Development of a Microvascular Network in a Microfluidic Model

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ABSRACT

The development of a physiologically relevant, *in vitro* microvasculature network platform is an important goal across a variety of applications including perfusable tissue graft development, physiologically relevant drug screening platforms and the modeling of dysfunctional vasculature in disease states such as cancer and diabetes. Vascular network development and formation is enhanced through a defined extracellular matrix composition, specific combinations of growth factors and flow control in a microfluidic device. The objective for the project outlined here is develop a working model of vasculogenesis and to further the understanding of how various factors can influence the growth and development of a microvasculature network. Here, a three-channel microfluidic device was designed and optimized to provide an adequate environment to foster vessel development. A range of factors were tuned including ECM composition, endothelial cell density, fluid flow control and growth factor conditions. The importance of the work presented here is the resulting functional model of a microvasculature network that can be implemented in future studies in the Wood Lab to make platforms more physiologically relevant.

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Chapter 1

Introduction to Vasculogenesis

Section 1.1 – What is Vasculogenesis?

During human embryonic development, the cardiovascular system is the first functional organ system to develop (Patel-Hett & D'Amore, 2011). This is vital as every other organ system to sequentially develop will completely depend on the functionality of the circulatory system as it is a vast, complex network of blood vessels responsible for a wide range of functions. Every cell relies on the vasculature to permeate all tissues of the body to deliver essential nutrients and carry out vital biological processes they need to survive. Blood vessels are necessary to maintain homeostasis in the body through gas exchange, cellular and biochemical transport, temperature regulation, nutrient delivery, and waste removal (Kim et al., 2013).

The foundation of this complex system are endothelial cells, the cells that line blood vessels in a monolayer forming the endothelium (Fig. 1a) (Tronolone & Jain, 2021). Intercellular signaling promotes the growth and development of the endothelial cells into mature blood vessels. These cells can expertly adapt to various biochemical and biophysical signals from the surrounding microenvironment. For example, mechanoreceptors on the endothelial cell surface can sense the shear stress from blood flow. The endothelial cells signal this information to the surrounding cells and the blood vessel can change its wall thickness and diameter to adapt to blood flow conditions. Cells can also rapidly respond to neural signals for vessel dilation or expansion. The endothelial cells will release nitric oxide gas to relax smooth muscle cells and dilate the vessels (Lewis et al., 2002). The cardiovascular system is heavily dependent on endothelial cells for maintaining homeostasis throughout the body and for growth and repair of blood vessels.

In normal, healthy adult tissues cycles of remodeling and reconstruction often occur periodically. Endothelial cells are needed both for the growth of new vessels and the repair of existing vessels. Endothelial cells have the capacity to proliferate and migrate to these areas of the body by responding to cellular signaling produced by the tissue they are being recruited to (Lewis et al., 2002). One of the most important cellular signaling

mechanisms are the release of growth factors. While the signaling pathway is complex, the key part of vessel growth and development depends on vascular endothelial growth factor (VEGF). VEGF induces a chemotactic response from the endothelial cells (Patel-Hett & D'Amore, 2011). Once at the site of tissue repair or growth, endothelial cells can proliferate and establish themselves in the tissue through endothelium repair or the formation of new vessels.

This vessel formation and development relies partly on a process called vasculogenesis. Vasculogenesis is the self-assembly of individual endothelial cells into dynamic, functional microvascular networks. (Fig. 1b). As cells grow and develop, they are able to connect to other cells, a process known as anastomosis, to form tubular structures in a cobblestone like structure, a vascular plexus (X. Wang et al., 2018). This process involves complex and dynamic interactions between the endothelial cells and the extracellular matrix (ECM) surrounding the cells. Chemical signals released by endothelial cells and physical factors such as ECM density dictate the formation and functionality of initial tube formation and the resulting vasculature. Endothelial cell behavior is impacted by ECM composition, structure, and mechanical properties. For example, the number and size of vessels that form is directly correlated to the density of the ECM material such as collagen or fibrin (Little et al., 2000).

Traditionally vasculogenesis was understood as a process that occurred in only embryonic development, crucial in the formation of a functional vascular network. In adults, it was thought that angiogenesis was the key process in wound healing and vessel growth. Angiogenesis is similar to vasculogenesis as it involves the creation of new blood vessels, however it differs in that it encompasses the process of sprouting from an already existing parent vessel (Tronolone & Jain, 2021). However, it has recently been discovered that vasculogenesis is a main contributor to tissue repair in adults as well as in cases of tissue ischemia (Parham et al., 2014). Tissue ischemia is a common stimulus for new vascular growth. Ischemia is defined as the occurrence of a decrease in blood supply to tissue. Consequently, oxygen and nutrient supplies are restricted in the affected area. A shortage of oxygen causes an increase in the intracellular concentration of hypoxia

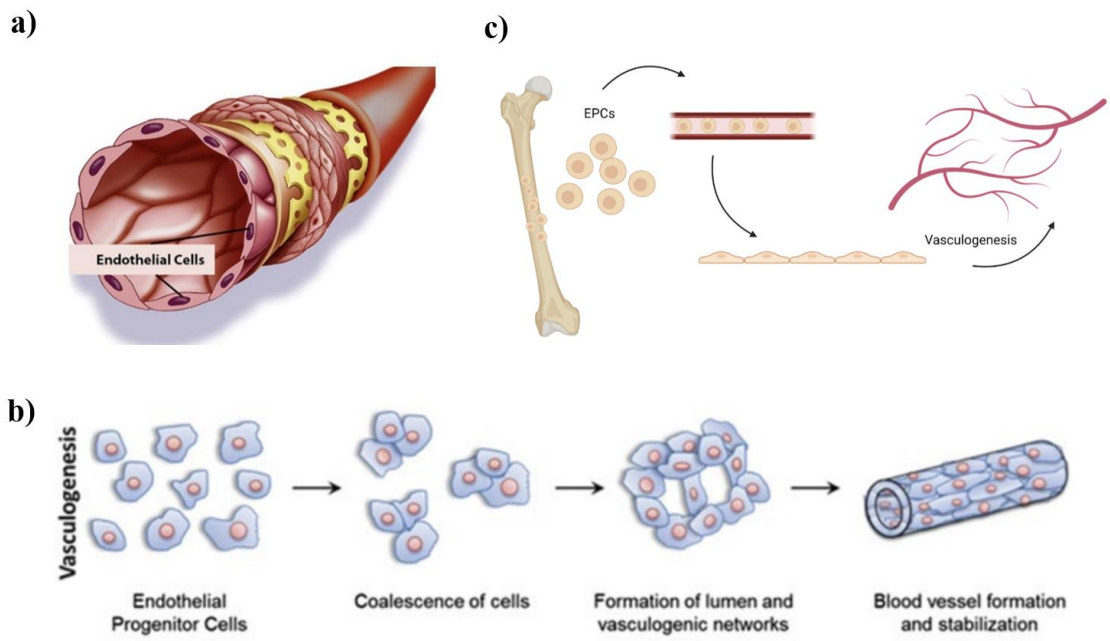

Fig. 1. a) Endothelial cells create the inner lining of blood vessels, the endothelium (Lifeline Cell Technology 2019). b) Endothelial cells self-assemble into microvasculature networks (Peak et al., 2015) c) EPCs can become mobilized from bone marrow to the site of tissue repair and undergo vasculogenesis. (Biorender).

inducible factor -1 (HIF-1). HIF-1 stimulates the production of VEGF causing an increase in VEGF production (Lewis et al., 2002). Nearby tissues respond by recruiting and incorporating endothelial cells into new functional vasculature. The recent discovery that vasculogenesis plays an important part in wound healing is supported by evidence that the local population of endothelial cells used for tissue repair is increased by endothelial cells recruited from the blood stream and bone marrow stem cell populations (Fig. 1c.) The endothelial cells are derived from bone marrow and are known as endothelial progenitor cells (EPCs) (Balaji et al., 2013).

Section 1.2 – Endothelial Progenitor Cells

Postnatal vasculogenesis is made possible by EPCs that are present circulating in the peripheral circulatory system and residing in the bone marrow. Most EPC populations are located in the stem cell portion of bone marrow and maintained in position by potent chemoattractants. During homeostatic conditions low numbers of EPCs are present in circulation. When tissue is injured or becomes ischemic, EPCs become mobilized and

are recruited to the tissue repair site directed by physical and chemical signaling (Balaji et al., 2013). Hypoxia is the most powerful physical stimuli for vasculogenesis. New vessel growth is necessary to restore oxygen delivery to the hypoxic tissue. The occurrence of ischemia causes cytokine release, an up-regulation in surface markers and the proliferation of mature endothelial cells in damaged tissues. Hypoxic conditions also cause growth factors such as VEGF, basic fibroblast growth factor (bFGF) and other chemoattractant signals to be released by cells in the damaged tissues. VEGF is the strongest chemoattractant that results from the hypoxic conditions. EPCS are able to respond to the increased VEGF signaling because they express strong angiogenic surface markers. VEGF signaling overcomes chemoattractant signaling keeping the EPCs in the bone marrow, mobilizing EPCs to damaged tissue (Spring et al., 2005). EPCs migrate through blood vessels and ECM until they arrive at the site of injury where they are activated by tissue specific chemokines. As EPCs differentiate into endothelial cells, they adhere to ECM structural components through integrin-mediated adhesion. This adhesion is accomplished by fibronectin, a multifunctional adhesive glycoprotein. In wound healing, it's vital for EPCs to be able to adhere to the ECM to effectively repair the damaged tissue (Balaji et al., 2013). The EPCs then differentiate into mature endothelial cells and undergo vasculogenesis or regulate the assembly of pre-existing endothelial cells into vascular networks (Fig. 1c).

The success of this neovascularization is dependent on the ability of the EPCs to respond to molecular cues. EPCs need to be able to interact with bone marrow, peripheral blood, blood vessels and the site of tissue injury to be able to successfully remodel the damaged tissue and restore proper function. While this process of EPCs being recruited to areas of tissue injury to undergo neovascularization has been observed, the exact mechanisms of EPC recruitment and incorporation into new blood vessels in the ischemic tissue remain unknown.

Section 1.3 – Biomechanical and Cell Specific Inducing Factors

Collagen and fibrin are the most common biomaterials used in vasculogenesis assays due to their biological relevance and their tunable mechanical properties. Collagen and fibrin are both ECM proteins that act as excellent scaffolds for cells to adhere to.

Fibrin is formed by the interaction of fibrinogen, a soluble plasma protein, and thrombin, a clotting enzyme. The structure of fibrin makes it a key material in the formation of blood clots and promoting wound healing in damaged tissues. Fibrin also has bioactive properties that recruit other cells to the tissue injury site, triggering cell adhesion. It also secretes various growth factors beneficial to vessel growth (Laurens et al., 2006). Because fibrin plays a key role in vasculogenesis in the body, its inclusion in vasculogenic models is important. While collagen is a readily available material with promising results, fibrin is used more frequently most likely due to its unique properties that highly encourage new vessel growth (Peterson et al., 2014).

The density of the ECM is an important factor in terms of vessel morphology and functionality within a microvasculature network. In a fibrin ECM a higher concentration of fibrinogen results in an increased number of branching vessels but decreased average branch diameter and length. In contrast, a lower concentration of fibrinogen present in the ECM results in fewer branching vessels with larger lengths and diameters (X. Wang et al., 2018). These same results are recapitulated in collagen ECM as well (Edgar et al., 2014). Fiber alignment has an impact as well as it affects vessel alignment due to contact guidance. Cellular traction forces can create aligned ECM fiber bundles from a cell aggregate during initial growth and development. Individual cells can even have an impact on fiber alignment and organization (Little et al., 2000). This structured ECM can then guide cell migration and the resulting shape of the microvasculature network.

Vasculogenesis depends on different biomechanical forces generated by fluid flow. The most dominant biomechanical force is shear force from blood flow. This shear stress decreases vascular permeability by enhancing barrier functions of the endothelial cells. It can also help to narrow vessel walls, improving the overall stability of the vasculature. In general, forces generated from blood flow in the body can help determine the growth and shape of a vascular network. On a cellular level, shear forces from the blood flow can directly impact the cell morphology with cells elongating and orienting themselves along the axis of flow. The migration pattern of cells is also affected by fluid shear stress. Studies have shown that both intramural shear stress and transmural flow through the endothelium above a certain threshold can activate endothelial cells to begin

sprouting into the ECM independent of other outside factors (X. Wang et al., 2018). Gene and protein expression of endothelial cells can be affected by the forces of fluid flow as well. (Nakajima & Mochizuki, 2017).

Endothelial cell density plays a critical role in vessel development as well. There needs to be a high enough cell density for cells to adequately communicate and anastomose with nearby cells to create a perfusable microvascular network. Branch length, diameter and area fraction of a vascularized region all increase with increased endothelial cell density (Whisler et al., 2012). Other mechanical properties such as pore size, gel permeability and fiber diameter and stiffness can play a role in vessel formation.

