Published in final edited form as:

Exp Cell Res. 2013 October 1; 319(16): 2409–2417. doi:10.1016/j.yexcr.2013.06.006.

In Vitro Models of Angiogenesis and Vasculogenesis in Fibrin Gel

Kristen T. Morin¹ and Robert T. Tranquillo^{1,2,*}

¹Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN

²Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN

Abstract

In vitro models of endothelial assembly into microvessels are useful for the study of angiogenesis and vasculogenesis. In addition, such models may be used to provide the microvasculature required to sustain engineered tissues. A large range of *in vitro* models of both angiogenesis and vasculogenesis have utilized fibrin gel as a scaffold. Although fibrin gel is conducive to endothelial assembly, its ultrastructure varies substantially based on the gel formulation and gelation conditions, making it challenging to compare between models. This work reviews existing models of endothelial assembly in fibrin gel and posits that differences between models are partially caused by microstructural differences in fibrin gel.

Introduction

The assembly of endothelial cells (ECs) into microvascular networks is of interest for myriad reasons. Understanding of the basic biology and signaling required for such assembly provides insights into development and both normal and pathologic angiogenesis. Although *in vivo* models are highly relevant, it can be difficult to isolate specific phenomena, and animal studies can be quite expensive. Therefore, the development of *in vitro* models of endothelial assembly is extremely worthwhile. In addition to studying the mechanisms of endothelial assembly, fully developed models would enable the testing of anti-angiogenic drugs for cancer therapy, the study of leukocyte-vascular interactions, and, potentially, the fabrication of vascularized engineered tissues for disease treatment.

It is important to note that another method for creating models of vasculature exists besides EC assembly, which involves forming channels within the scaffold and lining them with ECs.^{1,2} Although this strategy is simple conceptually and results in a network of EC-lined tubes that can be easily connected to a flow circuit, the 3-dimensional microfabrication required makes it difficult to form small (capillary-sized) microvessels, and the microvascular patterning must be designed. In contrast, when ECs are allowed to self-assemble into vascular networks, which technically simple (in comparison to microfabrication), they form complex topologies patterned by nature with lumen sizes of the same order of magnitude as native capillaries. In addition, the mechanisms of microvascular

© 2013 Elsevier Inc. All rights reserved.

*Corresponding Author: 7-114 Nils Hasselmo Hall, 312 Church Street SE, Minneapolis, MN 55455, Phone: 612-625-6868, Fax: 612-626-6583, tranquil@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

assembly and lumen formation can only be studied by the latter models. For these reasons, models of microvasculature involving the self-assembly of ECs are preferred.

Fibrin has been long used as a matrix for the development of *in vitro* models of microvasculature. As the provisional matrix in wound healing, fibrin is naturally angiogenic, and growth factors included in the gel formulation are released over a several day period,³ stimulating endothelial assembly longer than if the factors were included in the culture medium. In addition, also likely due to its role in wound healing, fibrin promotes the cellular production of extracellular matrix (ECM) proteins including basement membrane, which is crucial for achieving stable microvascular networks, and collagen I,⁴ which is necessary for tensile strength if one is to construct engineered tissues from biopolymers.

Within endothelial assembly models, several methods exist, which are typically divided into angiogenic or vasculogenic models. Angiogenic models induce sprouting from existing monolayers of ECs, similar to sprouting angiogenesis *in vivo*. Several techniques have been used, including seeding ECs in a monolayer on a surface of the gel, entrapping EC-coated microcarrier beads within the gel, or entrapping EC spheroids, in which the outer layer of ECs behaves similarly to a monolayer, within the gel.⁵⁻⁷ Vasculogenic models begin with ECs dispersed throughout the scaffold, which then, under certain conditions, spread and associate into microvessels (linear EC structures with lumens of capillary diameter) and also interconnect to form a network; this is similar to the process of vascular formation during development.⁸⁻¹⁰ In both models, “support cells” that stabilize the microvessels are often included.¹¹⁻¹⁵ Additional details on all of these techniques are discussed below.

Many factors affect the biochemical and ultrastructural properties of a fibrin gel, which in turn affect cell behavior. This likely reflects cellular response to the varied gel mechanical properties associated with these biochemical and ultrastructural properties.¹⁶⁻¹⁸ Therefore, comparisons between experiments can be tenuous. For example, a search of Sigma-Aldrich products yields greater than 10 types of fibrinogen and greater than 10 types of thrombin, from a variety of human and animal sources. This does not include specialty fibrinogen products including plasminogen-free, ultra-pure, and others, yet alone products from other suppliers. In addition, the fibril diameter, porosity, and other ultrastructural gel properties are affected by such factors as the absolute and relative concentrations of fibrinogen and thrombin, calcium ion content, ionic strength (other than calcium), temperature, and pH during gelation. For example, Dubey and colleagues reported fibril diameters ranging from 150 to 510 nm by varying calcium ion concentration from 1.2 to 30 mM.¹⁹ Other studies have indicated that increasing the ionic strength, the thrombin concentration or the pH during gelation results in finer fibrils with a decrease in gel permeability.^{20,21} Although a decrease in temperature during gelation below room temperature affected the final gel properties, no differences were observed between gelation at 25°C and 37°C.²⁰ Additionally, the ultrastructure is affected by gel compaction, the extent and rate of which are controlled by numerous factors.¹³⁻¹⁵ Finally, any additional components of the gel including the solvents for fibrinogen and thrombin (e.g. saline solutions or basal medium) as well as cytokines, other ECM molecules, or other bioactive reagents can radically affect the behavior of the entrapped cells. Fibrin fibrils can also be aligned if fibrillogenesis occurs in the presence of a strong magnetic field,^{13,19} and fibrin gels can be stiffened by dityrosine formation using a cell-compatible photo-crosslinking method.^{22,23} All of these properties can be manipulated to study EC assembly behavior, which makes fibrin an attractive scaffold. However, the range of chemical and structural properties of fibrin gels makes it difficult to compare results across publications, even before the myriad types of ECs and support cells utilized are considered. This review will include details of each model system developed, and discern potential effects of fibrin gel ultrastructure, which may explain some of these differences.

