiogenesis experiments, less stiff, more deformable collagen gels do indeed contain more interconnected (measured as branch points) neovessels.

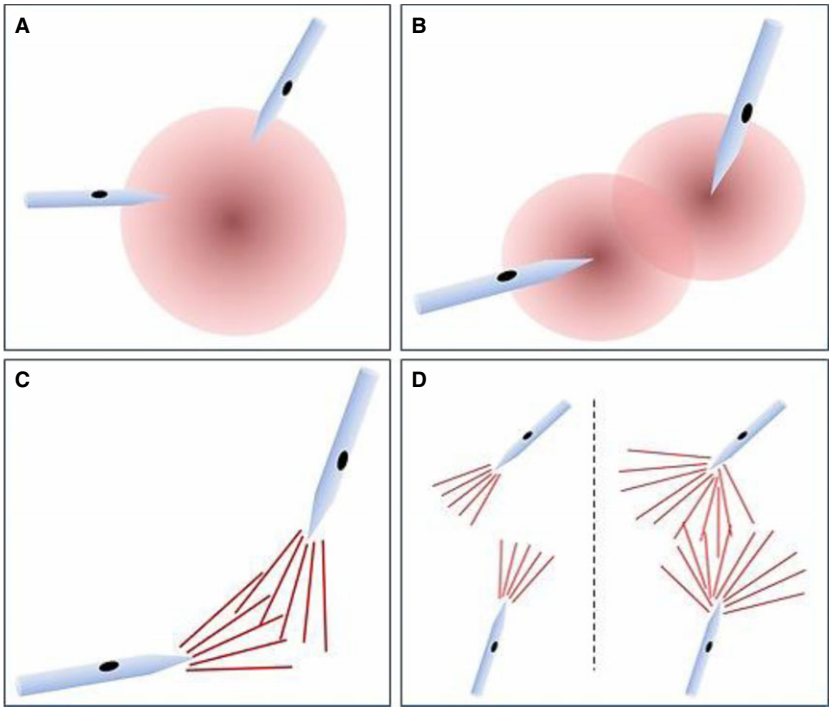

Figure 8. Diagrams of different means by which growing neovessels might locate each other. (**A**) An extravascular source of diffusible signal (graded pink) causes two neovessels (blue) to grow up the signal gradient incidentally approaching each other. (**B**) The tips of each growing neovessel produce a diffusible signal that forms a gradient the other neovessel recognizes and grows into. However, for this to occur, the signal from each neovessel would need to be a different molecule. (**C**) The "fan" of aligned matrix fibrils (red lines) that forms in front of each growing neovessel would act to "track" the neovessels toward each other when overlapping. (**D**) Consequences to neovessel location due to differences in the size of the fibril–alignment zone. A small fibril–alignment zone (due to stiffer matrix) would not readily overlap, while a larger zone (due to a less stiff matrix) would make overlapping of these tracks between neighboring neovessels more likely.

In the adult, the growing neovessel in angiogenesis navigates through the complex tissue space moving through the stroma between parenchymal compartments. Clearly, there is a large spectrum of diffusible and matrix-bound molecular signals critical to neovessel behavior. In addition, much is known concerning the intra- and extracellular force dynamics underlying endothelial cell interactions with the matrix related to angiogenic neovessel activity. Adding to this broad understanding, our recent body of evidence indicates that growing neovessels sense strain fields and not stress fields within the 3-D stromal space, the responses of which also influence microvascular network topology. Furthermore, the interplay between the growing neovessel and the physical behavior of the surrounding may contribute to neovessel guidance and growth direction. How these different molecular and physical stimuli are integrated to produce an effective microcirculation remains to be understood.

PERSPECTIVE

There is a dynamic interplay between growing neovessels and the surrounding stroma during angiogenesis in which growing neovessels actively remodel the matrix which in turn passively influences neovascular topology. The orientation and extent of neovessel growth is sensitive to the compressive strain or deformation within the stroma, which is influenced by the neovessels as they pull and remodel the matrix fibril structure. Differences in matrix compliance (i.e., stiffness) influences the extent of stromal deformation and therefore angiogenic neovessel organization. Thus, this back and forth dynamic between neovessels and stroma matrix may explain the different microcirculatory architectures present within the many different tissues of the body, and may be manipulated in the regeneration of vascularized tissues. In addition to the numerous molecular and cellular elements of the stroma influencing neovessel activity, it is becoming clear that the integrated physical aspects of the stromal matrix also strongly influence the growing neovessels and may act to guide neovessel growth.