Paracrine signaling from co-culturing the endothelial cells with other types of cells such as fibroblasts contribute to vascular network stability and vessel coverage area (Tronolone & Jain, 2021)**.** Other co-cultures with stromal cells such as pericytes have found that the number of branches, average branch length, percent vascularized area and average vessel diameter can be influenced as well (X. Wang et al., 2018) It has also been reported that when endothelial cells are co-cultured with other cell types, resulting microvasculature can remain viable for longer periods of time compared to microvasculature with only endothelial cells present (Whisler et al., 2012).

Section 1.4 – Growth Factors

The presence of growth factors can have a large effect on vessel development and growth into a perfusable network. They're secreted by endothelial cells to stimulate cellular growth, proliferation, and differentiation. (X. Wang et al., 2018). The specific response of endothelial cells to growth factors can be complex as each individual growth factor has its own unique role in the formation of new vessels. For example, VEGF is the most pro-angiogenic growth factor involved in the process of new blood vessel growth. It regulates endothelial cell migration and proliferation and is important the growth of and development of endothelial cells as it regulates sprouting and tube formation of the cells. VEGF molecules interact with cell surface receptors to initiate a complex signaling pathway. A cascade of downstream proteins is activated leading to the pro-angiogenic effects of VEGF. VEGF is the most important growth factor as its necessary for normal vascular morphogenesis to occur. Multiple studies have shown that when the VEGF gene

in mice is deleted, vascular defects are prevalent as well as various cardiovascular abnormalities. (Vailhé et al., 2001).While VEGF may be the most important growth factor in vasculogenesis, other growth factors can enhance vessel development when combined with VEGF.

Spingosine-1-phosplhate (S1P) is an endothelial cell chemoattractant involved in regulating endothelial cell proliferation and cell mobility. It promotes motility and migration of endothelial cells. S1P also plays a key role in endothelial cell assembly into tubular structures and cell-cell junction formation (Takuwa et al., 2010). Phorbol 12-myristate 13-acetate (PMA) is another growth factor that primarily aides in endothelial cell proliferation and overall vascular network formation in terms of lumen formation and stabilization (van Duinen et al., 2019). PMA also enhances morphological changes in endothelial cells and increases cell motility (Tanaka et al., 2000).

Based on results from angiogenic assays, combining growth factors has a synergistic effect on the forming vasculature improving vessel density, length, diameter and overall integrity of the vessel compared to the use of single growth factors. For example, since PMA causes endothelial cells to proliferate at a higher rate, S1P can act on more cells causing greater cell motility and an overall higher rate of anastomosis. However, to induce functional, multicellular sprouting growth factor amounts need to be balanced. Too much S1P compared to PMA can lead to too much cell migration without enough cells to form fully functional, intact vessels. The overall magnitude of the amount of balanced growth factors also plays a role in angiogenesis with larger magnitudes of S1P and PMA resulting in larger vessel diameter (W. Y. Wang et al., 2020). Other angiogenic studies have shown that when PMA and S1P were used in combination with VEGF, vessel sprouting was increased compared using VEGF with either PMA or S1P alone (van Duinen et al., 2019).

While the growth factors involved in the formation of new blood vessels have many similar effects on endothelial cells, each growth factor is molecularly different and can have varying unique effects on vascular development. When used in combination growth factors can combine their pro-angiogenic potential and increase vessel development and angiogenic sprouting compared to using growth factors individually or

in partial combinations. Limited studies have been done in vasculogenic assays, but results should be recapitulated from the angiogenic studies as angiogenesis and vasculogenesis are both processes that involve vessel growth and development.

Section 1.5 Vasculogenesis in Disease States

As with any complex biological process, cellular mechanisms can get disrupted and vasculogenesis can become dysregulated. One example of this dysregulation is proliferative vessel formation in cancerous tumors. Tumors and vasculature have a dynamic relationship as tumors are highly dependent on a functional blood vessel network for a constant supply of nutrients and oxygenation as well as a mode of transportation. As tumors grow, their need for nutrients and oxygen increases enormously. To keep up with this ever-growing need, tumors work to expand the microvasculature network in the tissue they reside in by expressing pro-angiogenic factors. Overexpression of these factors leads to disorganized, tortuous blood vessel networks (Siemann, 2011). The irregular vessel shape and diameter restricts blood flow to the tumor causing hypoxic conditions which triggers the recruitment of EPCs. Tumor cells as well as surrounding endothelial cells release higher concentrations of VEGF which further promotes the migration of EPCs to the tumor site. (Dimberg et al. 2020) Higher levels of VEGF were found to correlate with the number of recruited EPCs (Balaji et al., 2013). Once at the tumor site, EPCs work to remodel the tumor environment with a chaotic, ever expanding network of microvasculature.

Research in tumor blood vessel formation via angiogenesis comprises a majority of the research in the vascularization of tumors. The study of vasculogenesis is largely neglected in this context. This is because tumors normally under-go vascularization by angiogenesis. However, when angiogenesis is inhibited, tumors actively recruit EPCs and undergo vasculogenesis. A myriad of anti-angiogenic drugs has been developed to combat tumor vascularization. While these drugs show promise, tumors become resistant to anti-angiogenic therapy with time. Since tumors can also form a blood supply from EPCs, they do not have to completely rely on angiogenesis. Vasculogenesis is the systematic backup plan for tumor vascularization. This has yet to officially be established but evidence suggests that CD11b+ myeloid cells, which are key in the vasculogenic

pathway, are responsible for resistance to some of these anti-angiogenic therapies (Brown, 2014). While less common, vasculogenesis in tumor vasculature development is still an important area of research to better understand how tumors and their vasculature work.

Tumors do not remain static, as cancer cells can metastasize all over the body. Cancer cells can break away from tumors and enter the circulatory system though the process of intravasation. Once in the circulatory system, they can travel to other regions of the body. Once a cancer cell attaches to a blood vessel wall it can extravasate out into a secondary tissue site (Fig. 2). Here the cancer cell will start to divide and multiply until another tumor is formed (Tsai et al., 2017). How successful cancer cells are at metastasizing or how well tumor growth and development can occur is dependent on how well tumor cells can recruit normal tissue cells, such as endothelial cells. It is vital to understand the complex relationships between normal tissue cells and tumor cells so we can develop more informed approaches to disease management.

Vasculogenesis is also dysregulated in diabetes through tissue ischemia. Diabetes reduces the number of EPCs and their functionality (Georgescu, 2011). Hyperglycemia and insulin resistance promote this endothelial dysregulation by blocking specific signaling pathways responsible for the mobilization, migration, and ability of EPCs to

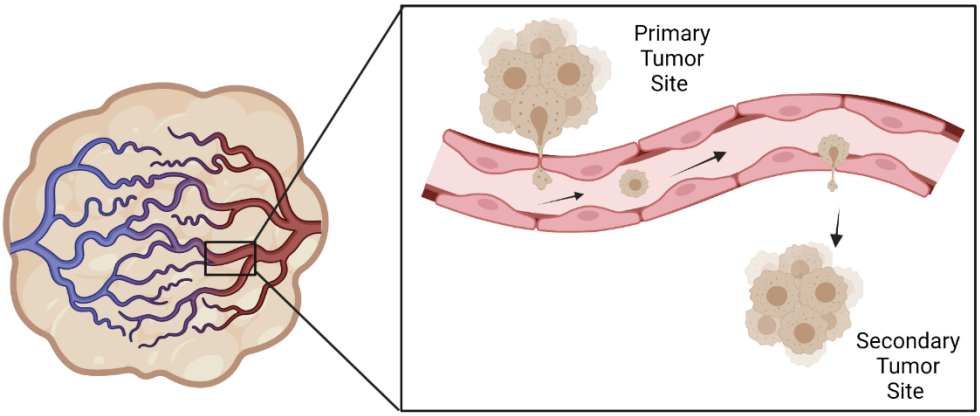

Fig. 2 A cancerous tumor with proliferative vessel dysregulation and the metastatic process of tumor cells. (Biorender).

undergo vasculogenesis. Several *in vitro* studies have shown EPC populations from diabetic patients have a reduced capacity to form tubes. This complicates the neovascularization of ischemic tissues and other damaged tissues. Endothelial dysfunction results which can lead to cardiovascular complications such as myocardial infarction, peripheral vascular disease and ischemic stroke. Vascular complications such atherosclerosis can occur as well. (Kolluru et al., 2012).

The dysregulation of vasculogenesis plays a part in a variety of disease states. In cancer vessel formation can become greatly increased while in diabetes vessel formation is severely restricted. There is a clear need to improve our understanding of vessel development in the context of the dysregulation of vasculogenesis.

Chapter 2

Current Models of Vasculogenesis

Section 2.1 Current Vasculogenic Models

Endothelial cells were first demonstrated to form capillary like structures when plated on top of a reconstituted basement membrane extracellular matrix more than 20 years ago (Arnaoutova et al., 2009). The endothelial cells attached themselves to the ECM and rapidly formed capillary like tubules over a two-dimensional surface. Since then, this tube forming assay has been used in a variety of applications including angiogenic factors, wound repair and tumor growth. This tube forming assay spurred the development of more advanced models of *in vitro,* three dimensional assays of blood vessel formation in across a wide range of applications including tissue engineering perfusable constructs, drug screening and development platforms and disease models of dysregulated vasculature.

Engineering fully-perfusable microvasculature for engineered tissue constructs is a major goal in current medical research. To ensure proper function of tissue constructs, the microvasculature needs to be fully intact. This is vital to ensure the proper diffusion of nutrients and oxygen transport throughout the tissue construct. However, development of these constructs remains limited due to the inability to fully vasculature the tissues.

(Peak et al., 2015). Vasculogenic models that can accurately recapitulate endothelial cell development into adult vessels are of great use in studying the development of engineered tissue constructs. Many groups of researchers have investigated several models and methods for producing engineered microvasculature in a variety of areas. A large area of interest is tissue constructs for vascular bypass applications to mediate various cardiovascular diseases. However, there are still limitations of these models.

Ischemic heart diseases are the leading cause of mortality and morbidity across the world resulting in 16% of the world's total deaths. (World Health Organization, 2020). Due to the huge burden of ischemic diseases, vascular conduits to reconstruct or bypass vascular occlusions are in high demand. Synthetic grafts for replacing the occluded vessels have been used since the 1950s but various complications routinely occur including low patency rates for smaller diameter vessels, increased risk of infection, repeated surgical interventions and restricted growth potential for the pediatric patients. The development of tissue engineered blood vessels (TEBV) helped to solve a lot of these problems (Song et al., 2018) . However, the fabrication methods used for larger vessels cannot be applied to the construction of microvasculature networks. These smaller vessels have lower blood flow velocities and different structural architecture requiring different design methods (Isenberg et al., 2006). For example, for larger vessels, a singular vessel is typically needed for the clinical application where its growth is precisely controlled in scaffold-based methods. When smaller vessels are needed it is typically an entire vascular network required for the application (Song et al., 2018). Developing an entire network is more challenging then just creating a new vessel due to complicated architecture and lack of precise control over spontaneous growth.

Recent methods in the creation of microvasculature networks can be categorized into two strategies. In the top-down method the geometry and architecture of the microvasculature network are pre-designed and prefabricated before the introduction of endothelial cells. The second method is a bottom-up method which relies on endothelial cells to develop into new vessels through physiological mechanisms such as vasculogenesis. Both methods are used commonly in the development of engineered cardiac tissue due to the prevalence of cardiovascular disease. However, due to their

physiologic relevance, bottom-up methods have been studied more extensively. For example, the bottom-up method was used to develop a functional small diameter arterial replacement graft. Arterial tissue and stem cells combined with synthetic scaffolds to make tubular structures have been a heavy area of focus. These constructs have various chemical and mechanical stimulation, such as growth factors and pulsatile fluid flow, applied to them to create a successful graft (Song et al., 2018).

It is important for the developed engineered microvasculature to exhibit clinically relevant characteristics. Two of these physiologically relevant characteristics that are not fully developed in *in vitro* applications are the alignment of microvessels and lumen density. Alignment of microvessels more closely recapitulates native microvessel architecture *in vivo* and allows for the creation of natural inlet and outlet areas for full perfusion through the vascular network. In this space, Tranquillo et al. were able to achieve strong microvessel alignment through mechanically constrained, cell-induced fibrin gel compaction after vasculogenesis was allowed to occur (Fig. 3a). This demonstrated that cell-induced compaction can be used to align microvessels in a vasculogenic model to create a more physiologically relevant model with full vessel perfusion and oxygen diffusion through the tissues. Additionally, the presence of interstitial flow through the engineered tissues resulted in increased microvessel density compared to a control sample with no interstitial flow. This highlights the importance of dynamic flow conditions in developing vasculature. Another important finding was that interstitial flow can impact the lumen density and overall microvessel formation (Morin et al., 2014).