Fibrin gel ultrastructural properties can be examined in a number of ways. Gathering data on gel turbidity is relatively simple and informative, as they can give estimates of fibril diameter.^{19-21,24} Permeability can also yield information on ultrastructure and fibril diameter, and it has been noted that thinner fibrils have much more effect on permeability than turbidity, so both measures may be important to understand.^{20,25} Confocal microscopy of gels made with fluorescently tagged fibrinogen and scanning electron microscopy can also produce visual information on fibril size, density, and topology (e.g. connectivity).^{19,21,26} It may be wise for those working with *in vitro* models of EC assembly to routinely assess the fibrin gel ultrastructure using at least one of these methods.

Endothelial Cell Types

A wide variety of EC types have been used to develop models of vascular assembly. Mature ECs have been the most commonly used, from a range of species sources including bovine, canine, porcine and human. Isolation locations include both large vessels (e.g. umbilical vein, jugular vein, aorta) and microvessels (e.g. dermis). Clearly differences in endothelial biology exist across species, but even within an individual, differences in expression patterns have been observed between arterial and venous ECs,^{27,28} and between large and small vessel ECs, even within the same organ.²⁹ Recently, human ECs have been used almost exclusively, because they are relatively easy to obtain and are the most relevant.

Human umbilical vein ECs (HUVECs) are the most widely studied human EC type, and are easily isolated and cultured, which explains their popularity. PubMed lists over 5000 publications on HUVEC isolation, phenotype, and behavior. Over half of the reported studies of endothelial assembly in fibrin gel have utilized HUVECs. Human dermal microvascular ECs (HDMECs) are the second most commonly used cell type in studies of EC assembly, also due to their ease of isolation and culture.

More recently, a variety of stem cell or blood-derived ECs have been studied. For example, several groups have used cells derived from the mononuclear fraction of cord or adult blood. These ECs are isolated via selection for CD31+ cells,³⁰ or by plating in EC-favoring medium and selecting the late outgrowth cells.^{13,31} Others have reported the isolation of EC-like cells from human blood using CD34 and CD133 as selection factors,³² but these have not as yet been used in models of EC assembly. Variation in isolation and culture methods makes it difficult to compare between cell types, but current research indicates that there are two distinct types of endothelial-like cells that can be isolated from blood, termed early and late outgrowth cells, due to the timing of their appearance in cultures of blood mononuclear cells. Early outgrowth cells appear within one week of culture, while late outgrowth cells appear after 2-3 weeks. The two cell types are distinct morphologically and proliferate at different rates (late outgrowth cells have a shorter doubling time), but both groups express a wide range of EC markers.^{33,34} However, early outgrowth cells also express hematopoietic markers and do not undergo tubulogenesis *in vitro* or incorporate into existing networks *in vivo*; the opposite is true for late outgrowth cells.³⁴ These results suggest that only late outgrowth cells are truly ECs.

Mesenchymal stem cells (MSC) exhibit an endothelial phenotype with certain stimuli, both *in vitro*³⁵⁻³⁹ and *in vivo*.^{40,41} Using vascular endothelial growth factor (VEGF) supplementation and/or endothelial growth medium, several groups have demonstrated induction of endothelial surface marker expression.^{35,37,40} Laminar shear stress has been shown to similarly induce endothelial markers,³⁸ with shear stress magnitude being critical for achieving enhanced expression.³⁶ Synergistic effects of shear stress and growth factor stimulation on the progression of endothelial differentiation have also been reported.³⁹

Recently, the differentiation of induced pluripotent stem (iPS) cells into ECs has also been reported, although these have not yet been used in EC assembly models. To induce endothelial phenotype, the iPS cells were cultured in either a cocktail of VEGF and fibroblast growth factor (FGF) or on a feeder layer of mouse fibroblasts, and then were sorted via flow cytometry to purify the population.^{42,43}

In developing models of microvasculature for the study of microvessel behavior, HUVECs and HDMECs are as useful as any other cell type. However, despite their popularity in research, HUVECs and HDMECs have limited potential to be autologous for disease treatment. Models of microvasculature using the other possible EC sources, especially circulating populations and ECs derived from MSCs, iPS cells, or other stem cells, are most promising for use in engineered tissues. However, preparing models of microvasculature using any of these cell types will require much additional research prior to clinical use, so it is as of yet unclear which cell type(s) will ultimately succeed. For example, endothelial-like cells derived from MSCs have not yet been shown to form microvascular networks; it is possible they are not truly ECs. Also, there is some recent evidence for developmental plasticity between venous and arterial ECs,⁴⁴ which provides additional impetus to continue research on HUVECs.