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REFERENCES

1. Adams RH, Eichmann A. Axon guidance molecules in vascular patterning. *Cold Spring Harb Perspect Biol* 2: a001875, 2010.
2. Annex BH. Therapeutic angiogenesis for critical limb ischaemia. *Nat Rev Cardiol* 10: 387–396, 2013.
3. Buettner M, Pabst R, Bode U. Stromal cell heterogeneity in lymphoid organs. *Trends Immunol* 31: 80–86, 2010.
4. Campagnola PJ, Clark HA, Mohler WA, Lewis A, Loew LM. Second-harmonic imaging microscopy of living cells. *J Biomed Opt* 6: 277–286, 2001.
5. Cardinal TR, Struthers KR, Kesler TJ, Yocum MD, Kurjiaka DT, Hoying JB. (2011). Chronic hindlimb ischemia impairs functional vasodilation and vascular reactivity in mouse feed arteries. *Frontiers Physiol* 2.
6. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473: 298–307, 2011.
7. Casteilla L, Planat-Benard V, Laharrague P, Cousin B. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. *World J Stem Cells* 3: 25–33, 2011.
8. Chang CC, Krishnan L, Nunes SS, Church KH, Edgar LT, Boland ED, Weiss JA, Williams SK, Hoying JB. Determinants of microvascular network topologies in implanted neovasculatures. *Arterioscler Thromb Vasc Biol* 32: 5–14, 2012.
9. Chappell JC, Wiley DM, Bautch VL. Regulation of blood vessel sprouting. *Semin Cell Dev Biol* 22: 1005–1011, 2011.
10. Diaz-Flores L, Gutierrez R, Madrid JF, Varela H, Valladares F, Acosta E, Martin-Vasallo P, Diaz-Flores L Jr. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol* 24: 909–969, 2009.
11. Edgar LT, Underwood CJ, Guilkey JE, Hoying JB, Weiss JA. Extracellular matrix density regulates the rate of neovessel growth and branching in sprouting angiogenesis. *PLoS ONE* 9: e85178, 2014.
12. Egginton S, Gaffney E. Tissue capillary supply–it's quality not quantity that counts!. *Exp Physiol* 95: 971–979, 2010.
13. Egginton S, Hudlicka O, Brown MD, Walter H, Weiss JB, Bate A. Capillary growth in relation to blood flow and performance in overloaded rat skeletal muscle. *J Appl Physiol* 85: 2025–2032, 1998.
14. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, Peri F, Wilson SW, Ruhrberg C. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 116: 829–840, 2010.
15. Freitas C, Larrivee B, Eichmann A. Netrins and UNC5 receptors in angiogenesis. *Angiogenesis* 11: 23–29, 2008.
16. Gianni-Barrera R, Trani M, Reginato S, Banfi A. To sprout or to split? VEGF, Notch and vascular morphogenesis. *Biochem Soc Trans* 39: 1644–1648, 2011.
17. Gresele P, Busti C, Fierro T. Critical limb ischemia. *Intern Emerg Med* 6(Suppl. 1): 129–134, 2011.
18. Hughes CC. Endothelial-stromal interactions in angiogenesis. *Curr Opin Hematol* 15: 204–209, 2008.
19. Jetten N, Verbruggen S, Gijbels MJ, Post MJ, De Winther MP, Donners MM. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis* 17, 109–118, 2013.
20. Kirkpatrick ND, Andreou S, Hoying JB, Utzinger U. Live imaging of collagen remodeling during angiogenesis. *Am J Physiol Heart Circ Physiol* 292: H3198–H3206, 2007.
21. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med* 13: e23, 2011.
22. Koh YJ, Koh BI, Kim H, Joo HJ, Jin HK, Jeon J, Choi C, Lee DH, Chung JH, Cho CH, Park WS, Ryu JK, Suh JK, Koh GY. Stromal vascular fraction from adipose tissue forms profound vascular network through the dynamic reassembly of blood endothelial cells. *Arterioscler Thromb Vasc Biol* 31: 1141–1150, 2011.
23. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. *CMLSI* 71, 549–574, 2013.
24. Korff T, Augustin HG. Tensional forces in fibrillar extracellular matrices control direc-