Epithelial tissue constructs are a large area of interest as well. Voytik-Harbin et al. developed a novel *in vitro* multi-tissue interface model featuring tissue spheres encapsulating endothelial cells. Specifically, they produced human endothelial colony forming cell (ECFC) encapsulated tissue spheres which were directly embedded into a surrounding tissue environment (Fig. 3b). This experiment demonstrated how independent controlling of the ECM through matrix composition and density and cell-cell interactions directly affected vascularization of the tissue sphere and vascularization of the surrounding tissue environment (Buno et al., 2016).

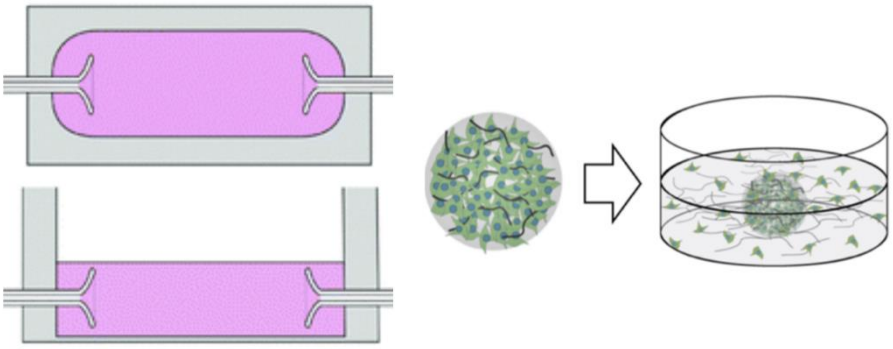

Fig. 3. Examples of current vasculogenic models a) Custom chamber for vascular constructs to measure gel compaction and interstitial flow on various microvascular characteristics most notably vessel alignment and lumen density (Morin et al., 2014). b) Design of a multi tissue spheroid with encapsulated endothelial cells surrounding by a tissue environment (Buno et al., 2016).

While vasculogenic models presented provide excellent platforms to develop more physiologically relevant tissue constructs through vascularization, more recent developments have been focused on vasculogenic assays in microfluidic platforms.

Section 2.2 Microfluidic Devices in Vasculogenic Modeling

Mechanical and chemical factors can be tightly controlled through microfluidic technology. The design, fabrication and application of microfluidic devices allows for the manipulation of fluids on a micrometer scale. The precise control of device geometry, biochemical gradients and mechanical stimuli gives microfluidic devices the ability to more closely mimic *in vivo* conditions.

Early *in vitro* studies of vascular physiology relied on cell culture plates and hydrogels to grow endothelial cells. While these studies greatly increased our knowledge and understanding of vascular system functionality in both healthy and diseased tissue states, they did not accurately portray a physiologically relevant microenvironment (Wong et al., 2012). Now with microfluidic devices, a more relevant model of microvasculature can be created. Microfluidic devices provide a variety of advantages in the development of functional vasculature including better control over geometry, biochemical gradients, and mechanical stimuli (Haase & Kamm, 2017). In microfluidic

device, a channel provides a three-dimensional (3D) surface for cells to adhere to and create a 3D extracellular matrix. A 3D structure allows for a more physiologically relevant microenvironment compared to a 2D structure. Cells in a 3D environment are morphologically different and their cellular responses are different as well. For example, cells cultured on a 2D surface are flatter and more elongated. This affects various cellular processes like proliferation, apoptosis and gene and protein expression (Kapałczyńska et al., 2018). To get the most accurate data regarding cellular response, the cells need to be in an environment that most closely recapitulates their native environment. Another key feature of microfluidics is the ability to continuously perfuse fluid across the developing vasculature. This continuous fluid flow allows for a more constant supply of nutrients and removal of waste products. By fine-tuning the fluid shear stress, cell morphology and orientation are precisely controlled, impacting the resulting overall microvascular network. This can help the network last over an extended time period.

Besides making *in vitro* models more physiologically relevant, microfluidic devices also operate on a small, higher throughput scale. This helps reduce consumption of expensive reagents and generation of waste. Most importantly, the small dimensions of the device helps to recreate the same temporal and spatial features found in vascular microenvironments (Hasan et al., 2014).

There are three main techniques in the in vitro vascularization of microfluidic devices 1) endothelial cell (EC) lining based methods 2) vasculogenesis and angiogenesis-based assays and 3) hybrid methods. Endothelial cell (EC) lining methods typically begin with a 3D printed vascular mold structure (Fig. 4a). Cells are injected into the preformed vessel structures and allowed to form a monolayer on the channels of the microfluidic device. Microvasculature geometry and dimensions can be tightly controlled. However, the complexity of microvasculature networks is not recapitulated in this method. It is also difficult to form small, intricate capillary networks. This is due to the small diameters of the capillaries and the high density of endothelial cells (X. Wang et al., 2018). When perfusing the channels with cells, the cells will block the small vessels eventually halting perfusion. The physiologic relevance is restricted in this type

of model because in vivo conditions cannot be completely replicated so they are limited when trying to uncover complex mechanisms of vessel development. The second method for *in vitro* vascularization is the process of vasculogenesis and angiogenesis, which can increase the complexity of a developed microvasculature network. Microstructures such as the preformed channels in the EC lining methods are not needed to help guide the formation of a microvasculature structure. Cells undergo self-assembly within the ECM similarly to the process *in vivo* and create a more physiologically relevant model of vasculature (Fig. 4b). While multiple vascular models have been successful in vessel development, models that allow vasculogenic self-assembly through the distribution of endothelial cells in a specified ECM have been the most promising. This is because through self-assembly, the endothelial cells can create a more physiologic vascular structure that more closely resembles in vivo conditions (X. Wang et al., 2018). As an example of this method, Kamm et al. designed a five-channel microfluidic device in which human umbilical vascular endothelial cells (HUVECs) were embedded in fibrin gels and cultured along human lung fibroblasts in the adjacent channels with channels of media running between them (Fig. 4b) (Whisler et al., 2012).

The final method of *in vitro* vascularization is hybrid methods. Hybrid methods combine several vascularization methods and offer more control over the vasculogenic process through the manipulation of factors within the microenvironment (X. Wang et al., 2018). For example, Wang et al. developed a microfluidic device model that incorporated multiple vascular development methods such as the EC lining method, vasculogenesis, angiogenesis and anastomosis. After sufficient vascular network development had occurred in the central tissue chamber via vasculogenesis, endothelial cell lining along the channels of the device was done. The outside channels were meant to represent the artery and vein of a capillary network. Angiogenesis was then allowed to occur which led to anastomosis between the endothelial cell lined outer channels and the capillary network ensuring interconnectivity (X. Wang et al., 2016).

Vasculogenic models using microfluidic platforms have important clinical applications. For example, they can be used to develop microtissues. Hsu et. al. designed a microfluidic model to develop multiple human microtissues in parallel by controlling

mechanical stimuli such as interstitial flow. They used the device to induce vasculogenesis to create continuous microvascular networks across the microtissues (Fig. 3c).

Besides tissue engineering applications, microfluidic devices have a wide variety of other applications in microvasculature related areas. For example, devices can also be used for drug testing applications and used for *in vitro* modeling of dysregulated

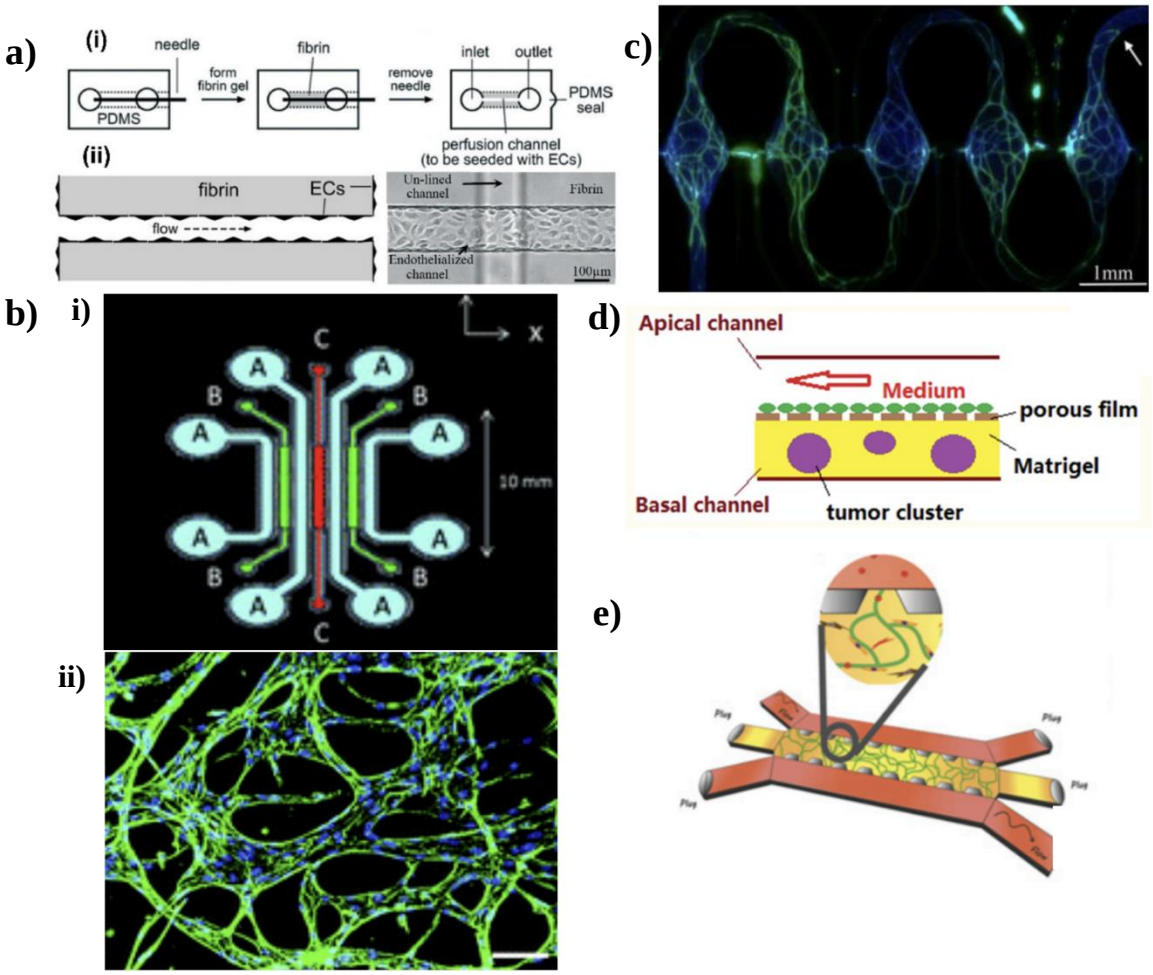

Fig. 4. Examples of current vascular modeling methods and applications using microfluidic platforms a) Endothelial cell lining method in preformed microfluidic channels (X. Wang et al., 2018). (b) (i) Multi-channel microfluidic device design that relies on vasculogenesis (ii) Developed, fully perfused microvasculature network. (Whister et al., 2012). c) Vascularized microtissues on a microfluidic model d) Microfluidic drug screening and development platform (Hsu et al., 2013) d) Biomimetic anti-tumor drug screening platform (Shi et al., 2019). e) Microfluidic device with organ specific vasculature for metastatic cancer cell studies. (Arrigoni et al., 2016).

screening platform for measuring the transportation of drug molecules through the microvasculature. (Fig. 4d). The objective of the study was to determine the effect the microvasculature had on the efficacy of the drugs. Previous studies done by this group indicate the endothelial cell monolayer is an obstacle for drug transportation (Shi et al., 2019). This is important as a physiological relevant model needs to incorporate this microvasculature component into the design of the platform. With more physiologically relevant models, drugs can be tested more accurately in a clinical setting to fully understand the effect of different drug concentrations and combinations of drug therapies before being introduced into the patient. Using microfluidics in drug studies such as this one can be advantageous due to high throughput nature of microfluidics. This allows many drug combinations can be tested simultaneously saving time and resources.

Microfluidic devices can model vasculature in disease states and help elucidate vascular dysregulation on a cellular level. Several studies have produced organ specific microvasculature to investigate tumor cell extravasation and the metastatic process (Fig. 4e) (Arrigoni et al., 2016). This type of model can help demonstrate how endothelial cells, immune cells and other cells along with the ECM can influence the progression of cancer. In a microfluidic device, different cell types can co-exist by compartmentalizing the different microenvironments. This co-culture design allows the complex process of metastasis to occur over the intricate, physiologically relevant environment. For example, breast cancer cells can be present in one microenvironment and connected to a bone microenvironment through a microvasculature network (Glaser et al., 2020).This allows the model to be more physiologically relevant as cancer cells would be traveling through a complex network of blood vessel during metastasis. To fully recapitulate the metastatic cascade, a functional microvasculature network needs to be incorporated along with the 3D tumor model. Metastasis relies on vasculature to carry tumor cells throughout the body. With an intricate model of tumor metastasis important stages of cancer progression such as cancer cell intravasation and extravasation can be closely investigated.