Support Cell Types

In vivo, capillaries are surrounded by a sparse layer of supporting cells termed pericytes (PCs). PCs are highly mobile and their processes extend to reach many ECs; primary processes extend along the capillary axis while secondary processes extend circumferentially.^{45,46} They are often located within the vascular basement membrane, and are directly connected to ECs via tight junctions.⁴⁵ During development these PCs are recruited to the forming capillaries, and this recruitment signals the end of the microvascular “plasticity window,” in which the microvessels freely grow and regress.^{46,47} ECs recruit PCs by releasing PDGF-B,⁴⁸ and PC recruitment can be blocked by knocking out PDGF or its receptor.^{48,49} The knockout phenotype includes lethality at birth due to microvascular hemorrhage and increased vessel diameter due to unchecked EC proliferation. Interestingly, however, there is no difference in vessel length or branching between knockout and wild type animals.⁴⁹ This suggests that PCs are responsible for regulating microvascular stability, permeability and diameter, but not microvessel patterning. The mechanism by which PCs have these effects is not fully known, but is thought to involve paracrine signaling from both soluble and insoluble factors. PCs release Ang-1, a vessel stabilizing protein, and in the absence of PCs, Ang-1 can improve microvessel stability, suggesting that the PC-derived Ang-1 is partially responsible for the stabilizing effects.⁵⁰ Insoluble factors include type IV collagen and laminin, among other components of the vascular basement membrane. PCs both produce and trigger ECs to produce basement membrane proteins, which are thought to be critical in the maintenance of capillary beds.⁴⁶

In vitro, it has consistently been reported that without the use of support cells microvessels will eventually regress. The support cell types used in the literature vary, including fibroblasts, smooth muscle cells, MSCs, and PCs.^{11,12,51,52} The use of these cell types has been met with varying degrees of success, but in general they tend to improve microvessel formation and/or stability. Fibroblasts and MSCs have been shown to behave similarly to PCs in that some of them become recruited to the microvascular niche and promote stability.^{11,12} Recent results suggest that the type of support cell used can drastically affect the EC networks that are formed.⁵³

Perhaps not unexpectedly, PCs excel at the role of stabilizing engineered microvessels, as shown in numerous publications from the laboratory of George Davis at the University of

Missouri as well as our laboratory. PCs co-entrapped with either HUVECs or blood-derived ECs in either type I collagen or fibrin gel recruited to the forming vessels, promoted the assembly of basement membrane, maintained microvessel diameter within the physiological range, and stabilized the networks for at least several weeks.^{14,15,52,54-56}

As with ECs, the clinical utility of a various support cell types is of concern. Lung fibroblasts and brain PCs have less potential for autologous use than dermal fibroblasts or MSCs. However, dermis may also be a source of PCs,⁵⁷ and the use of support cells derived from other types of stem cells have yet to be explored. Therefore only time will tell which support cell type(s) are the most clinically relevant.

Angiogenic Models

All angiogenic models involve the sprouting of ECs into the gel from a monolayer. However, the monolayer arrangement relative to the gel can vary: the monolayer can be formed on one of the gel surfaces, on the surface of microspheres embedded within the gel, or by the formation of EC spheroids (in which the surface of the spheroid behaves as a monolayer⁷) which are then entrapped in the gel.

The group of Victor van Hinsbergh of the Netherlands⁵ developed a method for inducing sprout formation from HDMECs seeded in a monolayer at confluence on the top surface of a fibrin gel via stimulation with a cocktail of growth factors. This system was used in several subsequent studies, one of which noted that decreasing the pH during gel polymerization created thicker fibrin fibrils and enabled greater total sprout length.^{58,59}

Volker Nehls of the University of Wurzburg in Germany⁶ developed another angiogenesis assay in which bovine pulmonary artery ECs (BPAECs) were grown to confluence on microcarrier beads and embedded in fibrin gel. Limited sprouting occurred unless fibronectin or pro-angiogenic cytokines were included in the gel formulation. Both the pH of gelation and the ionic strength affected the number of sprouts longer than 150 μm , with increases of either increasing sprout number.⁶⁰ This model was modified by Nehls and others to use other EC types, fibrin densities, and culture media (see Table 1).⁶¹⁻⁶⁷

The addition of support cell types to the microcarrier bead angiogenesis model improved sprout formation, although the level of improvement varied by support cell type. Support cell types that have been used with this model include porcine epicardial fibroblasts,⁶¹ NIH 3T3 fibroblasts,⁶¹ glioblastoma cells,⁶⁶ human dermal fibroblasts,⁶² human lung fibroblasts^{63-65,67} and human MSCs.⁶³ Recent studies have cultured gels containing HUVEC-coated microcarrier beads in endothelial growth medium (EGM)-2 (Lonza), which is supplemented with a variety of cytokines, yielding vastly increased sprout numbers and lengths.⁶²⁻⁶⁷ Despite the background cytokine levels from medium supplements and serum, support cells appear to produce chemical promoters of EC sprouting. Sprouting was observed to be reduced with increased fibrin density when fibroblasts were cultured in a monolayer on the gel surface, but this reduction was not present when the support cells were dispersed throughout the gel.⁶⁴ A reduction in sprouting was also observed as the separation distance was increased between the ECs and fibroblasts cultured on the gel surface.⁶²

Thomas Korff and Helmut Augustin of the University of Gottingen in Germany⁷ first described the culture of HUVECs as spheroids, in which the outer surface of the spheroid behaves similarly to a monolayer. They subsequently entrapped the HUVEC spheroids in fibrin gel. In the presence of VEGF or FGF-2, sprouts grew from the spheroids into the fibrin. Subsequent publications by others noted improvement in sprout lengths with the inclusion of support cells (dermal fibroblasts or preadipocytes),^{13,68} and in some cases the support cells were necessary to achieve sprouting.¹³ Additionally, the pH of fibrin gelation

drastically affected sprouting; gels polymerized at pH 6.8 induced single cell migration of ECs from spheroids, whereas gels polymerized at pH 7.0 induced sprout formation.⁶⁰

Vasculogenic Models

In contrast to angiogenic models, in which ECs sprout from existing monolayers, in vasculogenic models, ECs are entrapped in fibrin gel as single cells, which under certain conditions then associate with nearby ECs to form microvessels (linear EC structures containing lumens) that connect to form networks. In some cases, ECs will form networks in the absence of support cells, but typically support cells are required.