tional capillary sprouting. *J Cell Sci* 112(Pt 19): 3249–3258, 1999.
25. Krishnan L, Chang CC, Nunes SS, Williams SK, Weiss JA, Hoying JB. Manipulating the microvasculature and its microenvironment. *Crit Rev Biomed Eng* 41, 91–123, 2013.
26. Krishnan L, Hoying JB, Nguyen H, Song H, Weiss JA. Interaction of angiogenic microvessels with the extracellular matrix. *Am J Physiol Heart Circ Physiol* 293: H3650–H3658, 2007.
27. Krishnan L, Underwood CJ, Maas S, Ellis BJ, Kode TC, Hoying JB, Weiss JA. Effect of mechanical boundary conditions on orientation of angiogenic microvessels. *Cardiovasc Res* 78: 324–332, 2008.
28. Krishnan L, Weiss JA, Wessman MD, Weiss JA, Hoying JB. Design and application of a test system for viscoelastic characterization of collagen gels. *Tissue Eng* 10: 241–252, 2004.
29. Kutys ML, Doyle AD, Yamada KM. Regulation of cell adhesion and migration by cell-derived matrices. *Exp Cell Res* 319: 2434–2439, 2013.
30. Leblanc AJ, Krishnan L, Sullivan CJ, Williams SK, Hoying JB. Microvascular repair: post-angiogenesis vascular dynamics. *Microcirculation* 19, 676–695, 2012.
31. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol* 3, a005058, 2011.
32. Mammoto T, Mammoto A, Ingber DE. Mechanobiology and developmental control. *Annu Rev Cell Dev Biol* 29: 27–61, 2013.
33. Maruotti N, Annese T, Cantatore FP, Ribatti D. Macrophages and angiogenesis in rheumatic diseases. *Vasc Cell* 5: 11, 2013.
34. Masaki I, Yonemitsu Y, Yamashita A, Sata S, Tanii M, Komori K, Nakagawa K, Hou X, Nagai Y, Hasegawa M, Sugimachi K, Sueishi K. Angiogenic gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. *Circ Res* 90: 966–973, 2002.
35. McGettrick HM, Butler LM, Buckley CD, Rainger GE, Nash GB. Tissue stroma as a regulator of leukocyte recruitment in inflammation. *J Leukoc Biol* 91: 385–400, 2012.
36. Mierke CT. The biomechanical properties of 3d extracellular matrices and embedded cells regulate the invasiveness of cancer cells. *Cell Biochem Biophys* 61: 217–236, 2011.
37. Molin D, Post MJ. Therapeutic angiogenesis in the heart: protect and serve. *Curr Opin Pharmacol* 7: 158–163, 2007.
38. Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 294: L152–L160, 2008.
39. Mow VC, Holmes MH, Lai WM. Fluid transport and mechanical properties of articular cartilage: a review. *J Biomech* 17: 377–394, 1984.
40. Murdaca G, Colombo BM, Cagnati P, Gulli R, Spano F, Puppo F. Endothelial dysfunction in rheumatic autoimmune diseases. *Atherosclerosis* 224: 309–317, 2012.
41. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 11: 723–737, 2011.
42. Nguyen TD, Jones RE, Boyce BL. A nonlinear anisotropic viscoelastic model for the tensile behavior of the corneal stroma. *J Biomech Eng* 130: 041020, 2008.
43. Owen JL, Mohamadzadeh M. Macrophages and chemokines as mediators of angiogenesis. *Front Physiol* 4: 159, 2013.
44. Peirce SM, Skalak TC. Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. *Microcirculation* 10: 99–111, 2003.
45. Pries AR, Cornelissen AJ, Sloot AA, Hinkeldey M, Dreher MR, Hopfner M, Dewhirst MW, Secomb TW. Structural adaptation and heterogeneity of normal and tumor microvascular networks. *PLoS Comput Biol* 5: e1000394, 2009.
46. Pries AR, Hopfner M, le Noble F, Dewhirst MW, Secomb TW. The shunt problem: control of functional shunting in normal and tumour vasculature. *Nat Rev Cancer* 10: 587–593, 2010.
47. Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 64: 403–434, 1995.
48. Ricca BL, Venugopalan G, Fletcher DA. To pull or be pulled: parsing the multiple modes of mechanotransduction. *Curr Opin Cell Biol* 25: 558–564, 2013.
49. Shimbori C, Gauldie J, Kolb M. Extracellular matrix microenvironment contributes actively to pulmonary fibrosis. *Curr Opin Pulm Med* 19: 446–452, 2013.
50. Shiu YT, Weiss JA, Hoying JB, Iwamoto MN, Joung IS, Quam CT. The role of mechanical stresses in angiogenesis. *Crit Rev Biomed Eng* 33: 431–510, 2005.
51. Troidl K, Schaper W. Arteriogenesis versus angiogenesis in peripheral artery disease. *Diabetes Metab Res Rev* 28(Suppl. 1): 27–29, 2012.
52. Underwood CJ, Edgar LT, Hoying JB, Weiss JA. Cell-generated traction forces and the resulting matrix deformation modulate microvascular alignment and growth during angiogenesis. *Am J Physiol Heart Circ Physiol* 2014. In press
53. Zoumi A, Yeh A, Tromberg BJ. Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. *Proc Natl Acad Sci U S A* 99: 11014–11019, 2002.