Modeling the process of vasculogenesis and optimizing conditions for functional vasculature is important to understand how blood vessel formation can help with the perfusion of engineered tissue constructs. Vasculogenic models have increased our

understanding of vascular biology and the formation of blood vessels in both embryonic development and adult vasculogenesis.

Vasculogenic models are application dependent. Vasculogenic models for use in tissue engineering discussed in Section 2.1 are excellent models for the development of larger tissue constructs. For smaller tissue constructs and applications outside of tissue engineering, microfluidics provides an excellent platform.

Section 2.3 Current Microfluidic Models on Factor Dependent Vasculogenesis

To create microfluidic models of microvasculature, there are several key components of neovascularization that can be directly manipulated inside a microfluidic device as discussed previously. Many studies have been conducted on these specific components in angiogenic assays, but limited studies have been conducted in vasculogenic assays. Kamm et al. analyzed HUVEC seeding density and fibrin density in microfluidic devices to help uncover factors that control network morphology of developing vessels. It was found that a higher cell seeding density allowed for increased branch length, diameter and area fraction of the vascularized region. Additionally, a higher fibrin concentration led to an increased average branch length and diameter of the vessels. Area fraction of the vascularized area seemed to be unchanged with changing fibrin concentrations (Whisler et al., 2012). By manipulating factors that affect vasculogenesis, the most ideal conditions can be determined for fully perfused vasculature.

Since angiogenic models have been studied more than vasculogenic ones, more angiogenic growth factors and consequently their effects on the vasculature have been understood. Song et al. identified two distinct growth cocktails that resulted in the most robust and multicellular endothelial cell development. They determined VEGF had a negligible effect on cell sprouting when used alone and S1P alone only triggered increased cell migration. However, when different growth factors were combined, cells produced greater multicellular sprouting (Song et al., 2018). In another angiogenic sprouting assay, Hankemeier et al. also tried to uncover ideal growth factor combinations, with VEGF, PMA and S1P. Again, as reported by Song et al, when acting alone each growth factor did not induce angiogenic sprouting. When combined, VEGF, PMA and

S1P induced angiogenic sprouting with the sprouts fully traversing the gel channel and showing clear lumen formation (van Duinen et al., 2019) Few vasculogenic assays have explored the effects of growth factors and their various combinations on vessel growth and development. Kamm et al. used VEGF and S1P to understand their effect on developing vascular networks. They found the addition of these two growth factors together resulted in branches that were shorter in length and smaller in diameter. With smaller vessels, less area was covered by the developing network. They also co-cultured the endothelial cells with fibroblasts which improved vessel development. An important finding was that when co-cultured together with fibroblasts and stimulated by VEGF and S1P together, network stability of the microvasculature network was greater and vascularized space was increased compared to a control with no growth factors. Using growth factors alone in vasculogenic assays, based on this one study, decreases vessel development, but when used in a co-culture with fibroblasts, vessel development increases (Whisler et al., 2012).

Some existing models of vasculogenesis, while able to produce fully functional microvasculature networks, lack the precise control over fluid dynamics, device geometry and biochemical gradients. Vasculogenic microfluidic models that do have this precise control have not been studied as closely as angiogenic assays and therefore a complete understanding of the process is lacking. For example, evidence on the temporal control of growth factors on developing vasculature remains to be fully elucidated.

The objective the project outlined here is to enhance the understanding of how various factors like endothelial cell density, ECM composition and density, growth factor solutions and flow control all influence the development of a microvasculature network within a single microfluidic platform. The motivation is also to use this platform in future endeavors in the Wood Lab where a physiologically relevant, fully perfusable, microvasculature is needed. Based off previous findings it is predicted that a fibrin ECM with a higher initial cell-seeding density and a growth factor solution of multiple growth factors will induce a fully perfusable microvasculature network. Additionally, it is predicted that a microfluidic device with fluid flow control through the channels will allow for increased vessel growth and development.

Chapter 3

Methods

Section 3.1 Cell Culture and Preparation

HUVECs were chosen due to their commercial availably, ease of use and proven success in vasculogenic assays. HUVECs were seeded and cultured on gelatin coated 75cm^2 cell culture flasks with Lonza EGM media in a 5 % $C0_2$ controlled incubator at 37°C. Cells at passages 4-6 were used. The Lonza EGM media contained endothelial cell basal media (500mL) and an assortment of additional components including 10mL of FBS, 0.2mL of hydrocortisone, 2mL of hFGF, 0.5mL of VEGF, 0.5mL of R3-IGF-1, 0.5mL of Ascorbic Acid, 0.5mL of hEGF, 5mL of GA-1000 and 0.5mL of Heparin. 5mL of Antibiotic-Antimycotic was added as well to prevent bacterial and fungal contamination. HUVECs were used due to their commercial availability, ease of use and previous success in vasculogenic assays.

Cells were used at or very close to 100% confluency. They were washed twice with Hank's Balanced Salt Solution (HBSS) and trypsinized to detach the cells from the bottom of the flask. Once neutralized with EGM media, the cell solution was centrifuged at 1000rpm for 5min. The supernatant was aspirated and cells were resuspended in 5mL of EGM media. 10uL of the cell solution and 10uL of trypan blue were mixed and placed on a cell counting slide. Once cells were counted, desired amounts of the cell suspension were aliquoted based on the number of cells needed and centrifuged again. Cell viability was always above 90%. Cells tested negative for mycoplasma.

Section 3.2 ECM Gel Fabrication

Collagen gels were prepared with 9.1mg/mL collagen stock, EGM media, DPBS and NaOH to get a final collagen concentration of 6mg/mL. The collagen mixture was kept on ice for 2 hours to avoid premature polymerization. Cells were then mixed into collagen aliquots in small tubes and contents were gently disturbed and inverted to combine. If being mixed with Matrigel, the collagen and cells would be added to the Matrigel and mixed for both the well plate and microfluidic devices. Well plates and devices were incubated for 30 minutes at 37°C to allow for gel polymerization. With

Matrigel only assays, Matrigel was pipeted into the well plate (50µl) and the cells were seeded directly on top.

Fibrin gels were created by first dissolving fibronectin (Sigma Aldrich, Bovine Plasma) in DPBS at a concentration of 10mg/mL. After allowing the fibrinogen to get into solution, the solution was sterile filtered with a .22µm filter to ensure sterility. After the aliquoted amount of fibrinogen solution was mixed with cells, it was added to a 0.6uL aliquot of thrombin (Sigma-Aldrich, Bovine Plasma) (100U/mL) and quickly loaded into a well plate or the center channel of the device. Final fibrin concentration was 9mg/mL. Well plates and devices were incubated for 30 minutes at 37°C to allow for gel polymerization.

Section 3.3 Device Design and Fabrication

The microfluidic device used in this work is a three-channel device with trapezoidal posts in between the media channels and the central gel channel. The gel channel is where the fibrin or collagen ECM and cells are injected. The two flanking media channels provide nutrient delivery and a pressure driven flow across the gel/media interface. The 200µL of media placed in the inlet channels of the device flows through the device and towards the empty media outlet channels. The flow is sustained until an equilibrium is each reached between the media in the inlet pipet tips and the outlet pipet tips.

The microfluidic device was designed using AutoCAD and negative pattern masks were printed (Fig. 5a and Fig. 5b). A 115µm layer of SU-8 photoresist was coated onto silicon wafers and the pattern was photopolymerized onto the wafers creating a raised design. PDMS and a curing agent were mixed at a 10:1 ratio and poured onto the wafers. They were placed in a degassing chamber for an hour and then baked for 2 hours at 75°C. Devices were cut out and biopsy punched to create inlet and outlet ports for the three channels. Tape was used to clean the devices of debris. Devices and glass slides were plasma treated for 2 minutes and quickly bonded together. They were placed on a hot plate overnight at 120°C (Fig. 5c). The goal with this device was to create a physiologically relevant model of endothelial cells undergoing vasculogenesis, with flow on each side (Fig. 5d).

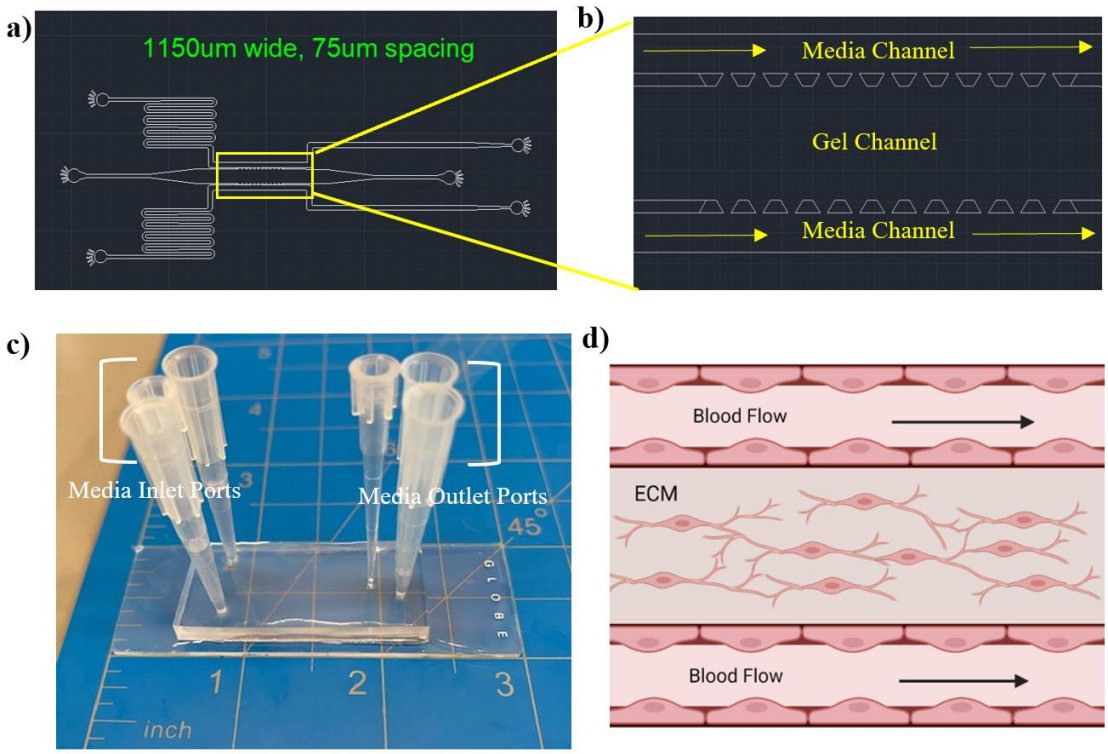

Fig. 5 Design of microfluidic device a) CAD design of the microfluidic device platform b) Magnified post design of the microfluidic device with direction of media flow through the device. c) Microfluidic device with PDMS device plasma bonded to a glass slide with inlet/outlet media channel and gel channel pipet tips d) Anatomical model of microfluidic device design (Biorender).

PDMS coated glass slides were made by spreading PDMS across a transparent sheet of paper and laying the glass slides down firmly on top of the PDMS layer to eliminate bubbles. Slides were left to sit overnight and baked the next day at 75°C for 2 hours. Devices were pulled apart and tape was also used to clean the slides of debris. PDMS coated glass slides and devices were bonded in the same manner as mentioned previously.

Several durations of plasma bonding were tested at two different power settings to determine the optimal bonding conditions for a PDMS coated glass slide and PDMS devices after bonding issues had occurred. Bonding groups at 50% power with a duration of 2 minutes and 1.5 minutes seemed to be the most ideal. Moving forward a plasma bonding time of 2 minutes at 50% power was used.

To create more resistance for fluid flow, leading to increased flow over a longer time period a secondary resistor was incorporated into a trial design. Two secondary resistors also made of PDMS following the same procedure as above, were attached to each inlet port of the devices by a 6cm length of 0.02mm diameter tubing and plasma bonded to glass slides. A 1mL pipet tip was inserted into each media outlet and each inlet of each of the secondary resistors. For devices without the secondary resistors, 200µL pipet tips were placed in the same manner in the devices. Due to the large amount of effort for one device, this design was not scalable for the number of devices needed. For the final design the second resistor was not included and only used as a trial design.

Section 3.4 Endothelialization of Media Channels

Vessel formation was allowed to occur for 96 hours before endothelialization of the media channels. The secondary resistor and tubing were disconnected from the inlets and 200µL pipet tips were inserted into the inlets of the device. 10µL of EGM media with 10µg/mL of fibronectin was loaded into both media channel inlets. Devices were incubated in static conditions for 1 hour to allow the fibronectin to fully coat the channels. Next 10µL of a 12 million cells/mL cell solution was loaded into each of the media channel inlets in all devices. Devices were incubated for 30 minutes to allow for cell adhesion to the channel walls. This was repeated for all devices again with 10µL of cell solution to ensure full cell coating of the channels. The 200µL pipet tips at the inlets were removed and the secondary resistors and tubing were reconnected with the devices. 1mL of media was added to each of inlet pipet tips.