The groups of Ernst Reichmann of the University of Zurich in Switzerland⁸ and Dylan Edwards of the University of East Anglia in England⁹ were both able to create vasculogenic systems in which support cells were not needed. In the first case, a small number of HDMECs were entrapped in a relatively high density fibrin gel and were cultured for an extended period of time before network formation (with lumens) was observed. The second system used HUVECs entrapped in low density fibrin gels, and microvessel formation occurred within 3 days in the presence of exogenous growth factors. Although additional differences were present between the systems (see Table 2), it appears that in the absence of support cells, a longer culture time is required if a smaller initial cell number or a higher gel density is used.

Bernhard Frerich and colleagues of the University of Leipzig in Germany¹⁰ developed a vasculogenic model in which adipose stromal cells were included but were prevented from fully interacting with HUVECs because they were adherent to microcarrier beads. The role of the adipose stromal cells was unclear because the authors did not report on conditions in which they were absent; however this system represents an interesting case to explore the function of support cells.

Finally, other groups have entrapped dispersed support cells with ECs, including MSCs (Jan Stegemann/Andrew Putnam, University of Michigan¹¹), human lung fibroblasts (hLFs; Steve George, University of California at Irvine^{12,30,69}), and human brain PCs (Robert Tranquillo, University of Minnesota^{14,15}/George Davis, University of Missouri¹⁵). In all cases, a relatively short culture period was required for network formation, and the support cells co-localized with the microvessels. A variety of unique characteristics have been incorporated into these systems such as interstitial flow,^{14,69} microvessel alignment,^{14,15} and defined medium.¹⁵

Potential Effects of Gel Ultrastructure

Tables 1 and 2 highlight the wide variety of gel components and cell types that have created successful models of endothelial assembly. It is clear that the gel ultrastructure can have a substantial effect on the resulting endothelial structures from studies in which these effects were directly tested. However, it is unclear whether the differences in gel ultrastructure between models can explain the variation in cell densities or cytokine concentrations that yield successful models. Although differences in variables (e.g. EC type, fibrin gel formulation, culture medium) often preclude firm conclusions, the comparison of factors known to affect gel ultrastructure across models may nonetheless explain some of the differences observed.

Among angiogenic models, those used by Steven George/Andrew Putnam and William Broadbush (highlighted in Table 1) are similar enough to compare. Both models used 2.5 mg/ml fibrin gel in which the EGM-2 was included in the gel formulation. HUVECs were seeded on microcarrier beads, and support cells (fibroblasts or glioma cells) were seeded on

the top surface of the gel. EGM-2 with aprotinin was used for culture. The only variable known to affect gel ultrastructure that was different between the models was the thrombin concentration (0.5 vs. 1.25 U/ml). All else equal, this difference in thrombin concentration would be expected to produce a 2 fold difference in permeability (lower thrombin concentration yields higher permeability).²¹ A comparison of the results demonstrates longer sprouts (~300 μm) were present in the gels polymerized with 0.5 U/ml thrombin relative to those (~150 μm) present in the gels polymerized with 1.25 U/ml thrombin. These results could be explained by the initial permeability differences of the gels. Of course, other differences in the matrix such as fibril diameter or stiffness could also explain the effects.¹⁶

Another example of potential gel ultrastructure effects can be seen in the model used by George and Putnam described above. Ghajar et al. noted that HUVEC sprout length decreased with increasing fibrin concentration when fibroblasts were seeded on the top surface of the gel.⁶⁴ However, when the fibroblasts were instead dispersed throughout the gel, the sprout length did not vary with fibrin concentration. The authors posited that the fibroblasts produced paracrine factors that diffused more slowly in the denser gels. While this is certainly a possible explanation, another explanation is that the HUVECs needed a relatively high permeability to grow long sprouts, and that the dispersed fibroblasts degraded the fibrin,⁷⁰ effectively increasing the permeability and enabling sprout growth. These explanations are, of course, not mutually exclusive.

A final example of potential effects of gel ultrastructure is apparent in vasculogenic models. The models used by Stegemann/Putnam, George, and Tranquillo are similar in several respects: the gel formulation included cytokines, the culture medium was EGM-2, and support cells were dispersed throughout the matrix. However, different fibrinogen, thrombin and cell concentrations were optimal in each model. Although several possible explanations exist, one possibility involves the differences in initial gel ultrastructure. The model used by George involved both the highest fibrinogen concentration and the highest thrombin concentration, indicating that initial gel permeability was the lowest in this model. The high concentration of fibroblasts found to be optimal in this model may be required to increase the gel permeability via fibrin degradation.⁷⁰ Another difference between models is that a relatively low concentration of HUVECs was used in the Stegemann/Putnam model, in which the lowest thrombin concentration was also used (more than 10-fold lower than the other models). The high gel permeability obtained via the low thrombin concentration may have enabled the HUVECs to assemble into a network more easily.

Despite the ultrastructural differences in fibrin gels that are likely present between model systems, a large number of similarities exist across these systems in the mechanisms of angiogenesis and vasculogenesis. For example, vasculogenesis occurs via the same steps observed *in vivo*, including cord formation, pinocytotic vesicle formation, and vesicle fusing.⁸ Many other studies have also reported that fibroblasts support angiogenesis and vasculogenesis in fibrin via cytokine release.^{61,62,64} A final example is the importance of MT1-MMP in endothelial assembly in fibrin gels, which has been reported by several authors.^{9,63}

Conclusion

A wide variety of *in vitro* fibrin-based models of EC assembly exist, spanning both angiogenic and vasculogenic models. In addition to differences in cell types and cytokines used, the variation in fibrin gel ultrastructure that can directly result from changes in fibrinogen, thrombin or calcium concentration, ionic strength, or pH, or indirectly result from gel compaction and fibrinolysis due to the ECs and support cells, make it difficult to replicate results of existing models or compare results between models. Additional studies

are needed to directly examine these effects. Nonetheless, fibrin provides a physiological substrate that is manipulatable and conducive to EC assembly, robustly leading to angiogenesis and vasculogenesis across a range of fibrin gel properties.