Section 3.5 Growth Factor Experimental Setup

Growth factor solutions were prepared 24 hours in advance according to the manufacturer's instructions. VEGF stock solution was prepared at .1µg/mL (murine VEGF, Peprotech) in cell culture water. S1P stock solution was prepared at 1mM in Methanol (Sphinogosine-1-Phosphate, Sigma Aldrich) and PMA stock solution was prepared at 2µg/mL in DMSO (Sigma Aldrich). Growth factors were all diluted in EGM-2 media with no VEGF added at the following concentrations: 50ng/mL for VEGF, 50ng/mL for PMA and 500nM for S1P.

The objective was to determine which combination of growth factors has the largest impact on vessel growth and development. As a negative control the VEGF negative media was used on a group of devices. It was prepared the same way as described previously, just without the 0.5ml of the VEGF to allow for absolute control over the growth factor addition to the experimental groups. Each experimental group (Table 1) had three devices and was replicated three times. After devices were injected with the fibrin/cell solution and allowed to polymerize, 10µL of respective media was

Table 1: Experimental Growth Factor Combinations

Group	Growth Factor Combination
1	Negative Control
2	S1P + PMA
3	VEGF + PMA
4	VEGF + S1P
5	VEGF + S1P + PMA

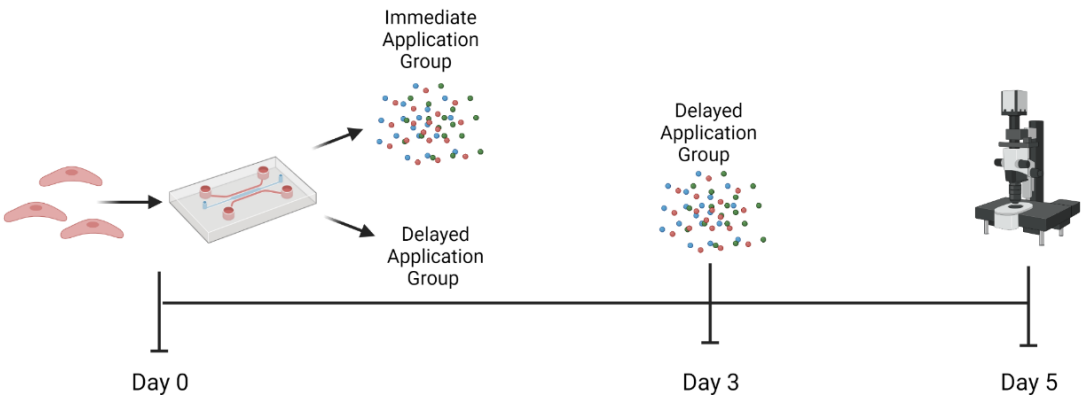

Fig 6. Experimental set up of growth factor experiments. Devices were loaded with fibrin ECM and HUVECs. Two experimental branches were set up, with one group receiving growth factor solutions right away at day 0 until day 5 and the other group at day 3 until day 5. Devices were fed twice at day until they were imaged on day 5. (Biorender).

pushed into both outer media channels until it reached the outlet. 200µL of respective media was added into each inlet pipet tip and the outlet pipet tips were left empty to create a pressure driven flow.

To test whether the timepoint at which growth factors are added to the devices impacts vessel development, the experiment was replicated with the exact same setup and conditions with growth factors being added 3 days after gel polymerization (Fig. 6). Up until day 3 devices in the delayed application group were given the control EGM. At day 3, after sufficient vessel development had occurred, the experimental groups in the second branch began to receive their respective growth factor solution instead of the standard VEGF negative media. Devices were kept in plastic boxes with 10µL of DPBS in the bottom of the box to prevent device dehydration. Respective media for each experimental group was replenished in devices twice a day for 5 days. After 5 days growth media was removed from all devices and stained with a Calcein solution to highlight the microvasculature network. Calcein stock solution (10µg) was diluted in 10mL of DPBS. To ensure proper dispersion of the Calcein across the gel channel, 200µL of the stain solution was placed in the inlet and outlet port on the same side of the device. This allowed for the Calcein stain to dissolve across the gel to the other media channel. The Calcein solution was left to sit on the devices for 30 minutes before imaging.

Section 3.6 Imaging and Statistical Analysis

Images were taken on a Zeiss Microscope and processed using ImageJ software. A standardized area of the central gel channel was used for all microvasculature network images where data was collected. After image processing, images were analyzed using Angiotool imaging software to map the microvasculature network.

The addition of growth factors experiment was replicated three times with 3 replicates per condition. The results are presented as mean + standard deviation. A one-way ANOVA test was performed for the comparison of the control to each individual growth factor. All statistical analyses were done on GraphPad Prism software. A p-value of less than 0.05 was considered to be statistically significant.

Chapter 4

Results

Section 4.1 Optimization of a Microvasculature Network

To fully understand the best conditions for vessel growth in an *in vitro* environment in a more streamlined manner, experiments were done first in well plates with the motive to move to microfluidic devices when a best method for vessel growth was uncovered.

Section 4.1.1 Fibrin Gels Create an Environment for Vessel Formation

Optimization in the well plate started with finding the ideal cell concentration conditions in a collagen ECM. Collagen was chosen for the first iteration of experiments because of its wide use and success in vasculogenic assays. Cell concentrations of 3 million cells/ml, 3.5 million cells/ml and 4 million cells/ml were tested. 50ul of collagen gel mixed with cells were placed in a 96 well plate with 100ul of media on top. Media was changed every other day and images were taken every 24 hours. In all of the conditions cells failed to adequately grow and anastomose even by the 96 hour mark. HUVECs were also seeded with fibroblasts in varying ratios (2:1, 4:1 and 5:1) but several experiments showed no change in growth of the HUVECs in terms of vessel development.

To show the cells had the ability to form vessels, cells were seeded on top of Matrigel at varying cell concentrations (4 million cells/ml and 8 million cells/ml). Matrigel is a murine tumor extract, composed of ECM material and basement membrane proteins (Stryker et al., 2019). In both concentrations, cells showed vessel network formation in the typical cobblestone like structure, at the 24-hour mark (Fig. 7a.) While promising, the use of Matrigel would not be feasible to use in the device design presented here for several reasons. First Matrigel is not as biologically relevant as other ECM materials as it was developed from a murine sarcoma. Second, when mixed with Matrigel, cells failed to adequately grow and anastomose to create vessels. To be incorporated into the device, the HUVECs would have needed to be mixed with the Matrigel. While it is not fully clear why the cells were able to undergo great vessel formation when seeded on top of the Matrigel and not mixed in, it can be speculated. We

hypothesize the cells were most likely able to form tubular structures more easily seeded on top because they were on a 2D surface. Matrigel assays were developed to imitate the ECM in a two-dimensional manner and are typically used in angiogenesis assays. The use of Matrigel in vessel development seemed unable to translate to a 3D environment for a vasculogenic assay. The cells also covered less of an area because of the dimensionally of a 2D surface and most likely had an easier time to anastomose.

To overcome the lack of cell growth in collagen, collagen and Matrigel were mixed in different ratios to see if a combination of the two would lead to vessel formation. Collagen and Matrigel were used at 100% collagen, ratios of 1:3, 1:1, 3:1 and 100% Matrigel in a tube formation assay. A ratio of 1:1 demonstrated the most potential to induce cell growth and vessel development. As evidenced in (Fig 7b.) tubular formation of the cells indicated a collagen and Matrigel combination led to more cellular growth and vessel development compared to collagen alone. While promising, the combination of collagen and Matrigel still did not show the vessel network development desired.

Fibrin was introduced as a new ECM material to use. Fibrin gels were created at concentration of 9mg/mL. Compared to a 1:1 ratio of collagen/Matrigel, the fibrin allowed the cells to grow more and undergo vessel formation (Fig. 7c). At 96 hours, gels were stained with Calcein and DRAQ5 to show the endothelial cell development into microvasculature. (Fig. 7d). Qualitatively the vessel diameter appeared to be increased as well in the fibrin compared to the collagen/Matrigel. Due to the nature of using bulk gels in a well plate, the 3D architecture of the assay made it difficult to obtain clear images of the vessel development and growth. It was hypothesized using a microfluidic device would decrease the thickness of the gel assay and result in much clearer images.

After successful optimization in the well plates with fibrin gels, vessel formation in a fibrin ECM in a microfluidic device was the next step. A 1:1 ratio of collagen and Matrigel was again tested against fibrin. Like results in the well plate, collagen and Matrigel failed to supply an adequate environment for vessel formation evidenced by the failure of cell growth into tubular structures (Fig. 8a). In stark contrast to the collagen/Matrigel bulk gel, in the fibrin ECM, vessel formation was very prevalent and

vessel network development seemed increased in the devices compared to the well plate (Fig. 8b). This is most likely the result of the microfluidic technology used, specifically the fluid flow through the media channels. Qualitatively the vasculature network appeared more developed and robust in vessel size in the device compared to in the well plate.

As an additional step in the optimization of vessel development in devices and to assess further areas of experimentation, a higher concentration of VEGF was added to the EGM. In the media supplemented in the devices, the VEGF concentration was 50ug/mL.

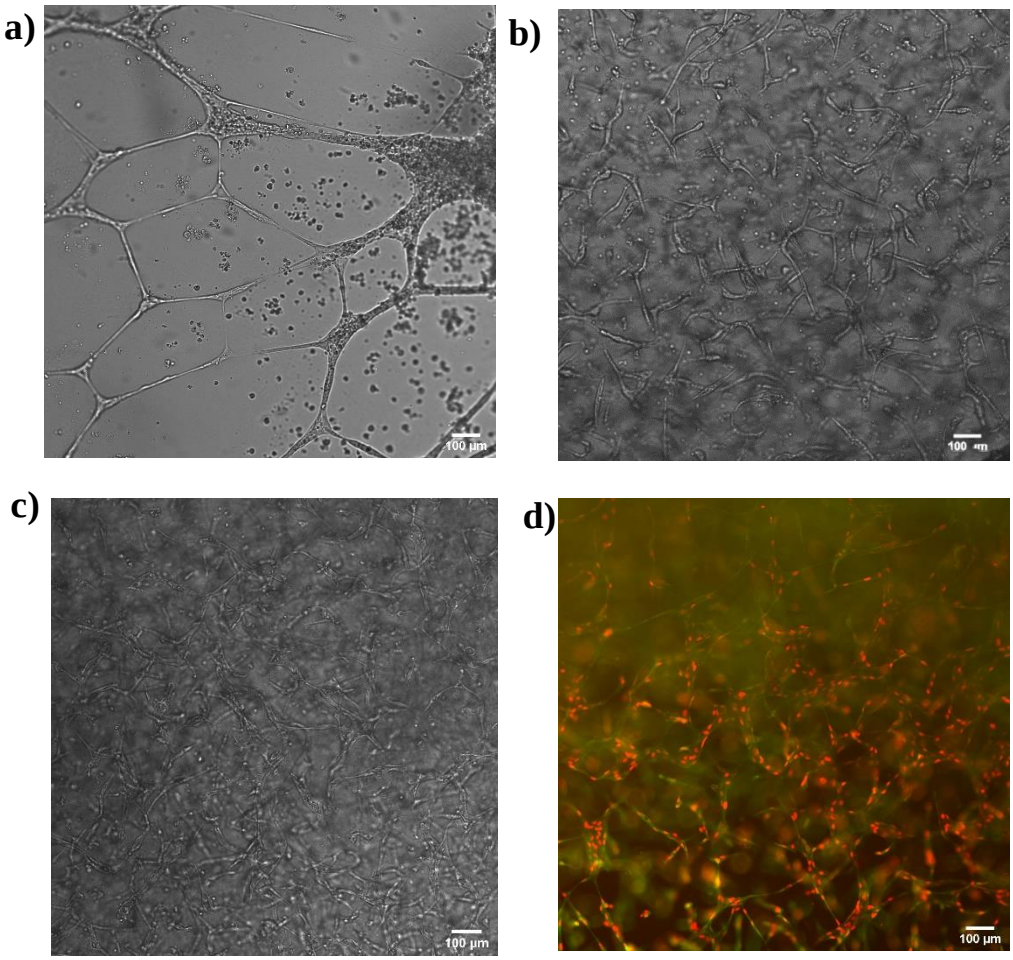

Fig. 7. Experimentation with different ECM compositions in a standardized well plate a) HUVECs seeded on top of Matrigel at a concentration of 8 million cells/ml b) Matrigel/Collagen combination in a 1:1 ratio with a HUVEC concentration of 9 million cells/ml c) Fibrin ECM with HUVECs at a concentration of 9 million cells/ml d) Fibrin ECM with HUVECs at a concentration of 9 million cells/ml stained with Calcein and DRAQ5 Scale bars = 100um

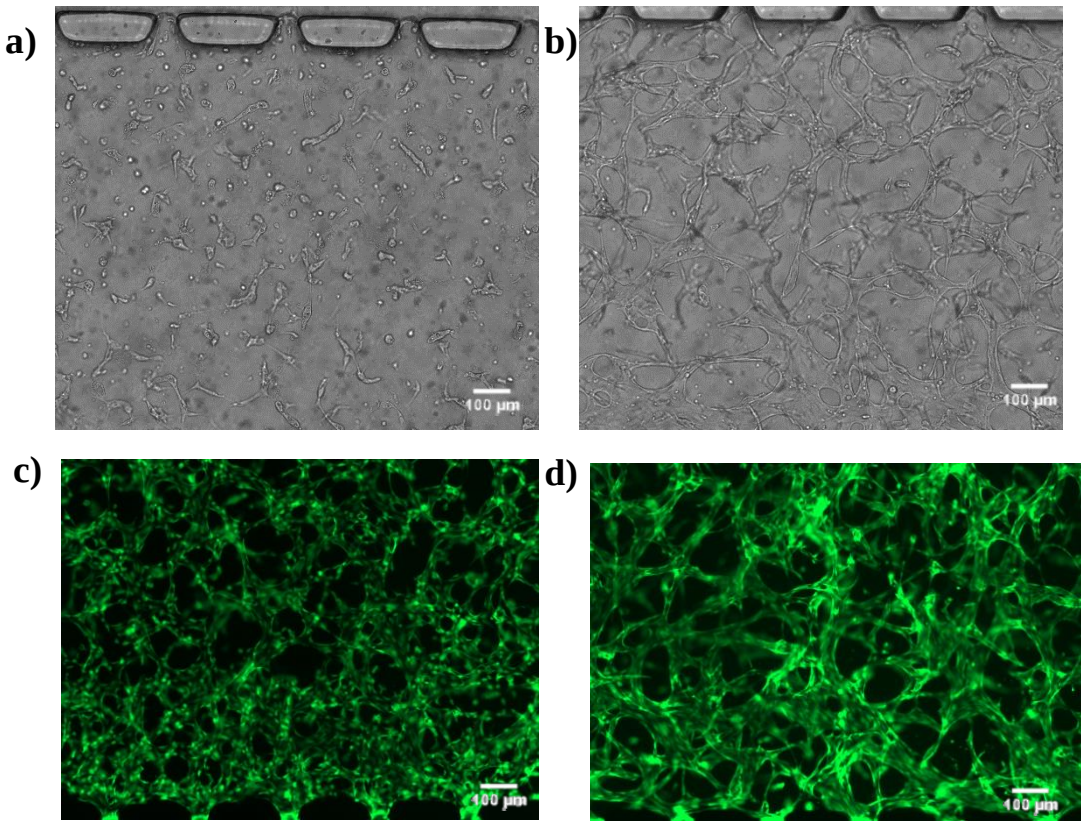

Fig. 8. Microfluidic device assays with 12 million cells/ml a) Collagen/Matrigel ECM where cells failed to form vessels b) Fibrin ECM supported the development of a microvascular network c) Microvascular network formation in fibrin ECM stained with Calcein d) Microvascular network formation in fibrin ECM with media supplemented with 50ug/ml of VEGF and stained with Calcein. Scale bars = 100um

Qualitatively, preliminary results showed increased vessel network formation with the addition of VEGF compared to a control (Fig. 8c and 8d) and a point of investigation for the effect of growth factors on vasculogenesis in a fibrin ECM. The control did not have any additional VEGF added to the media, just the VEGF present in the EGM.

To highlight the developed vasculature was able to form a perfusable network, a fluorescent bead flow assay was performed. Several beads were able to somewhat traverse the gel channel via perfused vessels, though the total number of beads able to traverse the entire gel channel was low. This is because beads were leaking underneath the gel from the media channels into the ECM.

Section 4.1.2 Endothelialization of Media Channels/PDMS Slides

To solve the issue of bead flow underneath the gels, media channels were endothelialized to create a fully perfused vessel from the inlet to the media/gel interface. With a fully intact vessel, beads would not be able to escape underneath the gel and the vessel development within the gel could be more accurately assessed. Fibronectin was used to help the cells adhere to the device walls. Compared to a control with no fibronectin present, media with 10ug/mL of fibronectin allowed for much more cell adhesion (Fig 9a and 9b). PDMS coated glass slides showed slightly improved cell adhesion over glass slides. However, the addition of fibronectin to the EGM made the largest improvement in cell adhesion (Fig 9c and 9d).

We hypothesized that using a PDMS coated slide might prevent bead flow underneath the gels and allow for better cell adhesion along the media channels. While it did help with some cell adhesion it remained inconclusive if it impacted bead flow underneath the gel. The PDMS devices failed to adequately adhere to the PDMS coated glass slides in every instance. This resulted in devices becoming unbonded in the middle of an experiment and caused DPBS to leak in. It was decided that since the difference of cell adhesion between the PDMS coated glass slides and uncoated glass slides was minimal, glass slides would be used for further experimentation to ensure proper bonding.

As a trial design for extended fluid flow, a two-resistor device set up was tested. The purpose of this design was to see if an extended time period of fluid flow from four hours a day to a fully 24 hours, impacted vessel development. Preliminary results indicated a negligeable difference in microvasculature development, but more testing needs to be done as the literature firmly states fluid flow leads to increased vessel network development.

After endothelialization of media channels, device design and PDMS coated slide experiments were completed we concluded that for the most ideal vessel formation and in

the interest of time, the channels would not be endothelialized for further experimentation and only glass slides would be used. However, the model described here could be developed further.

As the final points of optimization, cell seeding density and ECM density were fine tuned. Initial seeding in the devices was at 12 million cells/ml. It was hypothesized that a higher seeding density would aide in vessel development. The cell concentration was increased to 14 million cells/ml and vessel formation and vessel development

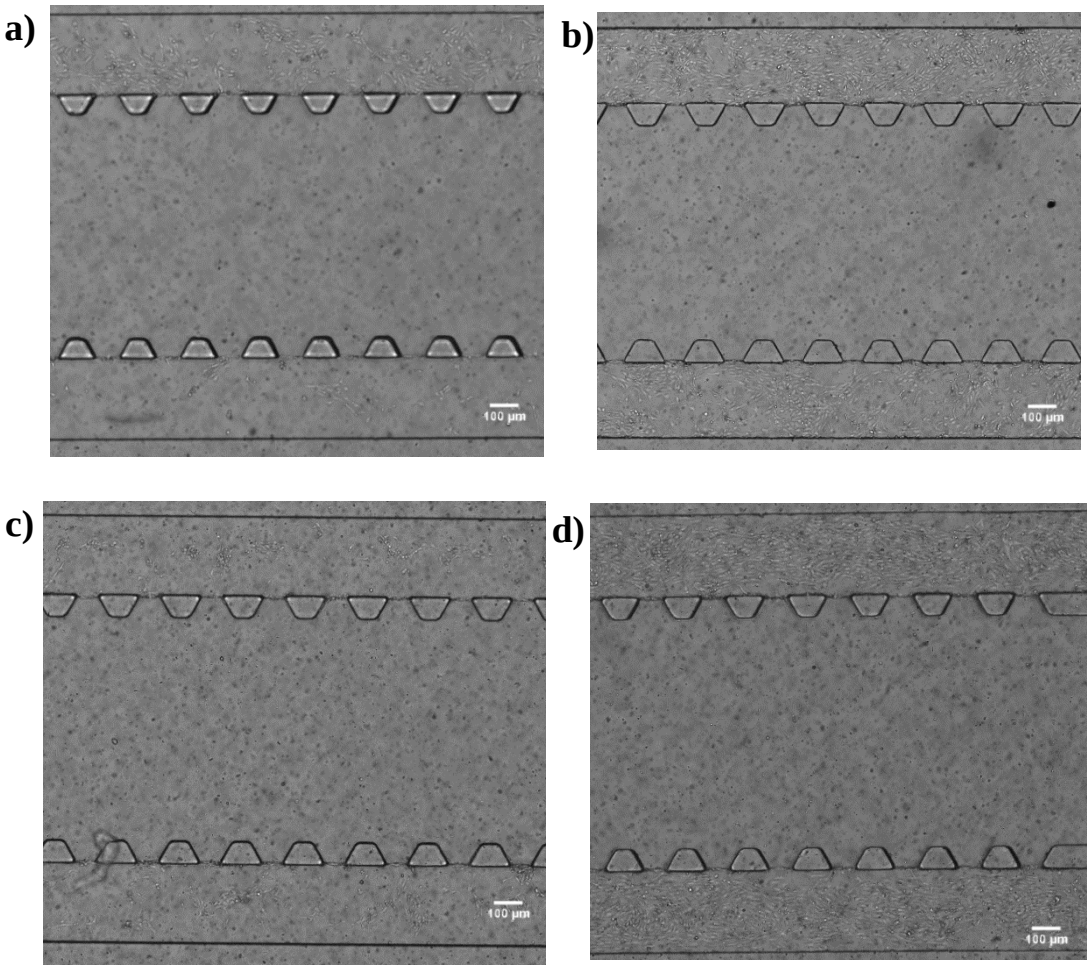

Fig. 9. a) Control. Glass slide with no fibronectin b) Glass slide with channels perfused with media supplemented with 10ug/ml fibronectin c) PDMS coated glass slide with no fibronectin d) PDMS coated glass slide with channels perfused with media supplemented with 10ug/ml fibronectin. Scale bars = 100um.

was observed to be increased. Data is not shown as this was just a point of optimization between experiments. To be certain that a higher seeding cell density impacts vessel formation and growth further experimentation would need to be done.

Section 4.2 Investigating the Effects of Growth Factors on a Microvasculature Network

Mixed results were seen in the images regarding proper calcein staining of the microvasculature. Specifically, the Calcein stain was not able to completely stain all the vessels throughout the gel channel in every device. It was hypothesized that the Calcein stain was not able to properly perfuse through the gel itself or the vessels were not developed enough or starting to degrade causing the calcein to not be able to travel through the gel via the vessels.

A permeability assay was performed to ensure that the Calcein stain was able to adequately perfuse through the fibrin gel. Fluorescent Dextran was diluted in 10mL DPBS and stained according to the Calcein staining protocol described in the methods section. Imaging showed that the dextran was able to fully perfuse through the gel, meaning the gel permeability was not the factor impacting the ability of the Calcein to stain the vessels.

It was also hypothesized that the leftover media inside the channels could be preventing the stain from permeating across the gel to reach all the vessels. With trials 2 and 3 of this experiment, 200µL of Calcein staining solution was pipeted into the upper left inlet port and placed in the incubator for 5 minutes. This allowed any remaining media in the device to be washed out. After this short incubation period 200µL of the Calcein stain solution was added to the corresponding upper outlet after remaining media was removed. With the calcein staining solution in one inlet-outlet pair, the calcein stain would be able to diffuse across the gel instead of just flowing through the media channels. To ensure complete staining of all the vessels in the gel channel, the same process was repeated in the lower inlet/outlet pair. Removing the media before staining with Calcein stain solution showed more complete staining of vessels than in trials where media was not removed.