Acknowledgments

This work was supported by NIH R01 HL108670 (to RTT) and American Heart Association predoctoral fellowship 11PRE7610056 (to KTM).

References

1. Leung AD, Wong KHK, Tien J. Plasma expanders stabilize human microvessels in microfluidic scaffolds. *J Biomed Mater Res A*. 2012; 100:1815–1822. [PubMed: 22489049]
2. Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A, Kermani P, Hempstead B, Fischbach-Teschl C, López JA, Stroock AD. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc Natl Acad Sci USA*. 2012; 109:9342–9347. [PubMed: 22645376]
3. Wong C, Inman E, Spaethe R, Helgersson S. Fibrin-based biomaterials to deliver human growth factors. *Thromb Haemost*. 2003; 89:573–582. [PubMed: 12624643]
4. Grassl ED, Oegema TR, Tranquillo RT. Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent. *J Biomed Mater Res*. 2002; 60:607–612. [PubMed: 11948519]
5. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, van Hinsbergh VW. Cooperative effect of TNF α , bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J Cell Biol*. 1996; 132:1177–1188. [PubMed: 8601593]
6. Nehls V, Drenckhahn D. A novel, microcarrier-based in vitro assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. *Microvasc Res*. 1995; 50:311–322.
7. Korff T, Augustin HG. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol*. 1998; 143:1341–1352. [PubMed: 9832561]
8. Montañó I, Schiestl C, Schneider J, Pontiggia L, Luginbühl J, Biedermann T, Böttcher-Haberzeth S, Braziulis E, Meuli M, Reichmann E. Formation of human capillaries in vitro: the engineering of prevascularized matrices. *Tissue Eng Part A*. 2010; 16:269–282. [PubMed: 19702510]
9. Lafleur MA, Handsley MM, Knauper V, Murphy G, Edwards DR. Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). *J Cell Sci*. 2002; 115:3427–3438. [PubMed: 12154073]
10. Frerich B, Lindemann N, Kurtz-Hoffmann J, Oertel K. In vitro model of a vascular stroma for the engineering of vascularized tissues. *Int J Oral Maxillofac Surg*. 2001; 30:414–420. [PubMed: 11720044]
11. Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. *Angiogenesis*. 2012; 15:253–264. [PubMed: 22382584]
12. Chen X, Aledia AS, Ghajar CM, Griffith CK, Putnam AJ, Hughes CC, George SC. Prevascularization of a fibrin-based tissue construct accelerates the formation of functional anastomosis with host vasculature. *Tissue Eng Part A*. 2009; 15:1363–1371. [PubMed: 18976155]
13. Morin KT, Tranquillo RT. Guided sprouting from endothelial spheroids in fibrin gels aligned by magnetic fields and cell-induced gel compaction. *Biomaterials*. 2011; 32:6111–6118. [PubMed: 21636127]
14. Morin KT, Dries-Devlin JL, Tranquillo RT. Engineered Microvessels with Strong Alignment and High Lumen Density via Cell-Induced Fibrin Gel Compaction and Interstitial Flow. Submitted.
15. Morin KT, Smith AO, Davis GE, Tranquillo RT. Aligned Human Microvessels Formed in 3D Fibrin Gel by Constraint of Gel Contraction. Submitted.