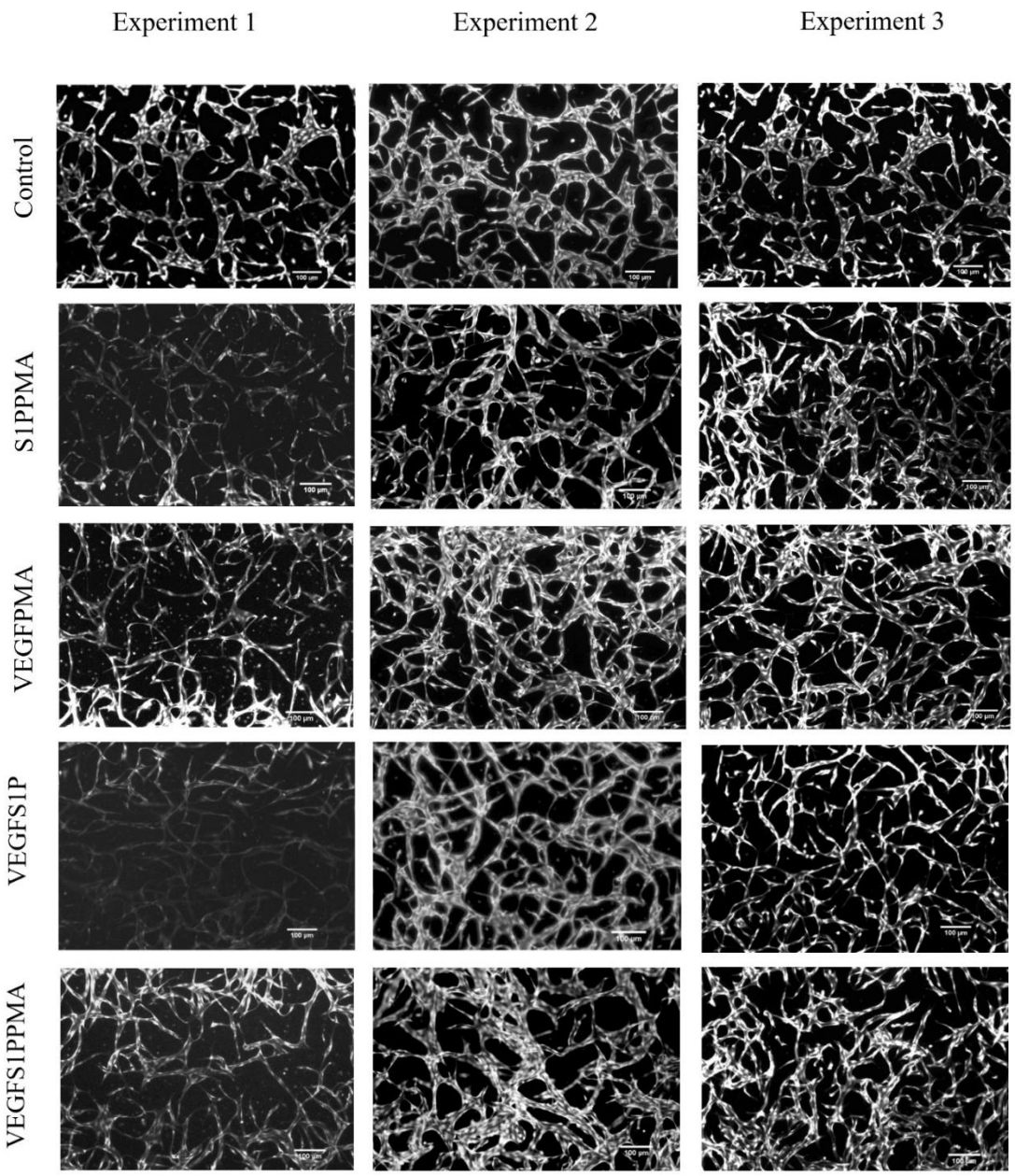

Fig. 10. Microvascular network development over a control group and four growth factor conditions in three different experimental trials. Scale bar = 100um

Data collection was focused on vessel percent area, average vessel length and total length. Microfluidic devices in the delayed application group where the growth factors were not added until after sufficient vessel development (day 3) showed greater vessel coverage area, average vessel length and total vessel length compared to the immediate application group (Fig. 10 and Fig. 11). This indicates that growth factors can

have a larger impact on vessel development if they are introduced later in endothelial cell growth. In experiment 1 in the immediate application group both average and total length were greater in the condition with the growth factor combination of S1P + VEGF. Results are not as clear as to which growth factor impacts vessel percent area the most. In the delayed application group vessel percent area and total vessel length were greater in the conditions with the growth factor combinations of VEGF + PMA and VEGF +S1P + PMA. The growth factor combination of VEGF + S1P +PMA resulted in the greatest average vessel length (Fig. 11b). In experiment 2 in the immediate application group results indicate the growth factor condition VEGF + S1P + PMA results in the most vessel growth in term of vessel percent area and both average and total vessel area compared to the other growth factor conditions. However, the vessel development in the growth factor conditions did not significantly surpass the vessel development in the control which had no growth factors. In the delayed application group, all growth factor conditions except S1P +PMA seemed to increase vessel percent area, average and total vessel length. These results are also represented qualitatively (Fig. 9) Finally, in experiment 3 in the immediate application group, results were not completely conclusive, but they did indicate that all the growth factor conditions except S1P + PMA had slightly increased vessel development compared to the control. In the delayed application group growth factor condition VEGF + PMA resulted in most increased vessel percent area and average vessel length. Results were inconclusive for the total vessel length.

A one-way ANOVA statistical test showed that in vessel % area, the growth factor combinations of VEGF + PMA and VEGF + S1P + PMA were statistically significant from the control in the delayed application. They were also statistically significant from the immediate application to the delayed application (Fig. 13a). VEGF + PMA was also statistically significant for average vessel length and total vessel length in terms of the control and immediate to delayed application (Fig. 13b and Fig. 13c). Overall, these results suggest a common overall trend that VEGF and PMA in combination is needed for complete vessel development in a microvasculature network. In most cases without VEGF and PMA together, growth factor conditions failed to produce vasculature with increased vessel percentage area, average vessel length and

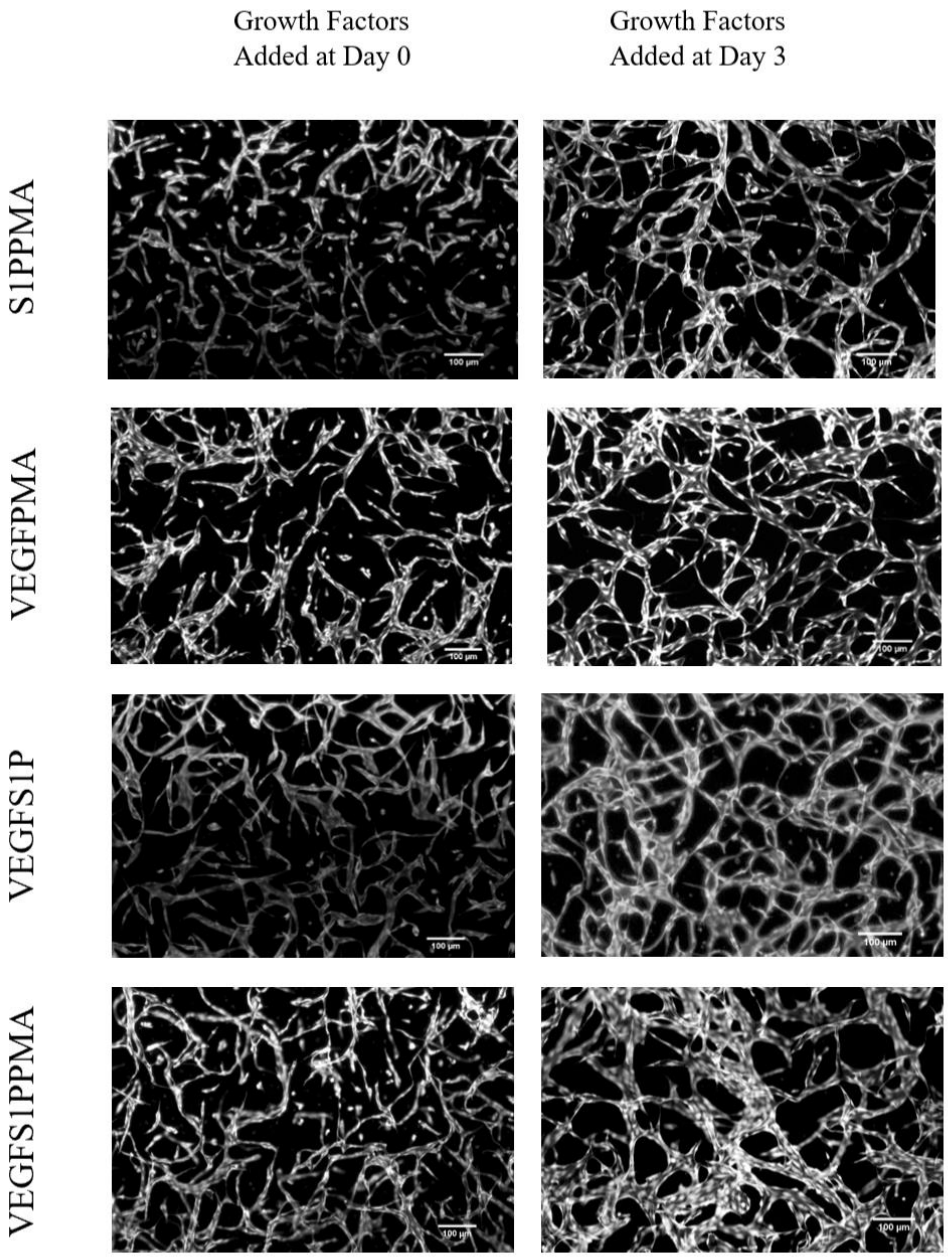

Fig. 11. Vessel development compared between two groups 1) Growth factor solutions added at Day 0 and 2) at Day 3 for all four growth factor conditions. Scale bar = 100um

total vessel length compared to conditions that did have VEGF and PMA together. The exact combinations of growth factors that lead to the most vessel development is unclear, but combinations of VEGF and PMA seem to repeatedly have a positive impact on vessel development.

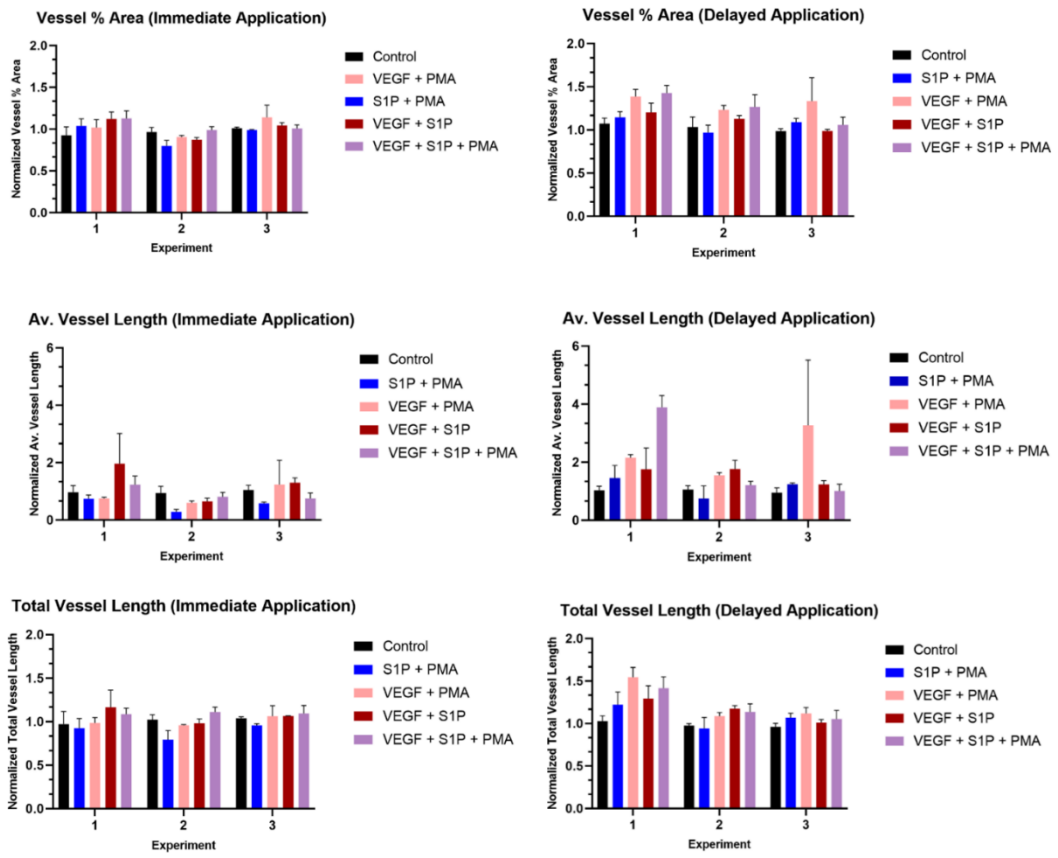

Fig. 12. Experiments 1-3 in the immediate application of the growth factor combinations on day 0 and in the delayed application of growth factors on day 3 as well as the individual performance of growth factors (n= 3) for a) vessel % area b) average vessel length and c) total vessel length. Graphs are presented as mean + standard deviation.

In the conditions where PMA was present, more cell proliferation occurred in the media channels. On day 4 a few cells that had escaped the gel channel were seen in the media channel. By day 5, those few cells had proliferated to fill the entire channel with cells. In the conditions where PMA was not present, cell proliferation in the media channels occurred but not at the same magnitude as the conditions with PMA. This is an important factor to consider when endothelializing media channels.