16. Mason BN, Starchenko A, Williams RM, Bonassar LJ, Reinhart-King CA. Tuning three-dimensional collagen matrix stiffness independently of collagen concentration modulates endothelial cell behavior. *Acta Biomater.* 2013; 9:4635–4644. [PubMed: 22902816]
17. Califano J, Reinhart-King CA. A balance of substrate mechanics and matrix chemistry regulates endothelial cell network assembly. *Cell Mol Bioeng.* 2008; 1:122–132.
18. Francis-Sedlak ME, Moya ML, Huang J-J, Lucas SA, Chandrasekharan N, Larson JC, Cheng M-H, Brey EM. Collagen glycation alters neovascularization in vitro and in vivo. *Microvasc Res.* 2010; 80:3–9. [PubMed: 20053366]
19. Dubey N, Letourneau PC, Tranquillo RT. Neuronal contact guidance in magnetically aligned fibrin gels: effect of variation in gel mechano-structural properties. *Biomaterials.* 2001; 22:1065–1075. [PubMed: 11352087]
20. Nair CH, Shah GA, Dhall DP. Effect of temperature, pH and ionic strength and composition on fibrin network structure and its development. *Thromb Res.* 1986; 42:809–816. [PubMed: 3726801]
21. Blombäck B, Carlsson K, Hessel B, Liljeborg A, Procyk R, Aslund N. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. *Biochim Biophys Acta.* 1989; 997:96–110. [PubMed: 2752057]
22. Syedain ZH, Bjork J, Sando L, Tranquillo RT. Controlled compaction with ruthenium-catalyzed photochemical cross-linking of fibrin-based engineered connective tissue. *Biomaterials.* 2009; 30:6695–6701. [PubMed: 19782397]
23. Bjork JW, Johnson SL, Tranquillo RT. Ruthenium-catalyzed photo cross-linking of fibrin-based engineered tissue. *Biomaterials.* 2011; 32:2479–2488. [PubMed: 21196047]
24. Carr ME Jr, Hermans J. Size and density of fibrin fibers from turbidity. *Macromolecules.* 1978; 11:46–50. [PubMed: 621951]
25. Shah GA, Ferguson IA, Dhall TZ, Dhall DP. Polydispersion in the diameter of fibers in fibrin networks: consequences on the measurement of mass-length ratio by permeability and turbidity. *Biopolymers.* 1982; 21:1037–1047. [PubMed: 7093433]
26. Lai VK, Frey CR, Kerandi AM, Lake SP, Tranquillo RT, Barocas VH. Microstructural and mechanical differences between digested collagen-fibrin co-gels and pure collagen and fibrin gels. *Acta Biomater.* 2012; 8:4031–4042. [PubMed: 22828381]
27. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell.* 1998; 93:741–753. [PubMed: 9630219]
28. Aitsebaomo J, Portbury AL, Schisler JC, Patterson C. Brothers and sisters: molecular insights into arterial-venous heterogeneity. *Circ Res.* 2008; 103:929–939. [PubMed: 18948631]
29. Gebb S, Stevens T. On lung endothelial cell heterogeneity. *Microvasc Res.* 2004; 68:1–12. [PubMed: 15219415]
30. Chen X, Aledia AS, Popson SA, Him L, Hughes CCW, George SC. Rapid anastomosis of endothelial progenitor cell-derived vessels with host vasculature is promoted by a high density of cotransplanted fibroblasts. *Tissue Eng Part A.* 2010; 16:585–594. [PubMed: 19737050]
31. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest.* 2000; 105:71–77. [PubMed: 10619863]
32. Masuda H, Asahara T. Post-natal endothelial progenitor cells for neovascularization in tissue regeneration. *Cardiovasc Res.* 2003; 58:390–398. [PubMed: 12757873]
33. Murohara T. Cord blood-derived early outgrowth endothelial progenitor cells. *Microvasc Res.* 2010; 79:174–177. [PubMed: 20085776]
34. Medina RJ, O'Neill CL, Humphreys MW, Gardiner TA, Stitt AW. Outgrowth Endothelial Cells: Characterization and Their Potential for Reversing Ischemic Retinopathy. *Invest Ophthalmol Vis Sci.* 2010; 51:5906–5913.
35. Pankajakshan D, Kansal V, Agrawal DK. In vitro differentiation of bone marrow derived porcine mesenchymal stem cells to endothelial cells. *J Tissue Eng Regen Med.* 2012
36. Kim DH, Heo S-J, Kim S-H, Shin JW, Park SH, Shin J-W. Shear stress magnitude is critical in regulating the differentiation of mesenchymal stem cells even with endothelial growth medium. *Biotechnol Lett.* 2011; 33:2351–2359. [PubMed: 21805363]

37. Cho S-W, Lim SH, Kim I-K, Hong YS, Kim S-S, Yoo KJ, Park H-Y, Jang Y, Chang BC, Choi CY, Hwang K-C, Kim B-S. Small-diameter blood vessels engineered with bone marrow-derived cells. *Ann Surg*. 2005; 241:506–515. [PubMed: 15729075]
38. Dong J, Gu Y, Li C, Wang C, Feng Z, Qiu R, Chen B, Li J, Zhang S, Wang Z, Zhang J. Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts. *Acta Pharmacol Sin*. 2009; 30:530–536. [PubMed: 19417732]
39. Fischer LJ, McIlhenny S, Tulenko T, Golesorkhi N, Zhang P, Larson R, Lombardi J, Shapiro I, DiMuzio PJ. Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. *J Surg Res*. 2009; 152:157–166. [PubMed: 19883577]
40. Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem Biophys Res Commun*. 2005; 332:370–379. [PubMed: 15896706]
41. Yue W-M, Liu W, Bi Y-W, He X-P, Sun W-Y, Pang X-Y, Gu X-H, Wang X-P. Mesenchymal stem cells differentiate into an endothelial phenotype, reduce neointimal formation, and enhance endothelial function in a rat vein grafting model. *Stem Cells Dev*. 2008; 17:785–793. [PubMed: 18522495]
42. Taura D, Sone M, Homma K, Oyamada N, Takahashi K, Tamura N, Yamanaka S, Nakao K. Induction and isolation of vascular cells from human induced pluripotent stem cells—brief report. *Arterioscler Thromb Vasc Biol*. 2009; 29:1100–1103. [PubMed: 19423866]
43. Lin B, Kim J, Li Y, Pan H, Carvajal-Vergara X, Salama G, Cheng T, Li Y, Lo CW, Yang L. High-purity enrichment of functional cardiovascular cells from human iPS cells. *Cardiovasc Res*. 2012; 95:327–335. [PubMed: 22673369]
44. Red-Horse K, Ueno H, Weissman IL, Krasnow MA. Coronary arteries form by developmental reprogramming of venous cells. *Nature*. 2010; 464:549–553. [PubMed: 20336138]
45. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol*. 2005; 7:452–464. [PubMed: 16212810]
46. Allt G, Lawrenson JG. Pericytes: cell biology and pathology. *Cells Tissues Organs*. 2001; 169:1–11. [PubMed: 11340256]
47. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development*. 1998; 125:1591–1598. [PubMed: 9521897]
48. Bjarnegård M, Enge M, Norlin J, Gustafsdottir S, Fredriksson S, Abramsson A, Takemoto M, Gustafsson E, Fässler R, Betsholtz C. Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. *Development*. 2004; 131:1847–1857. [PubMed: 15084468]
49. Hellström M, Gerhardt H, Kalén M, Li X, Eriksson U, Wolburg H, Betsholtz C. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J Cell Biol*. 2001; 153:543–553. [PubMed: 11331305]
50. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res*. 2005; 97:512–523. [PubMed: 16166562]
51. Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S, Bischoff J. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood*. 2007; 109:4761–4768. [PubMed: 17327403]
52. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. *Blood*. 2009; 114:5091–5101. [PubMed: 19822899]
53. Grainger SJ, Carrion B, Ceccarelli J, Putnam AJ. Stromal Cell Identity Influences the In Vivo Functionality of Engineered Capillary Networks Formed by Co-delivery of Endothelial Cells and Stromal Cells. *Tissue Eng Part A*. 2013
54. Sacharidou A, Koh W, Stratman AN, Mayo AM, Fisher KE, Davis GE. Endothelial lumen signaling complexes control 3D matrix-specific tubulogenesis through interdependent Cdc42- and MT1-MMP-mediated events. *Blood*. 2010; 115:5259–5269. [PubMed: 20215637]