Chapter 5

Discussion

Vascular network development and formation is enhanced through a defined extracellular matrix composition, specific combinations of growth factors and flow

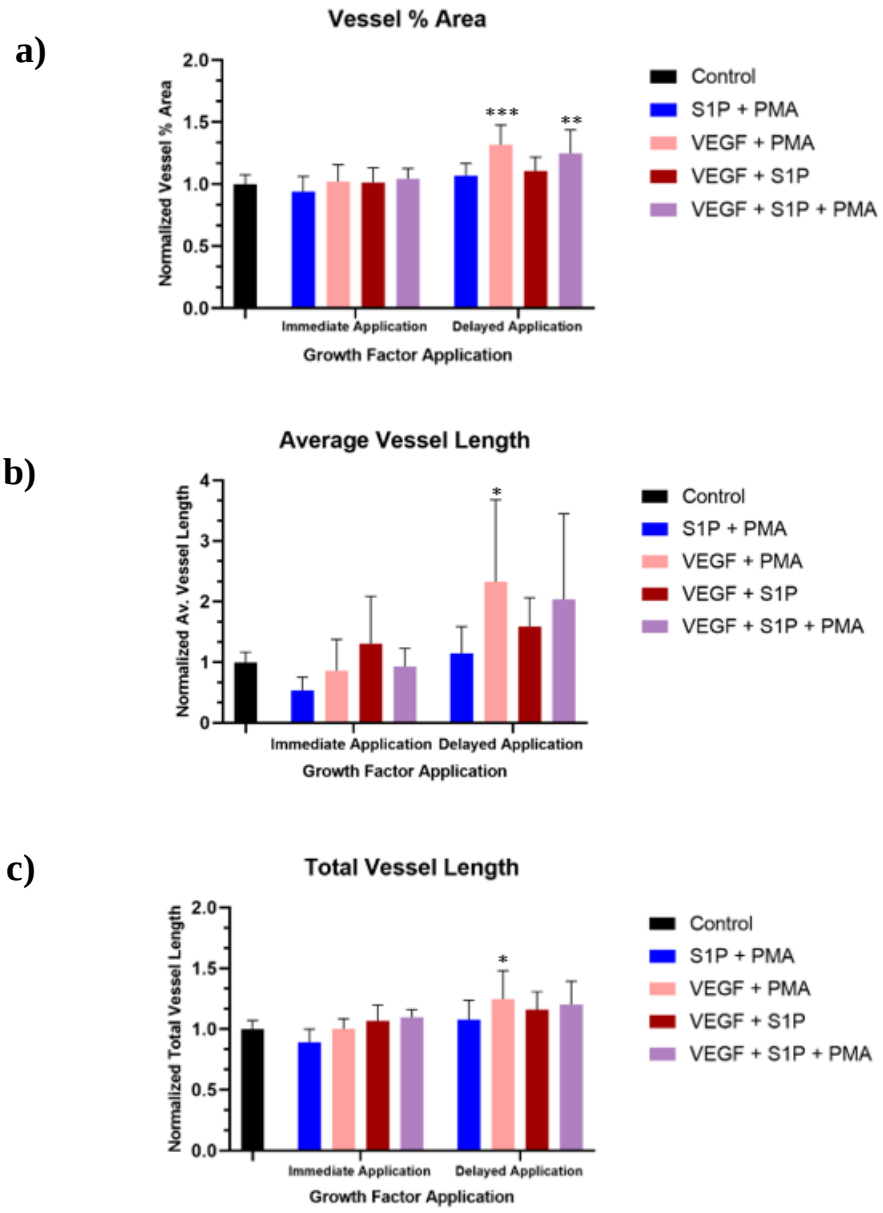

Fig. 13. Comprehensive analysis of vessel % area, average vessel length and total vessel length with a direct comparison between immediate and delayed growth factor application compared to a control. (n=9) Graphs are presented as mean + standard deviation. * Indicates statistical significance of ($p<0.05$), ** of ($p<0.01$), *** ($p<0.001$)

control in a microfluidic device. Studies done here were able to show the importance of developing and studying microvasculature networks in the context of microfluidic

devices. Experimentation demonstrated the finicky nature of endothelial cells in their development into a microvasculature network. Results can be used in the development of a fully perfusable, microvasculature network platform that can be used in experimentation in a variety of applications such as tissue engineering, drug screening and development or disease modeling.

It was apparent that a precisely defining the extracellular matrix in terms of density and material used was necessary in order to produce quality vasculature. In both a well plate and in a microfluidic device collagen mixed with Matrigel failed to produce vessel growth while fibrin showed successful vessel growth. While collagen and fibrin have both been successfully used to produce microvascular networks, only fibrin showed success in the platforms tested here. It is possible the collagen may have been incompatible with the specific HUVEC line used. Future experimentation with a different HUVEC cell line would indicate if this was the case. Interestingly a co-culture with fibroblasts in the collagen gels did not make a difference in cell growth. Again this could be due to compatibility issues with the collagen gel used. It would be interesting to do the co-culture in the fibrin gels in the microfluidic device to see if they would enhance vessel development.

It was also observed that increasing the initial endothelial cell concentration to 14 million cells/mL resulted in more vessel development compared to smaller concentrations which agrees with previous studies and the literature. Additionally, the concentration of the fibrin was experimented with, it was clear a set fibrin concentration of 9mg/ml as prepared was the most ideal for vessel growth. When the fibrin concentration was lowered to even 5mg/ml the fibrin was not able to stay contained inside the central gel channel. A concentration of 7.5mg/ml did stay inside the gel channel, but vessel development was not as strong as the 9mg/ml fibrin concentration. However, a clear picture of the effects of changing ECM density on the developing vessels was not able to be elucidated here.

Fluid flow control in a microfluidic model helped with vessel development. Vessel diameter and overall area was increased in the device platform compared to the standard well plate. While flow was present in the device, it only lasted for about two hours before

the pressure driven flow became equilibrated. This means each device was getting 4 hours of fluid flow through the device each day since media weas changed twice a day. The limited flow through the device was due to the size of the pipet tips used (200µL). While helpful, the flow was a limiting factor of the platform. Flow is important in the development of a microvasculature network because it controls growth and the over shape of the network. Blood flow in developing vasculature impacts cells in a variety of ways, for example through cell morphological changes and proliferation. These changes collectively impact the overall properties of the resulting microvasculature network and surrounding tissue. Without the incorporation of continuous flow in a vasculogenic model, the architecture of a microvasculature network would not completely recapitulate the physiological conditions inside the body.

In future iterations of this platform, fluid flow conditions could be connected to a fluid pump system where the flow rate could be tightly regulated, and a precise and physiologically relevant interstitial flow rate could be applied. Without continuous fluid flow, other forces are most likely affecting growth factors to permeate through the ECM such as mass transfer. The higher concentration of growth factors flowing through the media channels diffuse through the ECM to areas where no growth factors are present. Future experimentation with this platform could look more closely at this mass transfer effect in the absence of continuous fluid flow. With continuous fluid flow it would also be possible to see microvessel alignment in the direction of flow.

It was hypothesized that using a microfluidic device in place of a well plate would allow for clearer images to be taken of the resulting microvasculature networks. While still a 3D microenvironment, the ECM in the device was far less thick than the ECM in the well plate. Results showed using a microfluidic device platform did allow for clearer images to be taken.

It was found the addition of growth factors in different combinations and added at different timepoints affects the development of the resulting vasculature in vessel percent area, average vessel length and total vessel length. The results from this experiment fill a noticeable hole in the literature regarding the impact of the timing of the application of growth factors in models of vasculogenesis. Here two groups were presented with the

various growth factor combinations, one group receiving them right after gel polymerization on day 0 and the second after sufficient vessel development on day 3. Across all three experiments it was clear that waiting until day 3 to add the growth factors increased vessel percent area, average vessel length and total vessel length. Vessel networks visually looked more intact and stabilized as well (Fig. 9)This is interesting as growth factors *in vivo* are present as the endothelial cells are developing into vessels, not after they have already formed. More experimentation and analysis will need to be done to fully understand why waiting to add growth factors until vessel formation has occurred results in more developed vasculature.

Experiments demonstrated that specific combinations of growth factors can impact the quality and amount of vessel growth. Results here indicate that having VEGF and PMA present in a growth factor combination is important for the overall development of a microvasculature network. This agrees with the findings of previous vasculogenic assays that included VEGF and PMA performed to assess the effects of growth factors on a developing microvasculature network. Another interesting result showed that adding the growth factors right away on day 0 decreased vessel percent area, average vessel length and total vessel length compared to a control with no additional growth factors added. Initially these results contradict themselves as the addition of growth factors is supposed to induce more vessel growth and development, not reduce it. However, a few studies have shown that when growth factors were added to endothelial cells without a co-culture like in the case presented here, vessel development was decreased compared to endothelial cells that were co-cultured with other stromal cells (Whisler et al., 2012).

It was predicted that growth factors conditions containing VEGF would increase overall vessel development and formation. Conditions containing PMA were predicted to have a large impact on vessel % area due to its role in endothelial cell proliferation. Conditions with S1P were predicted to have more vessel network branching as S1P plays a key role in endothelial cell migration through the ECM.

Results indicated that when VEGF and PMA were present in a condition together, they were able to induce the most vessel development in terms of vessel % area, average vessel length and total vessel length. In this vasculogenic platform, S1P did not seem to

play a significant role in vessel development. To form any concrete observations further testing would need to be done. However, it is clear that the addition of growth factors has an impact on vessel % area, average vessel length and total vessel length.

It's important to note that specific growth factor combinations have different biological effects on developing vasculature. Vessel development is complex, and many factors contribute to the structure of a microvasculature network. Different organ systems also have their own unique vascular topology for organ-specific functions (Kur et al., 2016). A vascular network with a higher vessel density will have increased oxygen and nutrient deliver to its tissues. For example, cardiac tissue has much higher metabolic needs compared to adipose tissue and therefore will have a much denser vascular network. The overall functionality of a microvasculature network depends on its structure and topology. A sufficient supply of oxygen and nutrients results from an adequate amount of branching and spatial distribution of vessels throughout the tissue. (Kopylova et al., 2017). Depending on the application different attributes of vasculature might be desired. For example, if the goal is to fully perfuse a tissue graft with a microvasculature network, vessel percent area would be a very important metric to measure. Depending on the application of the vasculature, certain features of the vessels will be desired and specific combinations of growth factors can be used to achieve those topologies. There is no cumulative, best growth factor combination to achieve the most ideal vasculature. Different growth factors need to be strictly refined and managed for a specific application.

A large amount of variability was present between each individual experiment. Besides cells being dynamic and extremely sensitive to their culture conditions inside a microfluidic device, there does not appear to be a concrete explanation for the variability. However, factors like cell confluency, cell passage number and small differences in fibrin gels could have impacted the response of the endothelial cells. Further testing to generate a large data set would be beneficial to uncover more clear trends in how different combinations of growth factors impact the vessel formation. With the limited amount of data collected in this work, it is difficult to form concrete conclusions on the growth factor combination that produces the most robust microvasculature network. This

platform can be used for a wide variety of experimentation. For example, this model may be useful to demonstrate cancer cell extravasation. Results from the growth factor experiments were expected to influence the protocol for induced vasculogenesis in the model. Using the growth factor combination that would be expected yield the desired metric of the vessels, with vasculature that has fully developed into a fully perfusable network, cancer cells can be injected through the inlet ports and released into the vasculature. This experiment could allow for the observation of cancer cell extravasation into the ECM. This platform can also be used to simulate cancer metastasis from the primary site to the secondary site using the engineered vasculature

In tissue engineering engineered grafts often face complications such as poor nutrient and oxygen perfusion. This can greatly decrease their viability post transplantation. There exists a huge need to develop more reliable methods to vascularize tissue grafts. By fully understanding the conditions needed for the development of fully perfusable microvasculature network, a more viable method for creating vasculature can be created. A fully working model of vasculature can also aide in other applications such as drug development and screening. The more physiologically relevant a microvasculature network is, the more we can rely on these models to predict drug efficacy and potential side effects. Lastly, many disease states within the body are systemic and rely significantly on the vascular system. It is important then to incorporate this into any working model of a disease state to get the most accurate results as possible.

Chapter 6

Conclusions and Future Directions

The primary goal of this project was to create a functional model of vasculogenesis to further understand the ideal conditions needed to develop functional vasculature. Specific conditions are needed for optimal vessel development such as the composition of the ECM. In this model a fibrin ECM with a density of 9mg/ml was to foster the most developed microvasculature networks. Cell specific factors such as initial seeding density play a key part as well with a higher density of cells, specifically 14 million cells/mL in this case, leading to better network formation. Fluid flow across the

developing vessels was an important component of the platform as well. In the microfluidic model developed here fluid flow control allowed for an increase in vessel network formation. Finally, specific combinations of growth factors can have varying effects on vessel percent area, vessel length and overall development and stability of the network. Across the three metrics measured here, the growth factor combination VEGF + PMA resulted in the most developed vasculature.

Several modifications could be made to the device to make it more biologically relevant and therefore impact the growth and development of the endothelial cells. One modification is the incorporation of the secondary resistor to increase the fluid flow over the developing vasculature. Constant fluid flow would more closely recapitulate *in vivo* conditions. The design of the microfluidic device presented here was limited in its capacity for vasculogenic studies involving EPCs. In future work, this device could be modeled to incorporate a microenvironment containing bone marrow. Here the recruitment of EPCs to the site of vessel repair could be shown in detail.

There remains a large space for further development for vasculogenic assays specifically in microfluidic device applications. With a thorough understanding of the mechanisms behind vasculogenesis and the conditions in which it occurs most successfully, advances in modeling cancer metastasis and vascularizing tissue engineered grafts can be made. Areas such as tissue engineering, drug development and screening and disease models that highlight the dysfunctional occurrences of vasculature can all benefit from a fully perfusable, physiologically relevant, microvascular network platform. Specifically, this platform can be used in the Wood Lab where a physiologically relevant, fully perfusable microvasculature in needed. This is significant as a working model of vasculogenesis did not previously exist.

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