55. Stratman AN, Schwindt AE, Malotte KM, Davis GE. Endothelial-derived PDGF-BB and HB-EGF coordinately regulate pericyte recruitment during vasculogenic tube assembly and stabilization. *Blood*. 2010; 116:4720–4730. [PubMed: 20739660]
56. Stratman AN, Davis MJ, Davis GE. VEGF and FGF prime vascular tube morphogenesis and sprouting directed by hematopoietic stem cell cytokines. *Blood*. 2011; 117:3709–3719. [PubMed: 21239704]
57. Helmbold P, Nayak RC, Marsch WC, Herman IM. Isolation and in vitro characterization of human dermal microvascular pericytes. *Microvasc Res*. 2001; 61:160–165. [PubMed: 11254395]
58. Collen A, Hanemaaijer R, Lupu F, Quax PH, van Lent N, Grimbergen J, Peters E, Koolwijk P, van Hinsbergh VW. Membrane-type matrix metalloproteinase-mediated angiogenesis in a fibrin-collagen matrix. *Blood*. 2003; 101:1810–1817. [PubMed: 12393408]
59. Collen A, Koolwijk P, Kroon M, van Hinsbergh VW. Influence of fibrin structure on the formation and maintenance of capillary-like tubules by human microvascular endothelial cells. *Angiogenesis*. 1998; 2:153–165. [PubMed: 14517471]
60. Nehls V, Herrmann R. The configuration of fibrin clots determines capillary morphogenesis and endothelial cell migration. *Microvasc Res*. 1996; 51:347–364. [PubMed: 8992233]
61. Nehls V, Herrmann R, Huhnken M, Palmethofer A. Contact-dependent inhibition of angiogenesis by cardiac fibroblasts in three-dimensional fibrin gels in vitro: implications for microvascular network remodeling and coronary collateral formation. *Cell Tissue Res*. 1998; 293:479–488. [PubMed: 9716738]
62. Griffith CK, Miller C, Sainson RC, Calvert JW, Jeon NL, Hughes CC, George SC. Diffusion limits of an in vitro thick prevascularized tissue. *Tissue Eng*. 2005; 11:257–266. [PubMed: 15738680]
63. Ghajar CM, Blevins KS, Hughes CC, George SC, Putnam AJ. Mesenchymal stem cells enhance angiogenesis in mechanically viable prevascularized tissues via early matrix metalloproteinase upregulation. *Tissue Eng*. 2006; 12:2875–2888. [PubMed: 17518656]
64. Ghajar CM, Chen X, Harris JW, Suresh V, Hughes CC, Jeon NL, Putnam AJ, George SC. The effect of matrix density on the regulation of 3-D capillary morphogenesis. *Biophys J*. 2008; 94:1930–1941. [PubMed: 17993494]
65. Griffith CK, George SC. The effect of hypoxia on in vitro prevascularization of a thick soft tissue. *Tissue Eng Part A*. 2009; 15:2423–2434. [PubMed: 19292659]
66. Chen Z, Htay A, Dos Santos W, Gillies GT, Fillmore HL, Sholley MM, Broaddus WC. In vitro angiogenesis by human umbilical vein endothelial cells (HUVEC) induced by three-dimensional co-culture with glioblastoma cells. *J Neurooncol*. 2009; 92:121–128. [PubMed: 19039523]
67. Grainger SJ, Putnam AJ. Assessing the permeability of engineered capillary networks in a 3D culture. *PLoS ONE*. 2011; 6:e22086. [PubMed: 21760956]
68. Xue L, Greisler HP. Angiogenic effect of fibroblast growth factor-1 and vascular endothelial growth factor and their synergism in a novel in vitro quantitative fibrin-based 3-dimensional angiogenesis system. *Surgery*. 2002; 132:259–267. [PubMed: 12219021]
69. Moya ML, Hsu Y-H, Lee AP, Hughes CCW, George SC. In Vitro Perfused Human Capillary Networks. *Tissue Eng Part C Methods*. 2013
70. Ye Q, Zünd G, Benedikt P, Jockenhoevel S, Hoerstrup SP, Sakyama S, Hubbell JA, Turina M. Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering. *Eur J Cardiothorac Surg*. 2000; 17:587–591. [PubMed: 10814924]

Highlights

Reviews existing *in vitro* models of angiogenesis and vasculogenesis in fibrin gel.

Notes that many differences exist between models; for example cell densities or gel formulations.

Postulates that differences in models may be attributed to ultrastructural differences in the fibrin gels.

Table 1

Details of angiogenic models. The highlighted rows are compared in the text.

Angiogenic Models											
Group	Fibrinogen	Thrombin	Other Gel Components	Ionic Strength	pH during gelation	Gel Size ¹	ECs	Support Cells	Medium	Growth Time	References
van Hinsbergh	human 2 mg/ml	undecleared 0.1 U/ml	5 U/ml Factor XIII, 2 mg/ml Na-citrate, 0.8 mg/ml NaCl, 5 µg/ml plasminogen, M199	undecleared	7.4 (7.0 yielded thicker fibrils and longer total sprout length)	300 µl - 48 well plate, 600 µl - 24 well plate	HDMECs, monolayer on top of gel	none	M199, 10% human serum, 10% NCF, 4 ng/ml TNFα, 50 ng/ml bFGF, and 100 ng/ml VEGF	8-10 days	5
Nehls	bovine 2.5 mg/ml [6] or 1.5 mg/ml [52]	bovine 0.625 U/ml	100 µg/ml fibronectin, PBS (with 200 U/ml aprotinin for [6] only)	140 mM NaCl (160 mM yielded more sprouts longer than 150 µm)	7.4 (7.6 yielded more sprouts longer than 150 µm)	1.5 ml - 35 mm dish	BPAECs, monolayer on beads	none	DMEM, 20% FCS, (with 200 U/ml aprotinin for [6] only)	6 days	6, 60
Nehls	porcine 1 mg/ml	bovine 0.625 U/ml	PBS	140 mM NaCl	7	2 ml - 35 mm dish	PAECs, monolayer on beads	Porcine epicardial 1 fibroblasts or NIH 3T3 fibroblasts (on beads)	DMEM, 1 µg/ml insulin, 1 µg/ml transferrin, 1 ng/ml selenium	9 days	61
George/Putnam	undecleared 2.5 mg/ml (higher concentrations only work with dispersed fibroblasts)	undecleared 0.5 U/ml	5% FBS, EGM-2 MV	undecleared	undecleared	500 µl - 24 well plate	HUVECs, monolayer on beads	hDFs or nhLFs (monolayer on top of gel), or hMSCs, 0.05 M/ml dispersed	EGM-2 (with 0.15 U/ml aprotinin for [54] only)	7 days	62-65, 67
Broadbuss	undecleared 2.5 mg/ml	undecleared 1.25 U/ml	EGM-2, 0.15 U/ml aprotinin	undecleared	7.4	500 µl - 24 well plate	HUVECs, monolayer on beads	human glioma cells (VEGF- producing instead of VEGF in medium)	EGM-2, 0.15 U/ml aprotinin, 3 ng/ml VEGF	5 days	66
Korff	bovine 2.5 mg/ml	bovine 1 U/ml	10% FCS, DMEM	undecleared	undecleared	24 well plate	BAECs, 750/spheroid	none	DMEM, 10% FCS, and 50 ng/ml VEGF or 30 ng/ml FGF-2	3 days	7
Greisler	human 2.5 mg/ml	human 0.32 U/ml	M199	undecleared	7.8	300 µl - 24 well plate	BAECs or CJVECs, 20,000/spheroid	none	M199, 10% FBS, 5 U/ml heparin, 1 ng/ml FGF-1, 100 KIU aprotinin	23 days	68
Tranquillo	bovine 3.3 mg/ml	bovine 1.25 U/ml	EGM-2 (no FBS), HEPES-saline	undecleared	undecleared	250 - 750 µl - slabs	HBOECs, 500/spheroid	nhDF, 0.25 M/ml	EGM-2+	7 days	13

¹ Unless otherwise noted, the gel filled the entire well or dish.

Table 2

Details of vasculogenic models. The highlighted rows are compared in the text.

Vasculogenic Models										
Group	Fibrinogen	Thrombin	Other Gel Components	Ionic Strength	pH during gelation	Gel Size ¹	ECs	Support Cells	Medium	References
Reichmann	bovine 10-11 mg/ml (other concentrations not successful)	undecleared 1 U/ml	NaCl	undecleared	undecleared	1 ml - 6 well insert	HDME Cs, 0.03 M/ml	none	EGM-2 MV	8
Edwards	human plasminogen and uPA free 2.5 mg/ml	undecleared 0.5 U/ml	basal medium purchased from supplier (no FBS)	undecleared	undecleared	300 µl -24 well plate	HUVE Cs, 1.5 M/ml	none	purchased from supplier, contained 2% FBS, added 25 ng/ml VEGF and 10 ng/ml FGF-2	9
Frerich	bovine, 0.68 mg/ml	bovine, 25 U/ml	50 ng/ml VEGF, 10 ng/ml bFGF, 20 ng/ml EGF	undecleared	undecleared	440 µl -24 well insert	HUVE Cs, 0.6 M/ml	adipose stromal cells, on beads	IMDM/HAM F12, 1% BSA, transferrin, insulin, 50 ng/ml IGF- 1	10
Stegemann/Putnam	bovine, 2.5 mg/ml	bovine, 0.1 U/ml	DMEM, 10% FBS	undecleared	undecleared	24 well plate	HUVE Cs, 0.36 M/ml	MSCs, 0.24 M/ml	EGM-2	11
George	undecleared 10 mg/ml	Undecleared 4 U/ml	EGM-2, 5% FBS	undecleared	undecleared	540 µl -24 well plate	EPCs, 1 M/ml	nhLFs, 0.2-2 M/ml	EGM-2	12, 30
Tranquillo	bovine, 2.5 mg/ml	bovine, 1.25 U/ml	M199, HEPES-saline, 200 ng/ml SCF, IL-3, SDF	undecleared	undecleared	400 µl -custom well	BOECs 2 M/ml	PCs, 0.4 M/ml	EGM-2 + 8% FBS	14
Tranquillo/Davis	bovine, plasminoge n-free, 7.5 mg/ml	bovine, 2.75 U/ml	M199, 150 ng/ml SCF, IL-3, SDF	undecleared	undecleared	18 µl - 96 well A/ 2 plate	HUVE Cs 3 M/ml	PCs, 0.6 M/ml	M199, RSII, 40 ng/ml FGF, 50 µg/ml ascorbate, 2 KIU aproitinin	15

¹ Unless otherwise noted, the gel filled the entire well or dish.